Insulin-stimulated expression of *c-fos*, *fra1* and *c-jun* accompanies the activation of the activator protein-1 (AP-1) transcriptional complex

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The activator protein-1 (AP-1) transcriptional complex is made up of members of the Fos (c-Fos, FosB, Fra1, Fra2) and Jun (c-Jun, JunB, JunD) families and is stimulated by insulin in several cell types. The mechanism by which insulin activates this complex is not well understood but it is dependent on the activation of the Erk1 and Erk2 isoforms of mitogen-activated protein kinases. In the current study we show that the AP-1 complex isolated from insulin-stimulated cells contained c-Fos, Fra1, c-Jun and JunB. The activation of the AP-1 complex by insulin was accompanied by (i) a transient increase in *c-fos* expression, and the trans-activation of the ternary complex factors Elk1 and Sap1a, in an

Erk1/Erk2-dependent fashion; (ii) a substantial increase in the expression of Fra1 protein and mRNA, which was preceded by a transient decrease in its electrophoretic mobility upon SDS/PAGE, indicative of phosphorylation; and (iii) a sustained increase in *c-jun* expression without increasing c-Jun phosphorylation on serines 63 and 73 or activation of the stress-activated kinase JNK/SAPK. In conclusion, insulin appears to stimulate the activity of the AP-1 complex primarily through a change in the abundance of the components of this complex, although there may be an additional role for Fra1 phosphorylation.

INTRODUCTION

The ability of insulin to regulate gene transcription is central to several of its major metabolic effects (reviewed in [1,2]). The nature of the nucleotide sequences and transcription factors that confer the insulin response are beginning to become unravelled [1], as are the signalling pathways involved. For example, the inhibition of expression of the phosphoenolpyruvate carboxy-kinase gene requires phosphatidylinositol 3-kinase but not mitogen-activated protein kinase (MAP kinase) or p70S6 kinase [3]. On the other hand, the stimulation of hexokinase II gene expression is prevented by rapamycin, implicating the target of rapamycin (mTOR) and/or p70S6 kinase, but does not involve MAP kinase [4].

Insulin can stimulate activator protein-1 (AP-1) transcriptional activity in several cell types [5–7]. The AP-1 complex is typically composed of members of the Fos (c-Fos, FosB, Fra1 and Fra2) and Jun (c-Jun, JunB and JunD) transcription factor families [8]; however, the molecular basis by which insulin regulates its activity is not well understood. There are several potential mechanisms by which the activity of the AP-1 complex can be regulated. They are accounted for either by changes in the level of expression of individual components, or by their covalent modification. For example, serum and growth factors such as epidermal growth factor (EGF) can increase the level of expression of *c-fos* via the phosphorylation and activation of the ternary complex factor(s) (TCFs; e.g. Elk-1 and Sap1a) by the Erk isoforms of MAP kinases. These TCFs form a complex with the serum response factor (SRF) and bind to the Ets motif

flanking the serum response element (SRE) of the *c-fos* promoter [9–11]. Stress stimuli (e.g. UV irradiation and protein synthesis inhibitors) also stimulate the phosphorylation and trans-activation of Elk-1, but via the stress-activated MAP kinases Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) or p38 MAP kinase [12,13]. This phosphorylation is responsible, at least in part, for the upregulation of *c-fos* gene expression. In addition, growth factors, but not stress stimuli, stimulate the phosphorylation and activation of Sap1a [14,15]. Growth factors may also regulate the phosphorylation state of c-Fos itself through the MAP kinase-related enzyme, Fos-regulating kinase [16].

Growth factors and other stimuli may both increase the abundance of c-Jun and regulate the phosphorylation state of its transactivation domain on serines 63 and 73. It is generally accepted that the stress-activated kinase, JNK/SAPK, phosphorylates these residues, resulting in the transactivation of c-Jun. In addition, changes in the level of expression of other members of the Jun superfamily could potentially also contribute to activation of the AP-1 complex (e.g. see [17]).

The mechanism by which insulin regulates the AP-1 complex has received much less attention. We have demonstrated previously that the ability of insulin to stimulate AP-1 activity (as judged using the collagenase gene promoter as a reporter) is dependent on the activation of the Erk isoforms of MAP kinase [7]. Quite how MAP kinase regulates the activity of this complex in response to insulin is not known. Insulin is known to up-regulate *c-fos* expression and has been reported to promote changes in the phosphorylation states of c-Jun and c-Fos [6], but

Abbreviations used: AP, activator protein; MAP kinase, mitogen-activated protein kinase; JNK, Jun-N-terminal kinase; SAPK, stress-activated protein kinase; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; DSE, dyad-symmetry element; TRE, tetradecanoyl phorbol 13-acetate response element; EGF, epidermal growth factor; CHO, Chinese hamster ovary; MEK, MAP kinase/extracellular-signal-regulated kinase; MEKK, MEK kinase; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; colTRE, collagenase TRE motif.

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the consequences of these phenomena in regulating the specific activity of the AP-1 complex by insulin have not been investigated. In this study we have undertaken a detailed and systematic analysis of the regulation by insulin of the individual components of the AP-1 complex in Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Materials

Enhanced chemiluminescence reagents were from Amersham International (Little Chalfont, Bucks, U.K.). Stop and Glo® reagents, Tfx-50, and the plasmids pGL3 and pSV40.RL were from Promega Corp. (Madison, WI, U.S.A.). The T669 peptide (KRELVEPLTPSGEAPNQALLR) was synthesized by the Molecular Recognition Centre (University of Bristol, Bristol, U.K.). Antisera towards the Fos family (pan-Fos), Fra1 and Fra2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). 948-4 rabbit anti-chicken c-Jun antibody was raised against the c-Jun C-terminal 82 amino acids. 730-5 rabbit anti-chicken c-Jun was raised against full-length bacterially expressed chicken c-jun. JunD and JunB antisera were as described in [18]. CHO.T cells were maintained and cultured as described previously [7]. Unless otherwise stated all other biochemicals were supplied by Sigma (Poole, Dorset, U.K.) and general-purpose laboratory reagents were of analytical grade and from BDH (Poole, Dorset, U.K.).

Plasmids

The plasmid pCol.Luc was prepared by cloning the 580 bp 5' region (−517 to +63) of the human collagenase (Col) promoter upstream of the firefly luciferase gene in the plasmid pGL3 (Promega) [7]. The plasmid pFos.Luc contained 711 bp of the full-length human c-fos promoter cloned upstream of the firefly luciferase gene in the plasmid pGL3. In the plasmid pFos-SRE.Luc, sequences between −353 and −276, which includes the *Sis* inducible element, SRE and AP-1 sites [11], were deleted. The transcription factor–GAL4 fusion plasmids were pSG424.-Elk1(83–428), pSG424.Elk1(83–428)^{S383A}, pSG424.Sap1a(82–431) and pSG424.cJun(1–104); these were used in conjunction with the luciferase reporter plasmid pGL3.G5E4Δ38, which possesses five GAL4-binding sites upstream of the firefly luciferase gene in the plasmid pGL3 (Promega). pSRE.Luc contains a single copy of the SRE upstream of the Ad2 E4 basal promoter (−38 to +38) in pGL3. pDSE.Luc contains a single copy of a dyad-symmetry element (DSE) in the same context. The DSE binds SRF but does not allow recruitment of TCFs (P. E. Shaw, unpublished work). pcJun.CAT possesses the c-jun promoter (−207 to +192) upstream of the chloramphenicol acetyl transferase (CAT) gene. Expression vectors containing wild-type MAP kinase/extracellular-signal-regulated kinase (MEK), dominant negative MEK (MEK^{S217/221A}) and wild-type CL100 and CL100^{C258S} are all described in [7]. The MEK kinase (MEKK) expression plasmid is described in [19]. It should be noted that whereas CL100 may dephosphorylate the stress-activated protein kinases, these enzymes are not regulated by insulin in CHO.T cells (see below).

Cell extractions, Western-blot analysis and mobility-shift assays

CHO.T cells were grown to approx. 80% confluence in 60 mm diameter dishes using Ham's F12 media supplemented with 5% fetal calf serum/10 mM Hepes (pH 7.2)/2 mM L-glutamine/50 units/ml penicillin/50 µg/ml streptomycin/200 µg/ml G418.

Cells were serum starved for 2 h prior to stimulation with 100 nM insulin. Cell lysates were prepared as described previously [20] and oligonucleotide labelling and mobility-shift assays were performed as described [21]. The oligonucleotides used in this study were AGCTGTGTCTGACTCATGCT (collagenase tetradecanoyl phorbol 13-acetate response element motif, colTRE) and CCCCCACCACGTGGTGCCTGA (CM-1-containing oligonucleotide that binds Myc:Max heterodimers [21]). Crude cell lysates were preincubated with antisera for 30 min if required, prior to incubation for an additional 30 min with the labelled oligonucleotide. The products were separated on a 7% SDS/PAGE gel.

mRNA extraction and Northern blotting

CHO.T cells grown on 60 mm diameter dishes were serum starved for 2 h before stimulation with insulin (100 nM) for the times indicated in the Figure legends. Cells were lysed in Tri reagent (Sigma) and total RNA was purified according to the manufacturer's instructions with the quantity and purity being determined spectrophotometrically. RNA samples (25 µg) were denatured in formamide/formaldehyde/Mops for 15 min at 65 °C and separated on a 0.8% formaldehyde/agarose gel [22]. The RNA was transferred to nylon membrane by capillary action overnight in 20×SSPE [3 M sodium chloride/0.2 M sodium phosphate (pH 7.4)/20 mM EDTA]. In addition, a separate lane was cut before transfer, and stained with ethidium bromide to reveal the 28 S rRNA and 18 S rRNA positions. This allowed approximate sizing of radioactive hybridized bands. The membrane was baked at 80 °C for 2 h and cross-linked (1200 J/cm², Stratalinker) before being pre-hybridized for 4 h at 42 °C in 4×SSPE/5×Denhardtts/0.1% SDS/4.5% formamide/0.2 mg/ml salmon sperm DNA/0.5% dextran sulphate. An Fra1 probe (prepared by reverse transcription-PCR of a 544 bp fragment of CHO Fra1 using the primers: sense 5'-ATGTAC-CGAGACTTCGGGGAACC-3', antisense 5'-GGGCTTCCAGCACCAGCTACCGGC-3') was ³²P-labelled using the Random Prime kit (Boehringer Mannheim, Mannheim, Germany) and hybridized to blots overnight at 42 °C. Blots were washed twice for 10 min 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 PhosphorImage analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Blots were stripped in boiling 1% SDS before subsequent reprobing with a similarly labelled 133 bp β-actin probe (prepared by reverse transcription-PCR of CHO cDNA using the primers: sense 5'-GGTTCCGCTGCCCTG-AGGCAC-3', antisense 5'-CACTGTGTTGGCGTAGAGG-TC-3').

Protein kinase assays

CHO.T cells were grown to approx. 80% confluence in 60 mm diameter dishes. Cells were serum starved for 2 h prior to the addition of insulin (100 nM) for the times indicated. Alternatively, if required, the cells were exposed to UV-C light (0.8 J·m²·s^{−1}) followed by an additional 60 min at 37 °C. Cell lysates were prepared by scraping into 0.5 ml of ice-cold extraction buffer [50 mM β-glycerophosphate (pH 7.4)/1.5 mM EGTA/1 mM benzamidine/1 mM dithiothreitol/0.5 mM Na₃VO₄/0.1 mM PMSF/1 µM microcystin/1 µg/ml each of pepstatin, antipain and leupeptin] and centrifugation at 10000 g for 15 min at 4 °C. The supernatants (20 µl) were assayed in triplicate (50 µl final volume) in 20 mM Mops (pH 7.4)/3 mM EGTA/10 mM MgCl₂/1 µM microcystin/1 µM cAMP-dependent pro-

tein kinase inhibitor peptide/200 μ M T669 peptide (KRELVEPLTPSGEAPNQALLR) as substrate. Reactions were initiated by the addition of [γ - 32 P]ATP (50 μ M; $\approx$ 500 c.p.m./pmol) and terminated after 15 min at 30 °C by spotting a 30 μ l sample on to P81 phosphocellulose paper squares. Papers were washed four times with 150 mM H_3PO_4 and counted in 10 ml of water by the Čerenkov method [23].

JNK was immunoprecipitated using anti-JNK antibodies as described previously [24], and the immunoprecipitates were incubated in the presence of [γ - 32 P]ATP and 5 μ g of glutathione S-transferase (GST)–Jun as substrate [19] for 30 min at 30 °C. The reactions were terminated by the addition of SDS/PAGE sample buffer, boiled for 3 min, then separated on a 10% SDS/PAGE gel. The gel was stained, destained, dried and the 32 P-labelled GST–substrate was detected and quantified using a Molecular Dynamics PhosphorImager.

Cell transfections

Cells were grown to approx. 80% confluence in 12 mm diameter dishes. The cells were washed in PBS and then incubated for 2 h with a mixture of the appropriate DNA and Tfx-50 transfection reagent (2.2 μ l of Tfx50 per μ g of DNA in 5% serum-free Ham's F12). The dishes were further incubated in Ham's F12 containing 5% fetal calf serum for 4 h, then washed with PBS and serum starved for 2 h. The cells were stimulated with 100 nM insulin for the times indicated in the Figure legends.

Reporter gene assays

For luciferase assays, the cells were extracted using Stop and Glo[®] lysis buffer (Promega) and assayed sequentially for the firefly and *Renilla* luciferases according to the manufacturer's instructions (Promega). In brief, 10 μ l of crude cell lysate was incubated with 50 μ l of luciferin reagent (LARII). After 15 s at room temperature the luminescence was recorded for 30 s in a Berthold Lumat LB9501 luminometer. Stop and Glo[®] reagent (50 μ l) was added and the specific luminescence from the *Renilla* luciferase was recorded for an additional 30 s.

CAT assays were performed by extracting cells in 200 μ l of lysis buffer. Lysate (100 μ l) was assayed by ELISA with a commercially available kit (Boehringer Mannheim) according to the manufacturer's instructions. The absorbance was measured at 405 nm with a reference control at 492 nm.

RESULTS

Effect of insulin on AP-1 DNA binding and composition in CHO.T cells

To identify possible mechanisms through which insulin might potentiate AP-1 transcriptional activity, we first examined the effect of insulin on AP-1 DNA-binding activity by gel retardation using a 32 P-labelled oligonucleotide containing the consensus TRE motif from the collagenase gene promoter (colTRE). As shown in Figure 1a, two distinct colTRE-binding complexes were present in CHO cells, both of which were abolished when an excess of specific (colTRE) but not non-specific (CM-1) competitor DNA was included in the binding reaction (Figure 1b). Treatment with insulin led to an increase in the intensity of the more slowly migrating complex but had only a small effect on the lower band (Figures 1a and 1b). The binding activity of the upper complex was increased approx. five-fold over control by 1 h and this elevated level persisted for at least 4 h after stimulation (results not shown), suggesting that insulin increased either the mass of this complex and/or its affinity for DNA.

Figure 1 Components of the insulin-stimulated AP1 complex

(a) Cell lysates (10 μ g of protein) from CHO.T cells treated with insulin for the times indicated (min) were incubated with a 32 P-labelled oligonucleotide containing the colTRE. (b) Lysates from cells, treated in the absence (Basal) or presence of insulin (Insulin) for 60 min, were incubated with radiolabelled oligonucleotide and in the absence (Control) or presence of a 25-fold excess of an oligonucleotide containing the colTRE (TRE) or an unrelated CM-1 sequence (CM-1), or in the presence of anti-c-Jun antiserum 730-5 [c-Jun(1)], anti-c-Jun antiserum 948-4 [c-Jun(2)], anti-JunB, anti-JunD, anti-pan-Fos or non-immune serum, as indicated. (c) Cell lysates from CHO.T cells treated for 60 min with insulin were incubated in the presence of oligonucleotide plus non-immune serum, or anti-Pan-Fos, anti-Fra2 or anti-Fra1 antisera, as indicated. This Figure is representative of three independent experiments. For clarity, the region of the gel containing unbound oligonucleotide probe has been omitted. The arrow indicates the position of the conventional AP-1 complex.

To identify the components of these complexes, antisera specific for individual members of the Fos and Jun families of transcription factors were added to DNA-binding assays prepared using extracts from either untreated cells or cells treated with insulin for 1 h. As shown in Figure 1(b) (upper panel), two independent polyclonal antibodies demonstrated previously to recognize c-Jun [21,25] caused a substantial decrease in the intensity of the more slowly migrating species in untreated cells, whereas antibodies specific for JunB and JunD had little effect. Similar results were obtained using extracts from insulin-stimu-

lated CHO.T cells, except that in this case the upper complex was also substantially diminished by anti-JunB antibodies (Figure 1b, lower panel). The anti-JunB and anti-c-Jun antisera appear equally effective in removing > 50% of the upper band. The reasons for this are not clear, but whereas these antisera have been extensively characterized previously [21,25], we cannot exclude a small degree of cross-reactivity of these sera between hamster c-Jun and JunB.

An antiserum cross-reactive with c-Fos, Fra1 and Fra2 (pan-Fos) resulted in complete disruption of the upper band in both cases, indicating the presence of Fos-related proteins in essentially all of the AP-1 complexes in both treated and untreated CHO.T cells. In contrast, none of the antisera tested had any effect on the faster-mobility complex.

These results demonstrate that the more slowly migrating species represent a conventional AP-1 complex composed of both Jun- and Fos-related components. Furthermore, the composition of this complex was affected by stimulation with insulin; whereas c-Jun is a component both before and after stimulation, JunB is only present after treatment of the cells with insulin. The identity of the lower complex is not known; whereas its binding is prevented by excess TRE, it appears to be regulated by insulin to a significantly lesser extent than the upper band and does not comprise any member of the Jun or Fos family tested.

To further define which members of the Fos family might be present in these complexes, we investigated the effect of antibodies specific for Fra1 and Fra2. As shown in Figure 1(c), antibodies specific for Fra1 substantially abolished binding of the AP-1 complex isolated from insulin-treated cells. In contrast, antibodies specific for Fra2 had no effect, even though these are known to be effective in this assay (D. A. F. Gillespie, unpublished work). These results suggest that Fra1 is the predominant Fos-related component of insulin-stimulated AP-1 complexes in CHO.T cells. The minor population of AP-1 complexes that are non-reactive with Fra1 antiserum may contain c-Fos, since *c-fos* transcription is known to be upregulated by insulin in these cells ([27–29], see also below).

Studies on the regulation of expression of components of the AP-1 complex by insulin

To examine whether insulin altered the expression levels of individual Fos and Jun proteins, we performed Western blotting with specific antisera. An antiserum towards c-Jun showed that insulin caused a large and sustained increase in a protein of apparent molecular mass ≈ 46 kDa (which was absent from blots using pre-immune serum from the same rabbit; results not shown). The migration of this band is consistent with the known molecular mass of c-Jun (Figure 2a) and the presence of c-Jun in AP-1 complexes isolated from insulin-treated cells (Figure 1b). The increase in *c-jun* expression was first apparent at 30 min and peaked at approx. 60–90 min. Sustained levels of c-Jun were still apparent 36 h post-stimulation.

Western-blot analysis of extracts with the pan-Fos antiserum (Figure 2b) demonstrated an increased intensity of four bands. One of these bands initially migrated with an apparent molecular mass ≈ 55 kDa. We propose that this band corresponds to c-Fos, as its transient appearance is entirely consistent with the transient increase in *c-fos* mRNA in these cells, which has been reported previously in response to insulin [28]. A decrease in the electrophoretic mobility of the putative c-Fos band was also observed at the 1–1.5 h time points, an observation which could be explained by a slow increase in c-Fos phosphorylation induced by insulin, perhaps by the Ras-dependent Fos-regulating kinase,

Figure 2 Regulation of expression of members of the Fos and Jun families by insulin

CHO.T cells were incubated for the indicated times (h) and cell lysates (80 μ g) were separated for Western-blot analysis using 730-5 anti-c-Jun (a), anti-pan-Fos (b) or anti-Fra1 (c) antisera. The migration of the molecular-mass markers (kDa) are indicated on the right and the positions of c-Jun, c-Fos and the Fra1 triplet on the left. (d) Cell extracts prepared after the indicated time periods (h) of incubation with insulin (100 nM) were subjected to Northern blotting with probes specific for Fra1 (upper panel) or β -actin (lower panel). All the data shown are representative of three independent experiments.

FRK [16]. This mobility shift was followed by a rapid decrease in the levels of c-Fos.

The c-Fos band was a relatively minor reacting species compared with a triplet of bands of apparent molecular mass ≈ 35 –40 kDa. These proteins are likely to represent Fra1, as shown by Western blotting with an Fra1-specific antiserum (Figure 2c). At least three distinct species of Fra1 were apparent using the Fra1 antiserum, all of which were also seen with the pan-Fos serum. Insulin appears to cause an initial and rapid mobility shift of this protein to the most slowly migrating member of this triplet, an effect which is likely to be a result of its phosphorylation and was maximal within 15 min. This mobility shift then partially reversed during the next 45 min prior to an increase in the intensity of all three bands (Figures 2b and 2c). Again, a similar pattern was observed with both the Fra1 and pan-Fos antisera.

Figure 3 Time course of Erk1/Erk2 MAP kinase activation in CHO.T cells

The activity of the Erk1 and Erk2 isoforms of MAP kinase were measured at various time points after the addition of insulin (100 nM) to CHO.T cells, as described in Materials and methods. The data are expressed as means $\pm$ S.D. of two independent experiments, each of which was performed in triplicate.

Interestingly, the Fra1 mobility shift exhibited a similar, albeit delayed, initial transient time course to the activation profile of the Erk1 and Erk2 MAP kinases; at 2 h the activity of the MAP kinases were still significantly above basal (Figure 3). Gruda et al. [30] have demonstrated previously that Fra1 can be phosphorylated by Erk1/Erk2 MAP kinases and that this causes a reduction in the mobility of Fra1 upon SDS/PAGE. It is possible, therefore, that insulin causes an initial phosphorylation of Fra1 by Erk1/Erk2 leading to its increased DNA-binding activity and thus activation of the AP-1 complex. It is currently difficult to test this hypothesis, as the MEK inhibitor, PD98059, is ineffective at blocking insulin-stimulated Erk1/Erk2 activation in CHO.T cells (results not shown).

The increased expression of Fra1 protein was accompanied by an increase in expression of its mRNA (Figure 2d, upper panel). As with expression of the protein, an increase in *fra1* mRNA was first detectable at between 1 and 1.5 h, was maximal at 2 h, and was sustained at elevated levels for at least 36 h. Although there was also an effect of insulin on β -actin mRNA expression, this was small by comparison, and was not sustained over the 36 h of the experiment (Figure 2d, lower panel). In conclusion, the expression of *fra1* was somewhat slower than that of *c-fos* and was more sustained in nature.

Our results, taken together with those of others, suggest that insulin may cause an initial phosphorylation of pre-existing Fra1, but that in the longer term it causes an increased expression of *c-fos*, *c-jun* and *fra1*. Insulin may also alter the levels of *JunB* expression, but this was not determined directly in this study. We next investigated the mechanism by which insulin induces the expression of some of these proteins.

Mechanism of insulin-stimulated *c-fos* expression

As discussed above, it has been shown previously that insulin promotes a transient increase in *c-fos* transcription. This is the most likely explanation for the transient increase in c-Fos immunoreactive protein observed in our experiments (Figures 1 and 2b). To investigate the mechanism by which insulin stimulates

Figure 4 Activation of the *c-fos* promoter is dependent on the SRE and TCF binding

CHO.T cells were transiently transfected with pRL.SV40 in the presence of pFos.Luc or pFos-SRE (a), and pSRE.Luc or pDSE.Luc (b). After 24 h the cells were serum starved and treated without (open bars) or with insulin (black bars) for a further 8 h. The cells were lysed and the cell lysates assayed sequentially for the presence of firefly and *Renilla* luciferases. The data are expressed as the activity of the firefly luciferase relative to *Renilla* luciferase activity, and are the means $\pm$ S.E.M. of three independent experiments, each of which was performed in triplicate.

c-fos transcription, CHO.T cells were transfected with a plasmid placing the firefly luciferase cDNA under the control of the human *c-fos* promoter. Insulin caused an eight-fold increase in relative firefly luciferase expression after 8 h (Figure 4a) and this induction was decreased by approx. 75%, when the SRE within this promoter was deleted to prevent binding of the SRF (plasmid pFos-SRE.Luc; Figure 4a). This suggests that insulin mediates its effect on the *c-fos* promoter at least in part via the SRE.

We next transfected cells with a plasmid where the firefly luciferase gene was placed under the control of a minimal promoter possessing a single SRE (the plasmid pSRE.Luc). Insulin stimulated the activity of this promoter approx. five-fold (Figure 4b), an effect that was completely blocked by mutating the residues that comprise the Ets motif, preventing the binding of the TCF(s) but not SRE (the plasmid pDSE.Luc; Figure 4b). This suggests that insulin stimulates the *c-fos* promoter primarily through activation of the TCF(s) that bind the Ets motif. However, additional SRE-independent mechanism(s) may also contribute to the activation of the *c-fos* promoter (Figure 4a).

Elk-1 and Sap-1 are related TCFs that bind to the Ets motif of the *c-fos* promoter SRE. Both of these factors are phosphorylated by the Erk1 and Erk2 isoforms of MAP kinase [14,31]. We next investigated whether these factors were activated by insulin and,

Figure 5 Activation of the *c-fos* promoter by insulin requires MAP kinase activation and is accompanied by the activation of the TCFs Sap1a and Elk1

(a) CHO.T cells were transiently transfected with pRL.SV40 and pGL3.G5E4 Δ 38 in the presence of pSG424.Sap1a (Sap1a), pSG424.ElK1 (82-431) (Elk1) or pGAL4.ElK1^{S383A} (Elk1*) and with or without constitutively active MEK (stippled bars) as indicated. (b) CHO.T cells were transiently transfected with pRL.SV40 and pFos.Luc in the presence of empty vector (vector), or vector containing the MAP kinase phosphatase CL100 (CL100), a phosphatase-inactive CL100^{C258S} (CL100*), wild-type MEK (MEK) or dominant negative MEK (MEK*) as indicated. In a and b the cells were serum starved and treated without (open bars) or with insulin (filled bars) for 24 h. The cells were then lysed and the lysates assayed sequentially for the presence of firefly and *Renilla* luciferases. The data are expressed as the activity of the firefly luciferase relative to *Renilla* luciferase activity, and are the means $\pm$ S.E.M. of three independent experiments, each of which was performed in triplicate.

if so, whether this was mediated by the Erk isoforms of MAP kinases. As shown in Figure 5(a), insulin promoted a substantial activation of both GAL4-Elk1 and GAL4-Sap1a fusion proteins. In the case of Elk1, this activation was dramatically reduced by mutagenesis of serine 383, a target for phosphorylation by MAP kinase [11]. The effect of insulin was equivalent to or better than that induced by a constitutively active MAP kinase kinase (MEK^{S217/221E}), a potent activator of the Erk isoforms of MAP kinases.

As we have shown previously that insulin-stimulated AP-1 activation in CHO.T cells is dependent on the activation of the Erk isoforms of MAP kinase, we investigated whether this was also true for the induction of the *c-fos* promoter. As shown in Figure 5(b), over-expression of the MAP kinase phosphatase CL100 (either the wild-type or a C^{258S} mutant form, which is

phosphatase-inactive) or a dominant negative MAP kinase kinase, MEK^{S217A/221A}, blocked insulin stimulation of the human *c-fos* promoter. The phosphatase-inactive CL100 acts by binding very tightly to MAP kinase and preventing substrate access to the active site [7,32]. Over-expression of CL100 or dominant negative MEK also blocked the activation of a GAL4-Elk1 fusion protein (M. R. Griffiths and J. M. Tavaré, unpublished work).

Taken together, these data suggest that the ability of insulin to stimulate *c-fos* expression is at least in part the result of the phosphorylation and activation of the TCFs Elk1 and/or Sap1a, which bind to the Ets-motif within the *c-fos* promoter.

Regulation of *c-jun* transcription and transactivation by insulin

To investigate the possibility that insulin increases the expression of *c-jun* by increasing gene transcription, we determined whether insulin had any effect on the activity of the *c-jun* promoter. CHO.T cells transiently transfected with a *c-jun* promoter-CAT construct were treated with and without insulin, and the level of CAT expression determined by ELISA assays of resulting cell lysates. Insulin had no apparent effect on CAT activity (0.24 ± 0.03 compared with 0.25 ± 0.002 in the control; expressed as absorbance units $\pm$ S.D. for a representative experiment performed in triplicate). On the other hand, over-expression of a constitutively active MEKK (which is an upstream activator of JNK/SAPK) had an approx. four-fold effect (1.06 ± 0.02 absorbance units). The results suggest, therefore, that the increase in *c-jun* expression due to insulin treatment may be the result of an increase in mRNA translation and/or stability.

We also investigated whether the increase in AP-1 activity could also be due, in part, to transactivation of c-Jun through phosphorylation of serines 63 and 73 by the MAP kinases. Our data strongly argue against this possibility for several reasons. First, a GAL4-Jun(1-104) fusion protein was poorly activated (if at all) by insulin [0.64 ± 0.01 compared with 0.29 ± 0.02 in the control; luciferase:*Renilla* ratio (mean $\pm$ S.D.) in a representative experiment performed in triplicate] under conditions where it was activated almost 10-fold by a constitutively active MEKK (5.56 ± 0.25). Secondly, immune-complex assays of JNK (the most likely MAP kinase that phosphorylates c-Jun on serines 63 and 73), using purified GST-Jun as substrate, showed that this kinase was not activated by insulin under conditions where it was activated by UV irradiation (M. R. Griffiths and J. M. Tavaré, unpublished work). Thirdly, a c-Jun gel mobility shift, indicative of phosphorylation on serines 63 and 73, was not observed in response to insulin (Figure 2a).

DISCUSSION

Insulin stimulates the activity of the AP-1 complex in several cell types. We have demonstrated previously that the ability of insulin to stimulate the AP-1 complex that binds to the colTRE, is dependent on the activation of the Erk isoforms of MAP kinase [7]. However, previous studies have not addressed the identity of the components involved in the insulin-stimulated AP-1 complex, nor how they are themselves regulated by these MAP kinases.

In the current study we used a combination of Western-blot analysis and gel-retardation assays to demonstrate that the insulin-stimulated AP-1 complex of CHO.T cells is composed predominantly of c-Jun, JunB and Fra1 (Figure 1).

Insulin caused a rapid but transient increase in the expression of protein that has an appropriate molecular mass (55 kDa) for c-Fos (Figures 1c and 2b). This observation is consistent with

previous studies in this cell type where insulin was shown to increase the transcription of *c-fos* mRNA in a similarly transient manner and to stimulate the activity of a transiently transfected CAT reporter gene under the control of the *c-fos* promoter [28]. Fra1 was a relatively abundant component of the basal AP-1 complex; moreover, we found that insulin not only caused a dramatic increase in its amount in the cells (Figures 2b and 2c), but a rapid and transient retardation in its electrophoretic mobility upon SDS/PAGE analysis (Figures 2b and 2c). Indeed, the activation of the Erk isoforms of MAP kinases followed a rather similar transient time course (Figure 3). Gruda et al. [30] have demonstrated previously that Fra1 can be phosphorylated by MAP kinase and that this causes a reduction in the mobility of Fra1 upon SDS/PAGE [30]. On the basis of these observations we suggest that insulin causes an initial phosphorylation of Fra1 by MAP kinase, leading to its increased DNA-binding activity and thus activation of the AP-1 complex. This is followed by a decreased phosphorylation in parallel with the reduction in MAP kinase activity, observed during a phase of increased Fra1 synthesis (Figure 2c). Insulin induced a profound increase in *fra1* mRNA (Figure 2d), suggesting that the sustained phase of Fra1 protein synthesis is due to increased transcription, although we cannot completely exclude a role for an increase in *fra1* mRNA stability or translation.

We have not established how insulin stimulates Fra1 synthesis. However, it is of interest to note that the first intron of the *fra1* gene possesses a consensus AP-1 element [33]. Consistent with a possible role for the AP-1 complex, we found that the increase in *c-fos* and *c-jun* expression (Figures 2a and 2b), which was seen within 60 min of insulin treatment, precedes the increase in *fra1* expression, which was first apparent only at 90 min (Figures 2b and 2c).

Mohn et al. [34] have reported previously that insulin increases *c-jun* mRNA in Reuber H35 cells. In our studies, whereas insulin induced *c-jun* expression, it did not appear to stimulate CAT expression from a transiently transfected *c-jun* promoter–CAT reporter construct. Although this suggests that insulin may regulate *c-jun* translation or mRNA stability, rather than stimulating *c-jun* gene transcription, unequivocal proof would require more detailed analysis using nuclear run-on assays. A c-Jun–ATF2 complex is known to bind to the *c-jun* promoter TRE in the basal state [35]. However, a lack of stimulation of the *c-jun* promoter is also consistent with the inability of insulin to stimulate either the c-Jun or ATF2 transactivation domains when fused to the yeast transcription factor DNA-binding domain (M. R. Griffiths and J. M. Tavaré, unpublished work). Possibly, other transcriptional complexes are involved that bind elsewhere on the *c-jun* promoter.

JunB was also a component of the AP-1 complex that bound the colTRE (Figure 1b), but we have not investigated whether insulin also regulates *JunB* expression levels. Interestingly, Mohn et al. [34] have reported previously an increase in *JunB* mRNA upon insulin treatment of Reuber H35 cells. It is also of interest to note that in some cell types over-expression of JunB transrepresses the collagenase promoter under conditions where *c-jun* overexpression transactivates [17]. It remains to be established what role JunB plays in the regulation of AP-1 complex activity by insulin.

We investigated the mechanism by which insulin activates the *c-fos* promoter in these cells. Our data demonstrate that the effect of insulin was blocked upon deletion of the region of the *c-fos* promoter containing the SRE (Figure 4a). Insulin also stimulated the activity of a minimal promoter possessing a single SRE sequence (Figure 4b) and this effect was completely eliminated upon mutagenesis of the Ets motif, which prevents the binding of

the TCFs Elk1 and Sap1a. Our studies are consistent with those of several other groups using serum and growth factors as stimuli [36–38] but are hard to reconcile with those of Thompson et al. [29], who found that mutations in the TCF-binding sites of a *c-fos* promoter did not appear to affect the ability of insulin to stimulate CAT reporter expression. However, in their studies, also on CHO.T cells, mutations that blocked SRF binding to the *c-fos* promoter did not block insulin-stimulated CAT expression. It is possible, therefore, that in the studies of Thompson et al. [29] on CHO.T cells, much of the insulin effect on the *c-fos* promoter could have been mediated by a transcriptional complex(es) separate from SRF/TCF.

That stimulation of luciferase expression from a minimal promoter possessing an SRE is TCF-dependent suggests that insulin may regulate the TCF Elk1 and/or Sap1a. We confirmed that this was the case using fusions between the transactivation domains of these factors and the yeast GAL4 DNA-binding domain (Figure 5a). The activation of Elk1 was largely dependent on the presence of the MAP kinase consensus phosphorylation site serine 383 (Figure 5a). We also demonstrated that dominant negative MEK and a MAP kinase phosphatase, CL100, both blocked the activation of the human *c-fos* promoter (Figure 5b). Taken together, we propose that the mechanism by which insulin stimulates the *c-fos* promoter is primarily via phosphorylation and activation of Elk1 and/or Sap1a by the Erk1 and Erk2 isoforms of MAP kinases. Elk1 can also be phosphorylated and activated by JNK/SAPK; however, this cannot contribute to the insulin effect in CHO.T cells as JNK/SAPK was not activated, nor can JNK/SAPK activate a GAL4–Sap1a fusion [15].

In conclusion, we propose that insulin stimulates the activity of the AP-1 complex primarily through a change in the abundance of the complex and its components. Insulin upregulates the expression of *c-jun*, as well as *c-fos* and *fra1*. c-Fos induction is mediated via the activation of the Erk isoforms of MAP kinases and probably their ability to phosphorylate and activate the TCFs Elk1 and Sap1a. Insulin also promotes a rapid and transient phosphorylation of Fra1, which may itself be mediated by Erk1 and Erk2, resulting in Fra1 transactivation. However, this remains to be established directly. Finally, insulin does not modulate the activity of c-Jun by phosphorylation. Further studies are required to investigate the precise contribution that each of these changes makes towards the activation of the collagenase promoter.

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