# Dual role for mitogen-activated protein kinase (Erk) in insulin-dependent regulation of Fra-1 (*fos*-related antigen-1) transcription and phosphorylation

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Insulin regulates the activity of the AP-1 (activator protein-1) transcriptional complex in several cell types. One component of the AP-1 complex is the transcription factor Fra-1 (*fos*-related antigen-1), and we have demonstrated previously that insulin stimulates the expression of Fra-1 mRNA in CHO.T cells [Griffiths, Black, Culbert, Dickens, Shaw, Gillespie and Tavaré (1998) Biochem. J. **335**, 19–26]. Here we demonstrate that insulin stimulates the activity of a *fra-1* promoter linked to a luciferase reporter gene, indicating that the ability of insulin to induce expression of Fra-1 mRNA is due, at least in part, to an increase in gene transcription. Furthermore, we found that insulin induces the serine phosphorylation of Fra-1 and reduces its mobility during SDS/PAGE as a result of phosphorylation. The ability of insulin to induce the accumulation of Fra-1 mRNA, stimulate the *fra-1* promoter and stimulate phosphorylation of Fra-1 all require the mitogen-activated protein (MAP) kinase cascade, which leads to the activation of extracellular-signal-regulated kinase (Erk) 1/2. Consequently, our results demonstrate that the Erk cascade plays a dual role in the co-ordinated regulation of the transcription and the phosphorylation of Fra-1 by insulin.

Key words: AP-1 (activator protein-1) complex, Fos, Jun, mitogen-activated protein kinase (MAP kinase), signalling.

## INTRODUCTION

Alterations in gene expression underlie the ability of insulin to regulate metabolic processes and to control the rate of cell growth. The regulation of gene transcription by insulin is brought about largely through controlling the activity of transcriptional complexes that bind to a variety of insulin response elements within the promoters of target genes [1]. One such insulin response element, the activator protein-1 (AP-1) motif, comprises the consensus sequence TGA(G/C)TCA. This sequence binds the AP-1 transcriptional complex that is made up of members of the Fos [c-Fos, Fra-1 (*fos*-related antigen-1), Fra-2 and FosB] and Jun (c-Jun, JunB and JunD) families as either homo- or hetero-dimers [2]. Insulin stimulates the activity of the AP-1 complex, a phenomenon that explains the ability of insulin to increase transcription of the collagenase gene and malic enzyme [3,4].

Theoretically, insulin could stimulate the AP-1 complex either by increasing the expression levels of its component factors or by regulating their phosphorylation state, and thus control transactivation potential. Indeed, insulin increases the expression of the c-Fos and c-Jun mRNAs in several cell types (reviewed in [1]). The effect of insulin on c-*fos* expression, which is only transient (peaking at 30 min and returning to basal by 60 min; e.g. see [5]), is likely to be mediated via the serum-response element within the c-*fos* promoter, which binds a heterodimer between serum response factor and Elk1 [5,6]. Elk1, and the related factor Sap1a, are phosphorylated and activated by the mitogen-activated protein (MAP) kinases extracellular-signal-regulated kinase 1 (Erk1) and Erk2, in response to insulin [5]. However, in some cell types the effect of insulin on c-*fos* expression appears to be Elk1/Sap1a-independent [7].

In CHO.T cells, the induction of AP-1 complex activity is sustained for several hours beyond the time at which c-Fos returns to basal levels [5]. This suggests that the sustained stimulatory effect of insulin on AP-1 activity is c-Fos independent, and may require other components of the AP-1 complex. In CHO.T cells, we previously reported that insulin stimulates a substantial and sustained increase in the expression of Fra-1 as well as c-Jun protein [5]. Furthermore, insulin caused a retardation in the mobility of the Fra-1 protein on SDS/polyacrylamide gels, indicative of its increased phosphorylation in response to insulin. It is likely, therefore, that the sustained elevation of AP-1 complex activity requires an insulin-dependent increase in the levels of a Fra-1:c-Jun heterodimer.

The Fra-1 protein bears strong sequence identity to the other Fos-family members within its bZIP region, but does not contain any of the N- or C-terminal transactivation domains seen in c-Fos or FosB. Indeed, Fra-1 has been reported to lack any transactivation domain [8], although this has been disputed more recently [9]. Interestingly, Fra-1 does not possess transforming activity in focus formation assays, unlike c-Fos or FosB [10,11], although Fra-1 does display oncogenic potential, as overexpression of Fra-1 in fibroblasts leads to anchorage-independent growth of cells [12]. Fra-1 may mediate this effect through its ability to heterodimerize with c-Jun, thus providing an active

Abbreviations used: AP-1, activator protein-1; AdtTA, recombinant adenovirus expressing tTA; CMV, cytomegalovirus; Erk, extracellular-signal-regulated kinase; Fra-1, *fos*-related antigen-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/Erk kinase; MEK-A, dominant-negative MEK; MEK-E, constitutively active MEK; MOI, multiplicity of infection; p.f.u., plaque-forming units; RT-PCR, reverse transcription–PCR; TRE, tetracycline response element; tTA, Tet-Off tetracycline transactivator.

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AP-1 complex. Finally, the critical role of Fra-1 in development has been demonstrated through targeted inactivation of the murine *fra-1* gene, which results in an embryonic lethal phenotype due to placental vascularization defects [13].

The mechanism underlying the regulation of *fra-1* gene expression by hormones is not well understood. Unlike other members of the Fos family, it has been demonstrated that, under some conditions, the expression of *fra-1* is regulated by AP-1 activity itself [12]. In agreement with this, overexpression of c-Jun, c-Fos, FosB or Fra-1 itself leads to elevated expression of the *fra-1* gene [14], an effect that has been proposed to be mediated via AP-1 sites located within intron-1 of the *fra-1* gene [12].

The aim of the present study was to further investigate the molecular basis underlying the ability of insulin to elevate Fra-1 expression and promote a mobility shift on SDS/PAGE. In particular, we sought to investigate the signalling pathways involved, focusing on the role of the Erk protein kinase cascade, as this appears to be important in the induction of the AP-1 complex by insulin [5]. To do this we manipulated Erk activity either by using a regulated adenoviral expression system (Tet-off) expressing dominant-negative or constitutively active MAP kinase/Erk kinase (MEK) mutants, or by pharmacological inhibition of MEK.

## EXPERIMENTAL

### Materials

All reagents were from Sigma (Poole, Dorset, U.K.) unless otherwise stated. Anti-active™ Erk antibody was from Promega Corp. (Madison, WI, U.S.A.). Antibody against haemagglutinin (HA) and the Random Prime Kit were from Roche Molecular Biochemicals (Lewes, E. Sussex, U.K.). Hybond nylon membrane, and horseradish peroxidase conjugated to donkey anti-rabbit IgG or anti-mouse IgG, were from Amersham Biosciences (Amersham, Bucks., U.K.). [$^{32}$P]dCTP was from ICN Radiochemicals (Basingstoke, Hants., U.K.). The chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was a gift from Dr J. D. McGivan (University of Bristol, U.K.).

### Plasmids

The *fra-1* coding sequence was amplified by PCR from Chinese hamster ovary cell RNA (sense primer, ATGTACCGAGACTTCGGGGAACC; antisense primer, CACTGTGTTGGCGTAGAGGTC). Sequencing of the cloned PCR product (544 bp) showed it to be 92% identical to the published murine cDNA sequence. The Fra-1 cDNA was tagged at the N-terminus with the HA epitope by amplification using PCR (sense, TTTTAAGCTTGACGAGATGTACCCTTACGATGTGCCTGATTACGCTTACCGAGACTACGGG; antisense, TTTGAATTCTCACAAAGCCAGGAG). The resulting PCR product was cloned into the *Eco*RI/*Hin*dIII site of the mammalian expression vector pcDNA3, and sequenced to confirm integrity (Invitrogen), to yield the plasmid pHA-Fra-1.

The *fra-1* promoter (bases −794 to +2480 relative to the transcriptional start site) was amplified by PCR from murine 3T3-L1 adipocyte genomic DNA using PCR (sense, ATTAAAGCTTGTGATAGCTCCAGAG; antisense, TCGTAAGCTTTTGGCACAAGGTGGAACTTCTG). The resulting PCR product was cloned into the *Hin*dIII site of the pGL3-Basic luciferase reporter vector (Promega) to generate the plasmid pFra-1$^{(-794/+2480)}$-Luc.

### Cell culture

CHO.T cells (CHO cells stably expressing the human insulin receptor [15]) were routinely cultured in Ham's F12 medium containing 5% (v/v) foetal calf serum, 200 units/ml benzylpenicillin, 100 $\mu$g/ml streptomycin and 0.25 mg/ml G-418. Cells at approx. 70% confluence in 60 mm dishes were used for transfection and adenoviral infection experiments. HEK293 cells were routinely cultured in Dulbecco's modified Eagle's medium containing 5% (v/v) foetal calf serum, 200 units/ml benzylpenicillin and 100 $\mu$g/ml streptomycin.

### Generation of recombinant adenovirus and infection of CHO.T cells

Recombinant E1-deleted adenoviruses containing wild-type (AdTRE-MEK), constitutively active (AdTRE-MEK.E) and dominant-negative (AdTRE-MEK.A) MEK constructs were produced according to standard techniques [16,17]. In brief, the MEK cDNAs were tagged with the HA epitope at the 3′ end using PCR, and were inserted into the multiple cloning site of the plasmid ΔE1sp1a downstream from the tetracycline response element (TRE) and a minimal human cytomegalovirus (CMV) promoter. Recombinant virus was generated by homologous recombination with pJM17 in HEK293 cells [18], grown to high titre, and purified by CsCl density gradient centrifugation. Recombinant adenovirus expressing the Tet-Off tetracycline transactivator (tTA) (AdtTA) was generated by cloning tTA into pXCXCMV, thus placing it under the control of the CMV promoter [19]. All viral titres were achieved in the range $5 \times 10^9$–$1 \times 10^{10}$ plaque-forming units (p.f.u.)/ml.

CHO.T cells were infected in 60 mm dishes by incubation in 2.5 ml of growth medium (in either the presence or the absence of 1 $\mu$g/ml doxycycline) containing a 1:1 ratio of AdTRE-MEK virus:AdtTA at a multiplicity of infection (MOI) of 50 for each virus. Cells were incubated with the viruses for 32 h, after which time the cells were changed into 5 ml of serum-free medium in the presence or absence of 1 $\mu$g/ml doxycycline for a further 16 h.

### Northern blot analysis and reverse transcription–PCR (RT-PCR)

After serum starvation, the cells were treated with insulin (100 nM) for the times indicated. The cells were lysed in 2 ml of Tri-reagent and total RNA was purified according to the manufacturer's instructions. RNA samples were denatured and separated on a 0.8% formaldehyde/agarose gel. The RNA was transferred to a nylon membrane by capillary action overnight using 20 × SSPE (1 × SSPE: 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA). The membrane was baked at 80 °C for 2 h and cross-linked (1200 J/cm$^2$; Stratalinker) before being pre-hybridized for 4 h at 42 °C in 4 × SSPE, 5 × Denhardt's (1 × Denhardt's: 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA), 0.1% SDS, 4.5% formamide, 0.2 mg/ml salmon sperm DNA and 0.5% dextran sulphate.

$^{32}$P-labelled probes were prepared from cDNAs using a Random Prime Kit (Roche Molecular Biochemicals) and hybridized to blots overnight at 42 °C. Blots were washed twice at room temperature in 1 × SSPE/0.1% SDS, followed by two 10 min washes at room temperature in 0.5 × SSPE/0.1% SDS. Hybridized radioactive bands were visualized by analysis using a Molecular Dynamics PhosphorImager (Amersham Bioscience). Blots were stripped in boiling 1% SDS for subsequent reprobing.

For analysis of mRNA expression by RT-PCR, total RNA was isolated from 100 mm dishes of 3T3-L1 cells before and after insulin stimulation for 6 h. The cells were washed with 5 ml of ice-cold PBS and extracted with 1 ml of Tri-Reagent (Sigma). Total RNA was prepared from the aqueous phase by propan-2-ol precipitation according to the manufacturer's instructions. cDNA was prepared by reverse transcription of 3 $\mu$g of total RNA using oligo(dT)$_{30}$ primers and MMLV-RT (Promega). Fra-1 and GAPDH cDNAs were then amplified by 30 cycles of PCR using BioTaq polymerase (Bioline, London, U.K.) and the following primers. Fra-1: sense, 5′-ATGTACCGAGACTTC-GGGGAACC-3′; antisense, 5′-CACTGTGTTGGCGTAGA-GGTC-3′ (which gives a product of 544 bp); GAPDH: sense, 5′-GAACGGGAAGCTCACTGGCATG-3′; antisense, 5′-GTCC-ACCACCCTGTTGCTGTAG-3′ (which gives a product of 286 bp). The PCR products were separated on 1.2% (w/v) agarose gels by electrophoresis, and ethidium bromide-stained bands were visualized by UV illumination. Titration of reverse-transcribed template into the PCR reaction demonstrated a linear relationship between amount of template used and PCR product produced under the conditions used (results not shown).

### Transfection of CHO.T cells

CHO.T cells at 70% confluence were transfected in 60 mm dishes with 2 $\mu$g of each plasmid (HA-tagged Fra-1 and/or MEK constructs) using a charge ratio of 3 $\mu$l of Fugene 6 reagent (Roche Molecular Biochemicals) per $\mu$g of plasmid DNA, according to the manufacturer's instructions. After 36 h the cells were serum starved for 16 h prior to stimulation with insulin, as indicated in the Figure legends.

### Western blotting

The cells were serum starved for 2 h, treated with insulin (100 nM) for the times indicated as required, and then washed twice in ice-cold PBS before lysis by scraping into 500 $\mu$l of extraction buffer (50 mM $\beta$-glycerophosphate, 1.5 mM EDTA, 1 mM benzamidine, 0.5 mM $Na_3VO_4$, 1 mM dithiothreitol, 1 $\mu$g/ml pepstatin, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 0.1 mM PMSF, pH 7.28). Lysates were clarified by centrifugation at 10000 ***g*** and the supernatant was subjected to SDS/PAGE analysis on 10% (w/v) polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore, Watford, U.K.), and the transfected MEK and Fra-1 constructs were detected by Western blotting with the anti-HA antibody (0.5 $\mu$g/ml) and detection by Enhanced ChemiLuminescence (Amersham Biosciences). Active Erk, phosphorylated on both threonine and tyrosine, was detected in lysates by Western blotting using an antibody against active Erk (Promega Corp.; 25 ng/ml), as described by the manufacturer.

### Reporter gene assays

CHO.T cells, at 70% confluence, were transfected in wells of 12-well plates with 0.25 $\mu$g of pRL.SV40, 0.5 $\mu$g of the appropriate MEK plasmid and 0.5 $\mu$g of pFra-1$^{(-794/+2480)}$-Luc using Tfx-50 reagent (Promega Corp.). The cells were washed twice in ice-cold PBS, extracted in ice-cold passive lysis buffer (Promega Corp.) and assayed sequentially for firefly and *Renilla* luciferase activities using a Berthold Lumat LB9501 luminometer, according to the manufacturer's instructions (Promega Corp.). The ratio of firefly luciferase activity to *Renilla* luciferase activity provides a specific measure of *fra-1* promoter activity corrected for variations in transfection efficiency and cell viability.

### Metabolic labelling with [$^{32}$P]P$_i$

CHO.T cells in 60 mm Petri dishes, transiently transfected with the HA-tagged Fra-1 plasmid described above, were metabolically labelled for 3 h with 1 mCi of [$^{32}$P]P$_i$ in phosphate-free Dulbecco's modified Eagle's medium as described [20]. Lysates (0.5 ml) were prepared and the HA-tagged Fra-1 was immunoprecipitated with 15 $\mu$g of anti-HA monoclonal antibody and 2.5 mg of Protein A–Sepharose according to procedures described previously [20]. The immunoprecipitates were analysed by SDS/PAGE followed by autoradiography. The band corresponding to the $^{32}$P-labelled Fra-1 was identified, excised and subjected to phosphoamino acid analysis by thin-layer cellulose chromatography at pH 1.9 according to methods described previously [21].

### Incubation of anti-HA–Fra-1 immunoprecipitates with $\lambda$-phosphatase

HA–Fra-1 was immunoprecipitated from cell lysates prepared from CHO.T cells grown in 60 mm Petri dishes and transiently transfected with the HA-tagged Fra-1 plasmid described above. Immunoprecipitates were washed three times with 1 ml of ice-cold extraction buffer (50 mM $\beta$-glycerophosphate, 1 mM benzamidine, 1 mM dithiothreitol, 1 $\mu$g/ml pepstatin, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 0.1 mM PMSF, pH 7.28). Immunoprecipitates were then resuspended in phosphatase buffer (as supplied by the manufacturer) containing 2 units/$\mu$l $\lambda$-phosphatase (New England Biolabs, Hitchin, U.K.) and incubated at 30 °C for 1 h. Immunoprecipitates were subjected to SDS/PAGE analysis and Western blotting with the anti-HA antibody as described above.

## RESULTS

### Creation of doxycycline-inducible adenoviral MEK vectors to regulate Erk activation in CHO.T cells

In order to examine the role of the Erk cascade in the induction of Fra-1 mRNA expression, wild-type, constitutively active (MEK-E) and dominant-negative (MEK-A) MEK were tagged with an HA epitope at their C-terminus and expressed in CHO.T cells using adenoviral-mediated gene transfer. As expression of the MEK cDNAs was placed under the control of a TRE-containing promoter, in the simultaneous presence of tTA, expression of the MEK cDNA could be repressed by the addition of the tetracycline analogue doxycycline to the medium.

CHO.T cells were co-infected with adenovirus carrying the MEK constructs (AdMEK, AdMEK.E or AdMEK.A) and AdtTA. The MEK constructs were only expressed in the absence of doxycycline, as determined by Western blotting of the cell lysates with anti-HA antibody (Figure 1, lanes a, f, j and k for MEK, lanes m, p and q for MEK-A and lane b for MEK-E). By contrast, in the presence of doxycycline, expression of all three MEK constructs was essentially undetectable.

To assess the effects of the introduced viruses on activation of the MAP kinases Erk1 and Erk2, we Western blotted the cell lysates with an antibody against active Erk. The use of this antibody, which recognizes only active Erk1/Erk2 bis-phosphorylated on the activation loop Thr(P)-Glu-Tyr(P) motif, is well established as an indicator of Erk activity. In the absence of doxycycline, expression of MEK-E caused a clear increase in the bis-phosphorylation of Erk1 and Erk2 (Figure 1A, lane b), whereas wild-type MEK was without effect (Figure 1A, lane a). The presence of doxycycline, which repressed MEK-E expression, completely prevented the phosphorylation and thus activation of Erk1 and Erk2 (Figure 1A, lane d).

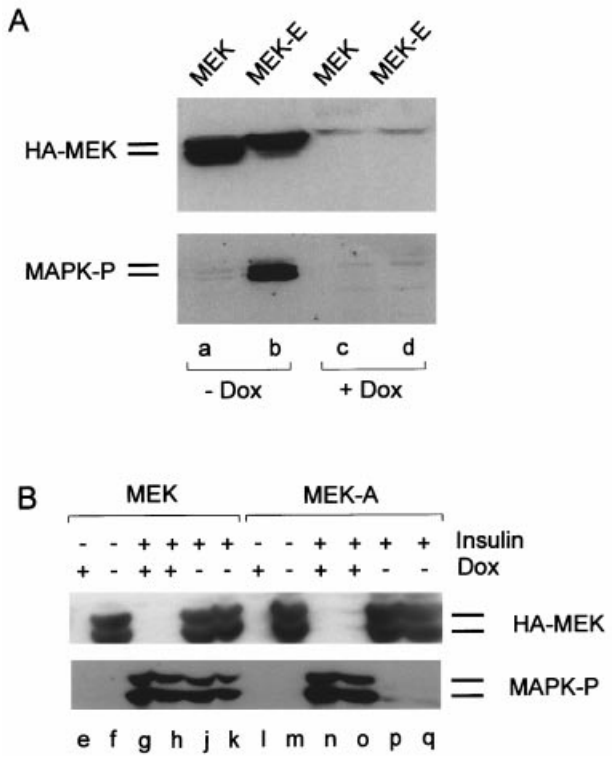


**Figure 1 Regulation of Erk activity by recombinant MEK adenoviruses**

(**A**) CHO.T cells were co-infected with either AdTRE-MEK (MEK) or AdTRE-MEK.E (MEK-E) and AdtTA at a MOI of 50 p.f.u./cell for each virus. Cells were infected in growth medium in either the presence or the absence of 1 $\mu$g/ml doxycycline (Dox) as indicated, and left for 32 h before serum starving the cells for 16 h in the further presence or absence of 1 $\mu$g/ml doxycycline. Expression of the HA-tagged MEK constructs was detected by Western blotting of cell lysates with the anti-HA antibody (upper panel). The blot was re-probed with the anti-active-Erk antibody to detect Erk activity (lower panel). (**B**) CHO.T cells were co-infected with either AdTRE-MEK (MEK) or AdTRE-MEK.A (MEK-A) and AdtTA at a MOI of 50 p.f.u./cell for each virus. The cells were cultured and serum starved as in (**A**) in the presence or absence of 1 $\mu$g/ml doxycycline (Dox), as indicated. The cells were then treated with or without insulin (100 nM) as shown. Expression of the MEK constructs was detected by Western blotting of lysates with anti-HA antibody (upper panel). The blot was re-probed with the anti-active-Erk antibody to detect Erk activity (lower panel). In both (**A**) and (**B**) the blots shown are representative of three separate experiments.

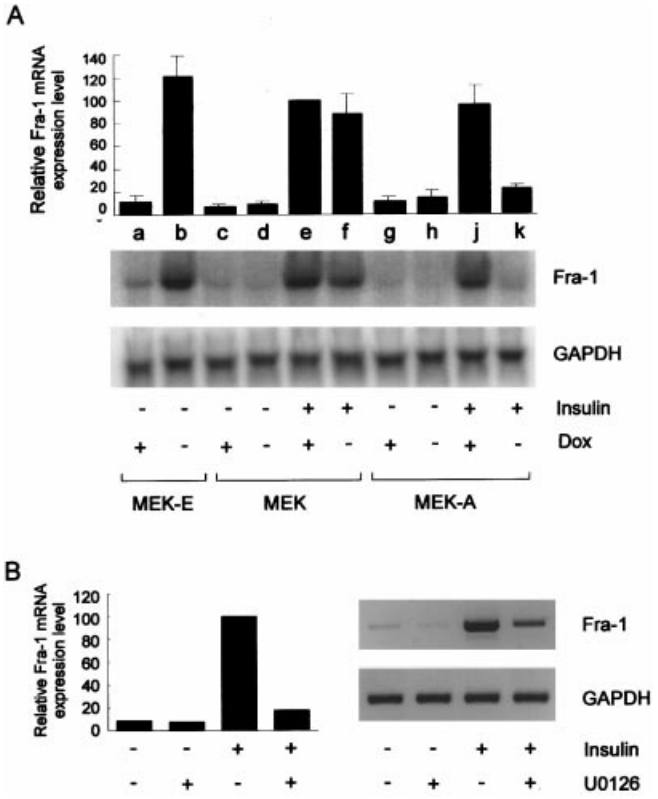


**Figure 2 Effects of recombinant adenoviruses on Fra-1 induction**

(**A**) CHO.T cells were co-infected with AdtTA plus AdTRE-MEK.E (MEK-E; lanes a and b), AdTRE-MEK (MEK; lanes c–f) or AdTRE-MEK.A (MEK-A; lanes g–k) at a MOI of 50 p.f.u./cell for each virus, and then incubated with or without doxycycline (Dox) as indicated. The cells were infected, cultured and serum starved as described in the legend to Figure 1, and were then either left unstimulated (lanes a–d, g and h) or treated with insulin (100 nM; lanes e, f, j and k) for 2 h as shown. Total RNA was isolated, separated by electrophoresis and transferred to a nylon membrane. The resulting blot was probed, stripped and re-probed sequentially with $^{32}$P-labelled probes for Fra-1 and GAPDH. The levels of mRNA were determined using a PhosphorImager and were quantified using ImageQuant software. The data are expressed as a percentage of the mRNA level seen for cells stimulated with insulin and infected with AdTRE.MEK plus AdtTA in the presence of doxycycline (lane e), and are displayed as means $\pm$ S.E.M. for three separate experiments. (**B**) 3T3-L1 adipocytes were pre-incubated in the absence or presence of U0126 (10 $\mu$M) as indicated for 30 min prior to further incubation in the presence of insulin (100 nM) as required for 6 h. The levels of expression of Fra-1 and GAPDH were assessed by RT-PCR as described in the Experimental section. The agarose gel was scanned, and the Fra-1 mRNA expression level is expressed relative to that of GAPDH. The results are representative of two separate experiments.

Insulin-stimulated Erk1/Erk2 activation (measured at both 5 min and 2 h) was almost completely blocked by an adenovirus containing MEK-A, but not by wild-type MEK (compare Figure 1B, lanes p and q versus lanes j and k), but this occurred only in the absence of doxycycline (compare Figure 1B, lanes p and q versus lanes n and o). This result suggests that the efficiency of infection of cells with the virus is high (indeed, adenoviral expression of a green fluorescent protein reporter suggests that in excess of 90% of CHO.T cells are infected using this method; results not shown). In contrast with the effect of MEK-A, overexpression of wild-type MEK had no significant effect on the activation of Erk1 or Erk2 (Figure 1B).

In conclusion, therefore, the use of the regulated expression system allows us to very effectively manipulate the level of expression of the transfected MEK using doxycycline and to control for potential non-specific effects of the infecting adenovirus, and thus to examine the direct consequences of MEK overexpression. This is important when attempting to express MEK mutants in cells over long periods of time (i.e. when looking at *fra-1* gene expression).

## MEK-E mimics, and MEK-A and U0126 block, the effect of insulin on Fra-1 mRNA induction

Insulin caused a substantial increase in the expression of Fra-1 mRNA in CHO.T cells, as determined by Northern blotting (Figure 2A, compare lanes c and e), and this was largely unaffected by the overexpression of wild-type MEK (Figure 2A, lanes d and f). In contrast, expression of the MEK-A construct, in the absence of doxycycline, almost completely blocked the effect of insulin (Figure 2A, compare lanes k and j). MEK-E, when expressed in the absence of doxycycline, promoted an increase in Fra-1 mRNA that was comparable with that seen in insulin-stimulated cells (Figure 2A, compare lanes b and e). The induction of *fra-1* expression by insulin is not specific to CHO.T cells, as we also found a similar effect in 3T3-L1 adipocytes;

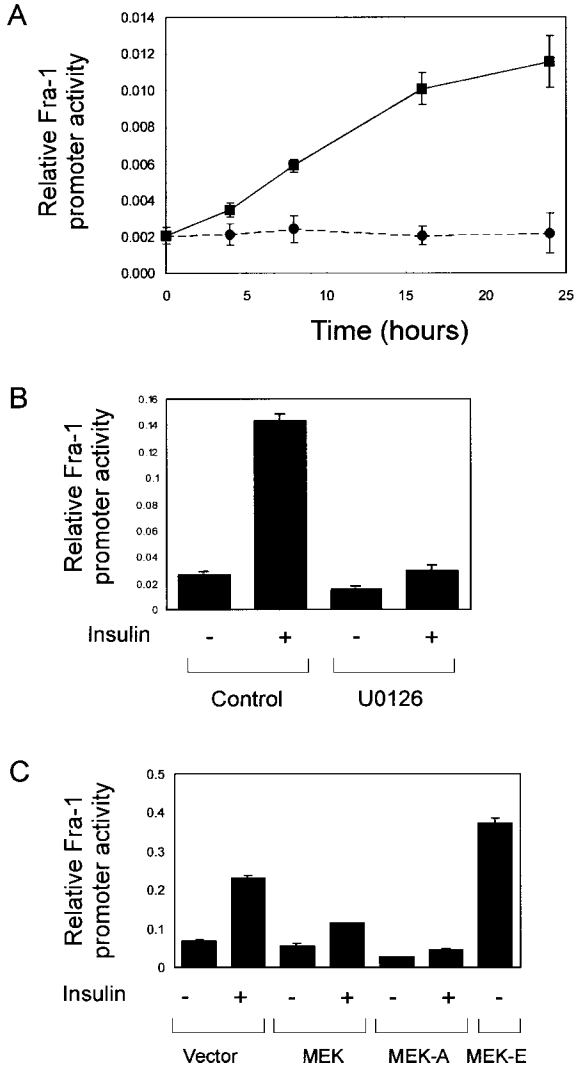


**Figure 3 Induction of *fra-1* promoter activity by insulin, and effects of manipulation of MEK activity**

(**A**) CHO.T cells were co-transfected with pFra-1$^{(-794/+2480)}$-Luc, containing the firefly luciferase reporter gene under the control of the murine *fra-1* promoter (bases −794 to +2480), and pRL.SV40, which contains the *Renilla* luciferase reporter gene under the control of the constitutive simian virus 40 promoter. The cells were serum starved for 16 h before stimulation with (■) or without (●) insulin (100 nM) for the indicated times (h). The cells were extracted and lysates were assayed sequentially for firefly and *Renilla* luciferase activities. Relative *fra-1* promoter activity is represented as the ratio of firefly to *Renilla* luciferase activity. Results are displayed as means ± S.D. and are representative of three separate experiments. (**B**) CHO.T cells were co-transfected with pFra-1$^{(-794/+2480)}$-Luc and pRL.SV40 as described for (**A**). The cells were serum starved for 16 h, followed by pre-incubation with either DMSO (control) or the MEK inhibitor U0126 (10 $\mu$M) for 30 min. After this time, the cells were incubated either with or without 100 nM insulin for a further 16 h as indicated. The cells were extracted and lysates were assayed sequentially for firefly and *Renilla* luciferase activities. (**C**) CHO.T cells were co-transfected with pFra-1$^{(-794/+2480)}$-Luc, pRL.SV40 and wild-type pMEK, pMEK.A or pMEK.E as shown. The cells were then serum starved for 16 h followed by incubation with or without 100 nM insulin as indicated. In both (**B**) and (**C**), relative *fra-1* promoter activity is represented as the ratio of firefly to *Renilla* luciferase activity (mean ± S.D.), and the data shown are representative of three separate experiments.

furthermore, this effect was blocked by the MEK inhibitor U0126 (Figure 2B).

Taken together, the results suggest that the Erk cascade is both necessary and sufficient for the effect of insulin on the induction of Fra-1 mRNA.

### Insulin stimulates a sustained increase in the activity of the *fra-1* promoter

Insulin could induce Fra-1 mRNA levels either by stimulating gene expression through a direct effect on the *fra-1* promoter, or by stabilization of the Fra-1 mRNA. To help distinguish between these possibilities, we cloned the *fra-1* promoter region encompassing nucleotides −794 to +2480. This region incorporates the transcriptional start site together with AP-1 sites located in intron 1 previously reported to be important for the regulation of *fra-1* gene expression [12]. This promoter was placed upstream of the firefly luciferase gene to give the plasmid pFra-1$^{(-794/+2480)}$-Luc.

Insulin stimulation of CHO.T cells transfected with pFra-1$^{(-794/+2480)}$-Luc resulted in a substantial (>5-fold) stimulation of the *fra-1* promoter that was sustained for at least 24 h (Figure 3A). These results indicate that the observed increase in Fra-1 mRNA levels in response to insulin was due, at least in part, to an effect on transcription. However, we cannot completely rule out an additional effect of insulin on mRNA stability.

### Activation of the Erk cascade is necessary and sufficient for insulin stimulation of the *fra-1* promoter

As activation of the Erk cascade is required for the stimulation of Fra-1 mRNA by insulin, we next asked whether this is also the case for the *fra-1* promoter. The ability of insulin to stimulate luciferase expression in CHO.T cells transfected with pFra-1$^{(-794/+2480)}$-Luc was blocked by the cell-permeant MEK1 inhibitor U0126 (Figure 3B). A similar inhibitory effect was seen in the presence of a co-transfected MEK-A plasmid (Figure 3C), and the effect of insulin was mimicked by a co-transfected MEK-E plasmid (Figure 3C). Interestingly, overexpression of wild-type MEK also had a dominant-negative effect on insulin-induced *fra-1* promoter activity in these experiments, although this was incomplete (Figure 3C), perhaps because it disrupts the ability of MEK1 to complex with the scaffolding protein MP-1 [22].

Taken together, these results suggest that the Erk cascade is both necessary and sufficient for the effect of insulin on activation of the *fra-1* promoter.

### Insulin stimulates the phosphorylation of a HA-tagged Fra-1 transiently transfected into CHO.T cells

We found previously that insulin caused a significant decrease in the mobility of Fra-1 on SDS/PAGE [5], an event that is indicative of phosphorylation. As basal levels of endogenous Fra-1 are very low and are subject to transcriptional regulation by insulin, in order to help determine the mechanisms involved, CHO.T cells were transiently transfected with an N-terminally HA-tagged Fra-1 under the control of the non-regulated CMV promoter.

As shown in Figure 4(A), the overexpressed Fra-1 protein was present as multiple closely migrating forms on SDS/PAGE gels in the serum starved state, as we have found previously for the endogenous Fra-1 protein [5]. Treatment of the cells with insulin caused a progressive decrease in the mobility of Fra-1, such that by 60 min only the most slowly migrating forms were apparent. This decrease in mobility was reversed by treating Fra-1 immunoprecipitates derived from insulin-stimulated cells with $\lambda$-phosphatase (Figure 4B), suggesting that the observed decrease in Fra-1 electrophoretic mobility was indeed due to phosphorylation.

To confirm that Fra-1 was phosphorylated in response to insulin, CHO.T cells transiently overexpressing HA-tagged Fra-1 were metabolically labelled with [$^{32}$P]$P_i$, followed by treatment

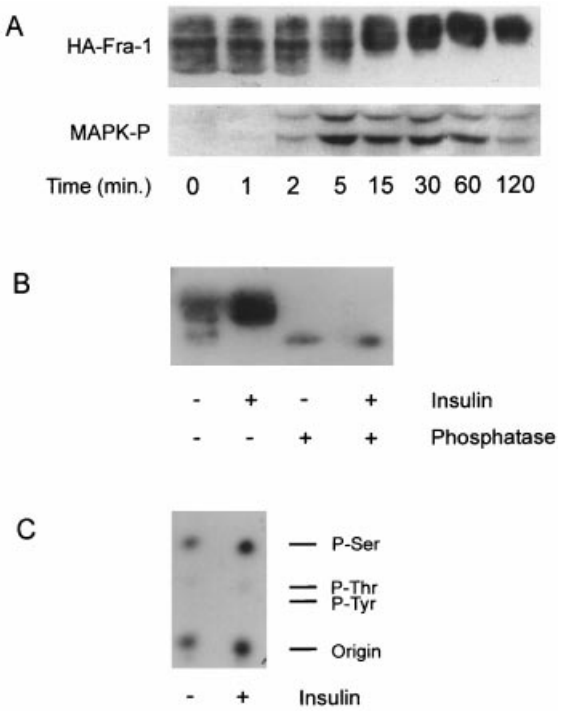


**Figure 4 Insulin stimulates Fra-1 phosphorylation**

(**A**) CHO.T cells were transfected with a vector expressing wild-type HA-tagged Fra-1 (pHA-Fra-1), serum starved for 16 h and then stimulated with insulin (100 nM) for the indicated times (min). The cells were extracted and the HA-tagged Fra-1 was detected by Western blotting lysates with the anti-HA antibody (upper panel). The blot was then stripped and re-probed with the anti-active-Erk antibody to measure Erk activity (lower panel). (**B**) CHO.T cells were transfected with pHA-Fra-1, serum starved for 16 h and then stimulated or not with insulin (100 nM) for 30 min as indicated. HA–Fra-1 was immunoprecipitated from cell lysates with anti-HA antibodies, and the immunoprecipitates were incubated in the presence or absence of 2 units/$\mu$l $\lambda$-phosphatase, as described in the Experimental section. HA–Fra-1 was detected by Western blotting with anti-HA antiserum. (**C**) CHO.T cells transfected with pHA-Fra-1 were metabolically labelled with [$^{32}$P]$P_i$ as described in the Experimental section. The cells were stimulated or not with insulin (100 nM) for 30 min, as indicated, before isolation of HA–Fra-1 by immunoprecipitation with anti-HA antibodies and separation by SDS/PAGE. $^{32}$P-labelled HA–Fra-1 was excised from the gel and subjected to phosphoamino acid analysis by TLC at pH 1.9. This panel shows an autoradiograph of the resulting thin-layer plate, showing the positions of migration of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), as well as the origin of sample application.

or not with insulin. As shown in Figure 4(C), phosphoamino acid analysis of $^{32}$P-labelled Fra-1 immunoprecipitated from the cells revealed that insulin indeed stimulated the phosphorylation of Fra-1, and that this phosphorylation was restricted to serine residues.

### Fra-1 phosphorylation in response to insulin is mediated by the Erk cascade

The insulin-induced phosphorylation of HA-tagged Fra-1 occurs almost in parallel with Erk phosphorylation and activation, although there is a detectable lag of the former behind the latter (Figure 4A). Co-expression of MEK-E with HA–Fra-1 induced an insulin-independent decrease in Fra-1 mobility on SDS/PAGE (Figure 5A, lane c). Taken together, these data are consistent with the Erk cascade mediating the effect of insulin on Fra-1 phosphorylation.

To determine more directly whether the Erk cascade is necessary for the ability of insulin to induce Fra-1 phosphorylation, CHO.T cells were co-transfected with MEK-A. This construct completely blocked the ability of insulin to induce Fra-1 phosphorylation (Figure 5A, compare lanes d and e versus f and g). Consistent with the inhibitory effect of MEK-A, we also found that insulin-stimulated Fra-1 phosphorylation was completely inhibited by the MEK inhibitor U0126 (Figure 5B).

These results confirm that the Erk cascade is necessary and sufficient for the induction of Fra-1 phosphorylation in response to insulin.

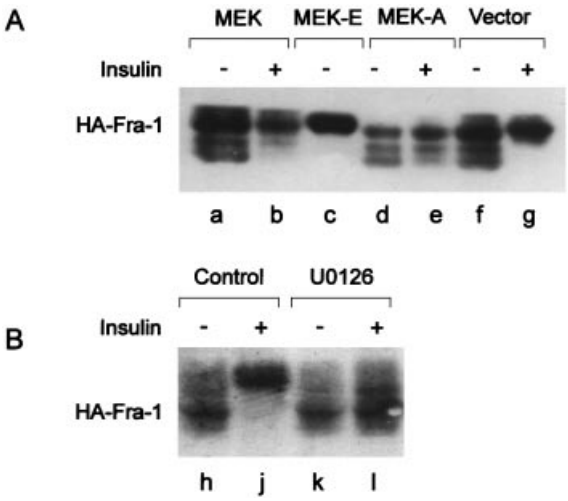


**Figure 5 Insulin stimulation of Fra-1 phosphorylation requires the Erk cascade**

(**A**) CHO.T cells were co-transfected with pHA-Fra-1 plus wild-type pMEK, pMEK-E or pMEK-A, as indicated. The cells were serum starved for 16 h and then treated with or without insulin (100 nM) for 30 min as indicated. The cells were extracted and expression of the HA-tagged Fra-1 construct was detected by Western blotting of cell lysates with anti-HA antibody. (**B**) CHO.T cells were transfected with pHA-Fra-1, serum starved for 16 h and then pre-incubated with DMSO (Control) or U0126 (10 $\mu$M) as indicated for 30 min. The cells were then treated with or without insulin (100 nM) as indicated for a further 30 min, and HA-tagged Fra-1 was detected by Western blotting of cell lysates with anti-HA antibody.

## DISCUSSION

The results presented in this study show conclusively that the activation of the Erk cascade is necessary and sufficient for the ability of insulin to stimulate Fra-1 mRNA expression, *fra-1* promoter activation and Fra-1 phosphorylation.

The ability of insulin to stimulate *fra-1* gene expression was mimicked by overexpressing MEK-E, and was blocked in the presence of MEK-A or of a pharmacological inhibitor of MEK, U0126 (Figure 2). This is consistent with recent studies showing that Erk was necessary and sufficient for *fra-1* induction during Ras-induced transformation, or in response to stimulation of Rat1 cells by lysophosphatidic acid [23,24].

The induction of Fra-1 mRNA levels by insulin could be due either to an effect of insulin on mRNA stability and/or to direct stimulation of transcription from the *fra-1* promoter. In the present study we have clearly demonstrated that insulin can promote a substantial and sustained stimulation of luciferase expression when this reporter gene is placed under the control of a *fra-1* promoter comprising bases $-794$ to $+2480$ (Figure 3). This promoter fragment includes distal AP-1 sites located in intron 1, reported previously to be important for the regulation of *fra-1* gene expression [12], although in our studies we have found that the insulin effect does not require these distal AP-1

sites, but appears to reside in the −392 to +244 region lacking intron 1 (T. W. Hurd and J. M. Tavaré, unpublished work).

The ability of insulin to induce the *fra-1* promoter was almost completely blocked in the presence of the MEK inhibitor U0126 (Figure 3B) or of MEK-A (Figure 3C). Furthermore, the effect of insulin was mimicked by MEK-E (Figure 3C). As MEK has no known *in vivo* substrates other than Erk1 and Erk2, we take the data to suggest that Erk1 and Erk2 are both necessary and sufficient for the induction of the *fra-1* promoter and the consequent stimulation of Fra-1 mRNA production (Figure 2). Whether insulin has an additional affect on Fra-1 mRNA stability will require further investigation.

As we have reported previously [5], insulin induces a decrease in the mobility of Fra-1 on SDS/PAGE, a phenomenon that lags slightly behind the activation of MAP kinase (Figure 4A). In the basal state, Fra-1 exists as up to four distinct species. While the abundance of each of these species appears to vary between experiments, perhaps as a result of differences in cell confluency, after insulin stimulation only the most slowly migrating form of Fra-1 is apparent. The decrease in mobility caused by insulin is almost certainly the result of phosphorylation, as treatment of Fra-1 immunoprecipitates with λ-phosphatase collapsed Fra-1 into the most rapidly migrating species (Figure 4B). This is consistent with the observation that Fra-1 phosphorylation changes during cell cycle progression [25]. In the present study we have demonstrated directly that insulin induces the phosphorylation of Fra-1, as determined by the increased incorporation of $^{32}P$ into serine residue(s) of HA-tagged Fra-1 (Figure 4C).

Insulin-stimulated Fra-1 phosphorylation is blocked by both the MEK inhibitor U0126 and MEK-A, and is mimicked by MEK-E (Figure 5). This strongly suggests that the Erk cascade is necessary and sufficient for insulin-stimulated Fra-1 phosphorylation. Interestingly, the serum-induced phosphorylation of Fra-2 has also been reported to be blocked by the MAP kinase inhibitor PD98059 [23], and Gruda et al. [25] have shown that purified Fra-1 is phosphorylated by MAP kinase *in vitro*.

Young et al. [9] have recently reported that PMA can stimulate the transcriptional activity of a fusion protein comprising the DNA-binding domain of Gal4 and the C-terminus of Fra-1 (amino acids 132–275), and that this can be blocked by PD98059 and mimicked by MEK-E. Using mutagenesis, this group proposed that Thr-231 of Fra-1 was required for PMA-stimulated Fra-1 transactivation, although no data were provided to prove that this site was indeed phosphorylated in response to PMA. Our data strongly support the notion that the insulin-stimulated phosphorylation site is a serine residue(s), and that threonine is not detectably phosphorylated (Figure 4C). In our hands the insulin-induced phosphorylation site appears to reside in the C-terminal 57 amino acids of Fra-1 (T. W. Hurd and J. M. Tavaré, unpublished work), and examination of this region of Fra-1 reveals only one serine residue (Ser-267), which lies in only a partial consensus site for phosphorylation by MAP kinase (PLG***S***P). However, site-directed mutagenesis of this site suggests that it is not the target for phosphorylation in response to insulin (T. W. Hurd and J. M. Tavaré, unpublished work).

Our data would be consistent with the possibility that a kinase downstream of Erk1/Erk2 [e.g. Rsk/MAPKAP-K1 (MAP kinase-activated protein kinase-1) or Msk] is responsible for the insulin-induced phosphorylation of Fra-1, although we have found that neither recombinant active Rsk nor Msk phosphorylates Fra-1 to any appreciable extent *in vitro* (T. W. Hurd, D. Alessi and J. M. Tavaré, unpublished work). On the basis of our data, we cannot rule out the possibility that MEK phosphorylates Fra-1 directly, although we believe that this is unlikely, as there are no known *in vivo* substrates for MEK other than Erk1 and Erk2.

Our results demonstrate that the Erk cascade is necessary and sufficient for the co-ordinated induction by insulin of Fra-1 mRNA expression (via the activation of the *fra-1* promoter), and of phosphorylation of the Fra-1 protein. While insulin activates the Erk cascade in most cell types, it is becoming clear that the phosphoinositide 3-kinase pathway is a major pathway in the regulation of metabolic events by this hormone. Furthermore, the Erk cascade may be activated to a greater extent in CHO.T cells than in hepatocytes, adipocytes or muscle cells. However, as we see Erk-cascade-dependent Fra-1 induction in adipocytes, this pathway is likely to be important in more physiologically relevant cells.

Insulin-induced Fra-1 mRNA expression and phosphorylation of the Fra-1 protein are both sustained for at least 24 h after insulin stimulation. While this is consistent with the fact that activation of the AP-1 complex by insulin is also sustained for at least 24 h [5], the precise contribution of Fra-1 induction and phosphorylation to the overall activation of the AP-1 complex remains to be established. Young et al. [9] have recently reported that PMA can transactivate Fra-1, but others have failed to find a transactivating domain on Fra-1 [8]. In addition, others have proposed that Fra-1, and the related transcription factor Fra-2, may act as repressors of the AP-1 complex [26,27]. Thus the precise role of Erk-dependent phosphorylation and induction of Fra-1 in the regulation of the AP-1 complex requires much further investigation.

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