Insulin regulation of mitogen-activated protein kinase kinase (MEK), mitogen-activated protein kinase and casein kinase in the cell nucleus: a possible role in the regulation of gene expression

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After insulin receptor activation, many cytoplasmic enzymes, including mitogen-activated protein (MAP) kinase, MAP kinase kinase (MEK) and casein kinase II (CKII) are activated, but exactly how insulin signalling progresses to the nucleus remains poorly understood. In Chinese hamster ovary cells overexpressing human insulin receptors [CHO(Hirc)], MEK, CKII and the MAP kinases ERK I and ERK II can be detected by immunoblotting in the nucleus, as well as in the cytoplasm, in the unstimulated state. Nuclear localization of MAP kinase is also observed in 3T3-F442A adipocytes, NIH-3T3 cells and Fao hepatoma cells, whereas MEK is found in the nucleus only in Fao and CHO cells. Insulin treatment for 5–30 min induces a translocation of MEK from the cytoplasm to the nucleus, whereas the MAP kinases and CKII are not translocated into the nucleus in response to insulin during this period. However, nuclear MAP kinase and CKII activities increase by 2–3-fold within 1–10 min after stimulation with insulin. By using gel-shift assays, it has

been shown that insulin also stimulates nuclear protein binding to an AP-1 site with kinetics similar to MEK translocation and MAP kinase and CKII activation. Treatment of the extracts *in vitro* with protein phosphatase 2A or treatment of the intact cells with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, a cell-permeable inhibitor of CKII, almost completely blocks the insulin-induced DNA-binding activity, whereas incubation of cells with a MEK inhibitor produces only a slight decrease. These results suggest that insulin signalling results in the activation of serine kinases in the nucleus via two pathways: (1) insulin stimulates the nuclear translocation of some kinases, such as MEK, which might directly phosphorylate nuclear protein substrates or activate other nuclear kinases, and (2) insulin activates nuclear kinases without translocation. The latter is true of CKII, which seems to regulate the binding of nuclear proteins to the AP-1 site, possibly by phosphorylation of AP-1 transcription factors.

INTRODUCTION

After insulin binding to and activation of its receptor tyrosine kinase, a number of cytoplasmic protein serine/threonine kinases including mitogen-activated protein (MAP) kinase, MAP kinase kinase (MEK), S6 kinase and casein kinase II (CKII) are activated, resulting in the phosphorylation of many cytosolic and nuclear proteins [1–5]. These kinases are known to play a role in many metabolic process and are essential in regulating cell growth, differentiation and gene expression [6–10]. The mechanisms by which the signals generated by these insulin-stimulated cytosolic serine kinases progress to the nucleus remain elusive. However, work from our laboratory and others suggests that insulin induces the phosphorylation of a number of nuclear proteins such as nucleolin, lamin, c-Jun, c-Fos and CREB on serine residues, and pp82, a Fos-related protein, on tyrosine residues [1,2,11,12]. Insulin also causes nuclear translocation of the insulin receptor and activates nuclear tyrosine kinase activity [13,14].

More recently it has been found that some MAP kinase might be located in the nucleus in quiescent HeLa cells, chicken embryo fibroblasts and mouse 3T3 fibroblasts, and on stimulation by serum or phorbol 12-myristate 13-acetate additional cytosolic MAP kinase is translocated to the nucleus [15–17]. Furthermore a number of potential nuclear substrates for the MAP kinase

have been identified, such as transcription factors of the AP-1 complex (c-Jun [18] and c-Fos [15]), c-Myc [19], NF-IL6 [20], ATF-2 [21], TAL1 [22] and p62^{TCF}/ELK-1 [23]. It has also been suggested that AP-1 transcription factors associate with MAP kinase-related proteins *in vivo* [24].

Activation of MAP kinase requires phosphorylation on Tyr-185 and Thr-183 by an upstream dual-specific protein kinase, MEK [25]. Full activation of MEK also requires phosphorylation by an upstream protein kinase on its serine and threonine residues. Several serine/threonine kinases have been shown to phosphorylate and activate MEK, including MEK kinase, c-Raf and c-Mos [26–30]. Although the subcellular distribution of MEK has not been fully elucidated, it has been reported that MEK might be localized exclusively in the cytoplasm of several cell lines and that treatment with epidermal growth factor and phorbol 12-myristate 13-acetate does not induce translocation of MEK into the nucleus [31].

CKII is another important serine/threonine protein kinase that is present in the nucleus. CKII has been shown to phosphorylate many nuclear growth-related proteins including c-Jun, leading to the proposal that CKII has a role in cell growth and gene expression mediated by insulin [32–34]. CKII exists as a heterotetramer consisting of α , α' and β subunits (42, 38 and 28 kDa respectively) in either $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ configuration [32]. The mechanism of CKII activation by insulin is poorly under-

Abbreviations used: CHO(Hirc), Chinese hamster ovary cells overexpressing human insulin receptors; CKII, casein kinase II; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; DTT, dithiothreitol; ERK, MAP kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase kinase; PP2A, protein phosphatase 2A.

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stood; however, one study has suggested that HSP90 interacts with CKII resulting in the activation of the kinase activity [35].

In the present study we investigated the activity and possible translocation of MAP kinase, MEK and CKII to the nucleus after stimulation with insulin, and have correlated these with the stimulation of the DNA-binding activity of AP-1 by insulin. Our results show that insulin induces nuclear translocation of MEK and stimulates nuclear MAP and casein kinases without translocation. The latter seems to be important in the regulation of the DNA-binding activity of AP-1.

MATERIALS AND METHODS

Materials

Cell culture medium was provided by the Joslin Tissue Culture Core Facility. Pig insulin was obtained from Elanco Products Co. (Indianapolis, IN, U.S.A.). The reagents used for PAGE were from Bio-Rad. Nitrocellulose filters (0.45 μ m) were purchased from Schleicher and Schuell. Antibody to the MAP kinase was kindly provided by Dr. John Blenis (Harvard Medical School, Boston, MA, U.S.A.) or purchased from Transduction Laboratory. Antibodies to CKII α , α' and β were kindly provided by Dr. David Litchfield (Manitoba Institute of Cell Biology, Winnipeg, Canada). MEK inhibitor (compound PD 098059) was a gift from Dr. Alan Saltiel (Parke-Davis Pharmaceutical Co., Ann Arbor, MI, U.S.A.). Protein phosphatase 2A (PP2A) was purchased from Upstate Biotechnology. The AP-1 oligonucleotides were synthesized in the Joslin Diabetes Center DNA Core Facility with the sequences as follows: sense, 5'-GATCCATCGTGACTCAGCGATCTCGTGACTCAGCGG-3'; anti-sense, 3'-GTAGCACTGAGTCGCTAGAGCACTGAGTCGCCCTAG-5'. The single-stranded oligonucleotides were annealed to generate a double-stranded AP-1 probe. All other chemicals were purchased from Sigma.

Cell culture

Chinese hamster ovary cells overexpressing human insulin receptors [CHO(Hirc)] cells were grown in F-12 media with 10% (v/v) fetal calf serum. Fao cells were grown in RPMI-1640 media with 10% fetal calf serum, and NIH-3T3(IR) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. These cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C. 3T3-F442A cells were grown in DMEM with 10% calf serum in a humidified 5% CO₂ atmosphere at 37 °C. For differentiation of 3T3-F442A cells to adipocytes, the medium was changed to DMEM supplemented with 10% fetal calf serum and the cells were grown in an atmosphere with 10% CO₂ for 10 days. Insulin (5 μ g/ml) was added for the first 8 days to accelerate the differentiation. Before experiments, insulin was removed from the culture medium for at least 48 h.

Isolation of nuclei

Nuclei were isolated as described previously [36,37]. Cells were serum-starved for 18 h and stimulated with 100 nM insulin for the indicated durations. Medium was removed and cultures were washed with ice-cold PBS. The cells were scraped into a buffer containing 20 mM Hepes, 50 mM MgCl₂, 25 mM KCl, 2 mM PMSF, 0.1 mg/ml aprotinin, 1 mM sodium vanadate, 1 mM sodium molybdate, 10 mM β -glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA and 0.25 M sucrose. Cells were disrupted by passing them five times through a 1 ml tuberculin syringe with a microfine IV needle. The cell homogenates were transferred to thick-walled polyallomer tubes and

centrifuged at 1000 *g* for 10 min at 4 °C. The pellets were resuspended in 1 ml of isolation buffer; 2 ml of isolation buffer supplemented with 1.6 M sucrose was layered below. After centrifugation at 100000 *g* for 35 min at 4 °C, the resulting pellets were subjected to a brief sonication at 4 °C to disrupt the nuclei and then stored at -70 °C.

Measurements of the activity of lactate dehydrogenase, a cytosolic marker enzyme, and the content of DNA in the nucleus and postnuclear preparations indicated that 95% of total lactate dehydrogenase was found in the postnuclear preparation, whereas 97% of the total DNA in the cell was recovered in the nuclear preparation.

MAP kinase and CKII assays *in situ*

Kinase assays in SDS/polyacrylamide gel were performed as described previously [38]. Samples were applied to SDS/polyacrylamide gel containing either myelin basic protein (MBP) or dephosphorylated casein at a concentration of 0.5 mg/ml. After electrophoresis the gel was incubated in a solution containing 20% 2-propanol and 50 mM Tris/HCl, pH 8.0, and subsequently equilibrated in a buffer containing 50 mM Tris/HCl, pH 8.0, and 5 mM 2-mercaptoethanol. The gels were subjected to denaturation by incubation in 6 M guanidinium chloride followed by renaturation in a buffer containing 50 mM Tris/HCl, pH 8.0, 0.04% Tween-20 and 5 mM 2-mercaptoethanol. The gel was then preincubated for 1 h at 23 °C in a buffer containing 40 mM Hepes, pH 8.0, 0.5 mM EGTA and 10 mM MgCl₂. The kinase reactions were performed in 10 ml of the same buffer containing 40 μ M ATP and [γ -³²P]ATP (50 μ Ci) for 1 h at 23 °C. The kinase reaction was stopped by washing the gel in a solution containing 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. The gel was dried and subjected to autoradiography. For some experiments, the nuclear and postnuclear preparations were first heat-denatured in the presence of 1% SDS and immunoprecipitated with anti-MAP kinase antibody (Transduction Laboratory). The immunocomplex was precipitated with Pansorbin and used to measure MAP kinase activity as described above.

Measurement of CKII activity with CKII peptide

CKII activities were measured as previously described [33]. Nuclear or postnuclear preparation (10 μ g) was mixed with a buffer containing 0.1 mM CKII peptide (Arg-Arg-Arg-Glu-Glu-Thr-Glu-Glu-Glu), 50 mM Tris/HCl, pH 7.6, 100 mM NaCl, 10 mM MgCl₂ and 0.1 mM [γ -³²P]ATP. The reaction mixture was allowed to incubate for 10 min at 30 °C. The phosphorylation reaction was terminated by spotting the assay mixture on Whatman p81 papers. After extensive washes with 150 mM phosphoric acid, the papers were dried and subjected to liquid-scintillation counting.

Gel-shift assay

Nuclei were isolated as previously described [39]. Briefly, cells were scraped into a buffer containing 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.75 mM spermidine, 0.15 mM spermine and 10 mM Hepes, pH 7.9. The cell suspension was kept for 15 min on ice and passed eight times through a microfine IV needle followed by vigorous vortex-mixing for 10 s. The homogenate was centrifuged for 30 s in a Microfuge at 4 °C. The resulting pellet was resuspended in 300 μ l of ice-cold buffer containing 10% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA, 1 mM PMSF, 0.2 mM EGTA and 20 mM Hepes, pH 7.9,

and rotated vigorously at 4 °C for 30 min on a shaking platform, followed by centrifugation for 5 min in a Microfuge at 4 °C. The supernatant was stored in aliquots at -70 °C until use. Gel-shift assays were performed as previously described [40]. Nuclear extract (5 μ l) was mixed with 5 μ l of buffer A [20 mM Hepes (pH 7.8)/10 % glycerol/0.2 mM EDTA/0.5 mM DTT] and 10 μ l of reaction mixture containing ^{32} P-labelled AP-1 (25000 c.p.m.), 1 μ l of 2 μ g/ml poly(dI)·poly(dC), 2 μ l of 50 % glycerol, 2 μ l of 10 $\times$ mobility shift buffer (100 mM Hepes (pH 7.8)/10 mM spermidine), 2 μ l of 2 % NP-40 and 1 μ l of 200 mM DTT, and incubated for 15 min at 23 °C. The reaction mixture was loaded on a 6 % (w/v) polyacrylamide gel and subjected to electrophoresis at 150 V. The gel was then dried and subjected to autoradiography.

Western blot analysis

Electrotransfer of proteins from the gels to nitrocellulose paper (Schleicher and Schuell) was performed for 1 h at 100 V (constant) as described by Towbin et al. [41]. The filter papers were preincubated for 1 h at 23 °C with PBS containing 0.1 % Tween-20 and 3 % (w/v) BSA and washed with PBS containing 0.1 % Tween-20 three times for 10 min each. The blots were probed with primary antibodies at the indicated dilution for 1 h at 23 °C, then washed with PBS containing 0.1 % Tween-20 three times for 10 min each at 23 °C. The blots were then incubated with horseradish peroxidase-conjugated anti-(rabbit IgG) for 30 min and washed with PBS containing Tween-20 five times for 10 min each. The detection of immobilized specific antigens was carried out by enhanced chemiluminescence (NEN).

Measurement of lactate dehydrogenase activity

Phosphate buffer, pH 7.5 (1 ml, 100 mM), 0.1 ml of 10 mM pyruvic acid, 25 μ l of 5 mg/ml NADH and 10 μ l of nuclear and postnuclear preparations were added to a cuvette, followed by measurements of the decreasing absorbance at 370 nm. The enzyme activity was calculated as $\Delta A_{370}/\text{min}$ per mg of protein.

Measurement of DNA

DNA content was measured by fluorimetric assay, essentially as described by Hopcroft et al. [42]. Nuclear or postnuclear preparation (40 μ l) was mixed with 10 μ l of a fluorochrome reagent Hoechst 33258 (10 mg/ml), and 1 ml of a buffer containing 0.05 M Na_2HPO_4 , 2 M NaCl and 2 mM EDTA, pH 7.4. A standard curve was prepared with herring sperm DNA. Relative fluorescence intensity was measured with a DNA fluorimeter, model TKO 100, from Hoefer Scientific Instruments.

RESULTS

Identification of MAP kinase, CKII and MEK in nuclear extracts

To determine whether MEK, MAP kinase and CKII were localized in the nucleus or might undergo translocation in response to insulin, Western blotting analysis of nuclear and postnuclear supernatants was performed with specific antibodies to each of these enzymes. All the cell lines tested contained a significant amount of MAP kinase (ERK I and ERK II) in the nucleus in the unstimulated state (Figure 1A), ranging from approx. 5 % in 3T3-F442A cells to 47 % in Fao hepatoma cells on the basis of blotting of equal protein concentrations (Table 1). In all but one case the levels of MAP kinase exceeded the 5 % estimate of maximal contamination of cytosolic enzymes in the nuclear fraction on the basis of LDH activity (see the Materials

Figure 1 Identification of MAP kinase, CKII and MEK in the nucleus

After treatment with insulin (100 nM) for the indicated durations, CHO(Hirc) cells were subjected to subcellular fractionation as described in the Materials and methods section. Equal amounts (20 μ g) of nuclear and postnuclear fractions were applied to a SDS/polyacrylamide gel, followed by electrophoresis and transfer to nitrocellulose membranes. The membranes were probed with (A) anti-MAP kinase, (B) anti-CKII or (C) anti-MEK antibody and detected with an enhanced chemiluminescence system in accordance with the protocol provided by the manufacturer (ICN). The content of MEK in the nucleus and postnuclear supernatant was quantified by scanning densitometry (D). The results are expressed as means $\pm$ S.E.M. for three separate experiments (* $P < 0.05$). Abbreviation in (D): ', min.

Table 1 Nuclear content of ERKs, MEK and CKII

CHO(Hirc), NIH-3T3(IR) and Fao cells and 3T3-F442A adipocytes were subjected to subcellular fractionation as described in the Materials and methods section. Nuclear and postnuclear fractions (20 μ g) were applied to an SDS/polyacrylamide gel, followed by electrophoresis and transfer to nitrocellulose membranes. The membranes were probed with anti-MAP kinase, anti-CKII or anti-MEK antibody and detected with an enhanced chemiluminescence system in accordance with the protocol provided by the manufacturer (ICN). The content of each kinase in the nucleus and postnuclear supernatant was quantified by scanning densitometry, and is expressed as a percentage of the total: $100 \times [(\text{nuclear}/(\text{postnuclear} + \text{nuclear}))]$. Abbreviation: n.d., not determined.

	Nuclear content (% of total)					
	ERK		MEK	CKII		
	I	II		α	α'	β
CHO(Hirc)	46 $\pm$ 3	38 $\pm$ 5	37 $\pm$ 7	30 $\pm$ 4	42 $\pm$ 11	28 $\pm$ 7
NIH-3T3-F442A adipocytes	12 $\pm$ 2	5 $\pm$ 1	0	n.d.	n.d.	n.d.
NIH-3T3(IR)	11 $\pm$ 4	10 $\pm$ 4	0	n.d.	n.d.	n.d.
Fao hepatoma	47 $\pm$ 3	43 $\pm$ 2	39 $\pm$ 1	n.d.	n.d.	n.d.

Figure 2 Activation of nuclear MAP kinase by insulin

After stimulation with insulin for the indicated durations, CHO(Hirc) cells were subjected to subcellular fractionation. Equal amounts (20 μ g) of nuclear or postnuclear fractions were applied to a 10% (w/v) polyacrylamide gel containing MBP (0.5 mg/ml) and subjected to electrophoresis. The gel was then incubated with a kinase reaction buffer in the presence of [γ - ^{32} P]ATP for 1 h as described in the Materials and methods section. After extensive washes, the gel was dried and subjected to autoradiography (A). Quantification of MAP kinase activity was performed by scanning densitometry (B). For MAP kinase activity, the nuclear and postnuclear fractions were first heat-denatured at 100 $^{\circ}$ C for 5 min in the presence of 1% (w/v) SDS and subjected to immunoprecipitation with anti-MAP kinase antibody (Transduction Laboratory). The immunocomplex was precipitated with Pansorbin, followed by an MBP-containing gel kinase assay *in situ* as described above. MAP kinase activity was detected by autoradiography (C). Abbreviation in (B): ', min.

and methods section). Stimulation with insulin for 30 min had little effect on the content of either ERK I or ERK II in the nuclear and postnuclear preparations in CHO(Hirc) cells (Figure 1A), 3T3-F442A adipocytes, NIH-3T3(IR) or Fao cells (results not shown). Significant quantities (28–42%) of CKII α , α' and β subunits were also present in the nucleus of CHO(Hirc) cells before stimulation with insulin by immunoblotting with specific antibodies (Table 1), and insulin did not alter the subcellular distribution of this enzyme (Figure 1B).

The situation for MEK was somewhat different. As with the ERKs and CKII, 37% of total MEK was localized in the nucleus in CHO(Hirc) cells in the basal state (Table 1). However, in this case, stimulation with insulin for 1–30 min resulted in an increase in the concentration of MEK in the nucleus (Figure 1C). The maximum increase in nuclear translocation of MEK was observed

Figure 3 Regulation of nuclear CKII activity by insulin

Nuclear and postnuclear fractions were isolated after the stimulation of CHO(Hirc) cells with insulin. Equal amounts (10 μ g) of each fraction were subjected to either filter paper CKII assays (A) or casein containing-gel kinase assays *in situ* (B) as described in the Materials and methods section. The results presented in (A) are expressed as means $\pm$ S.E.M. For the identification of CKII, both nuclear and postnuclear preparations were subjected to electrophoresis, followed by Western blotting analysis with anti-CKII antibodies (α , α' and β) and detected by enhanced chemiluminescence.

at 30 min and was approx. 75% over the basal level (Figure 1D). The increase in MEK content in response to insulin in the nucleus could be accounted for by a corresponding decrease in MEK concentration in the postnuclear preparation (Figure 1C). These results are consistent with the hypothesis that MEK translocates from cytoplasm to the nucleus in response to insulin, whereas insulin has little acute effect on the subcellular distribution of MAP kinase and CKII.

Interestingly, the distribution of MEK varied from cell type to cell type (Table 1). In the 3T3-F442A adipocytes and NIH-3T3(IR) cells, MEK was located exclusively in the cytoplasm, whereas in Fao hepatoma cells a large amount of MEK (39%) was present in the nucleus, as well as in the postnuclear preparation. In these cell types insulin did not cause any significant change in the subcellular distribution of MEK (results not shown).

Activation of nuclear MAP kinase

To measure MAP kinase activity in nuclear and postnuclear fractions, an MBP-containing gel kinase assay was used *in situ*. In both nuclear and postnuclear preparations MBP kinase activity was detected overlying a 44 kDa protein band that corresponded to ERK I and was stimulated by insulin (Figure 2A). As expected, the activity of cytosolic MAP kinase was stimulated 7-fold within 1 min of insulin treatment and decreased slowly to 4.5-fold over basal levels at 30 min (Figures 2A and 2B). Somewhat surprisingly, the nuclear MAP kinase activity was also stimulated within 1 min of insulin treatment and then decreased slowly to basal levels. Quantification by scanning densitometry revealed that insulin stimulated nuclear MAP kinase activity more than 3-fold at 1 min. In contrast with the

Figure 4 Stimulation of DNA binding activity of AP-1 by insulin

CHO(Hirc) cells were stimulated with 100 nM insulin for 1–30 min, after which nuclei were isolated for the gel-shift assay as described in the Materials and methods section. In some cases this followed a preincubation with a MEK inhibitor (50 μM) for 1 h or DRB (1 mM) for 10 min. The nuclear preparations (5 μg) were incubated with a radiolabelled AP-1 oligonucleotide for 30 min at 23 °C, followed by electrophoresis in a 6% (w/v) native polyacrylamide gel as described in the Materials and methods section. The protein–DNA complex was identified by autoradiography (A). Quantification of the stimulation of the DNA-binding activity of AP-1 by insulin was performed by scanning densitometry (B). The data are expressed as means $\pm$ S.E.M. for three separate experiments (* $P < 0.05$; ** $P < 0.01$).

cytosolic MAP kinase activity, nuclear activity showed a rapid decline to basal levels by 30 min (Figure 2B).

To confirm that the 44 kDa protein detected in Figure 2A was ERK I, we performed immunoprecipitation of denatured nuclear extracts with anti-MAP kinase antibody before the gel kinase assay *in situ*. Only the 44 kDa protein was immunoprecipitated with the antibody, indicating that it was ERK I (Figure 2C). Because there was no detectable translocation over this time course, the activation of MAP kinase in the nucleus by insulin must be due to the activation of a resident nuclear MAP kinase (possibly by translocation of MEK), rather than the translocation of activated cytosolic MAP kinase to the nucleus in response to insulin.

Activation of CKII by insulin

Insulin effects were even more striking on nuclear CKII. Thus insulin stimulated nuclear CKII activity by 75% within 1–10 min but had little effect on the postnuclear CKII activity over the same period (Figure 3A). The activation of nuclear CKII by insulin was also evident in a casein-containing gel kinase assay *in*

Figure 5 Insulin-induced increase in the DNA-binding activity of AP-1 is mediated by serine phosphorylation

CHO(Hirc) cells were stimulated with 100 nM insulin for 1–30 min, after which nuclei were isolated for the gel-shift assay as described in the Materials and methods section. The nuclear preparations (5 μg) were treated with or without PP2A (0.2 unit) in the absence or presence of okadaic acid (40 nM) for 30 min at 30 °C and then incubated with a radiolabelled AP-1 oligonucleotide for 30 min at 23 °C, followed by electrophoresis in a 6% (w/v) native polyacrylamide gel as described in the Materials and methods section. The protein–DNA complex was identified by autoradiography. Abbreviation: ', min.

situ (Figure 3B). Both α and α' subunits of the nuclear CKII were activated at 1–30 min after insulin treatment. Given that insulin did not induce nuclear translocation of CKII, activation of nuclear CKII by insulin must be caused by some mechanism other than translocation.

Regulation of DNA-binding activity of AP-1 by insulin

It has been previously shown that the DNA-binding activity of AP-1 is regulated via phosphorylation by protein kinases including MAP kinase and CKII [43], and that insulin stimulates the phosphorylation of AP-1 [11]. To determine whether insulin might regulate the interaction between AP-1 and its DNA-binding element, we performed gel-mobility shift assays in the absence or presence of a MEK or CKII inhibitor. Nuclear extracts of CHO cells contained a protein or proteins that bind to a radiolabelled AP-1 oligonucleotide (Figure 4A). DNA-binding specificity was confirmed by the inhibition of binding in the presence of unlabelled competitor oligonucleotide. The addition of anti-c-Fos antibody to the assay mixture caused a supershift in the gel-mobility of the protein–DNA complex, indicating that the nuclear factor contained c-Fos or a Fos-related protein. The addition of anti-c-Jun antibody disrupted the protein–DNA complex, suggesting that c-Jun was also present in the DNA–protein complex and that the AP-1 oligonucleotide-binding protein in CHO cells exists as a c-Jun·c-Fos heterodimer.

Insulin stimulated the DNA-binding activity of AP-1 complex in a time-dependent manner, reaching a peak activity of 2–3-fold over basal at 1–30 min (Figure 4B). The kinetics of DNA-binding activity of AP-1 followed with a slight delay the very rapid time course of activation of nuclear MAP kinase and CKII by insulin. Treatment of nuclear extracts with PP2A caused a marked decrease in the DNA-binding activity of AP-1, whereas incubation of the nuclear extracts with PP2A and okadaic acid resulted in a full recovery of the insulin-stimulated DNA-binding activity of AP-1 (Figure 5), indicating that serine/threonine phosphorylation of AP-1 is required for the insulin-induced DNA-binding activity of AP-1.

Table 2 Effect of MEK inhibitor and DRB on MAP kinase and CKII activities

After preincubation with or without the MEK inhibitor (50 μ M) for 1 h or DRB (1 mM) for 10 min, CHO(Hirc) cells were stimulated with 100 nM insulin for 10 min, after which cytosol and nuclei were isolated as described in the Materials and methods section. For immunoprecipitation of MAP kinase, the nuclear and postnuclear fractions were first heat-denatured in the presence of 1% (w/v) SDS and subjected to immunoprecipitation with anti-MAP-kinase antibody (Transduction Laboratory), followed by an MBP-containing gel kinase assay *in situ*. MAP kinase activity was quantified by scanning densitometry and is expressed in arbitrary scanning units. CKII activity was measured as described in the Materials and methods section. The numbers in parentheses represent the activities above basal remaining after inhibition, expressed as a percentage of their respective controls. The results are the means of two separate experiments. Abbreviation: n.d., not determined.

	MAP kinase activity (arbitrary units)		CKII activity (pmol/min per mg)	
	Nucleus	Cytosol	Nucleus	Cytosol
Basal	47	176	22	33
Insulin	1079	6027	61	34
Insulin after MEK1	729 (66)	3117 (50)	53 (79)	35 (100)
DRB	n.d.	n.d.	35 (33)	36 (100)

5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) is a cell-permeable CKII inhibitor [34]. DRB pretreatment of CHO(Hirc) cells decreased the insulin-stimulated nuclear CKII activity by 67% (Table 2). Simultaneously, the insulin-stimulated DNA-binding activity of the nuclear protein was almost completely inhibited (Figure 4), suggesting that CKII is involved in the DNA-binding reaction of AP-1. In contrast, pretreatment with a MEK inhibitor caused only a slight, but reproducible, decrease in the DNA-binding activity of AP-1. This modest effect of the MEK inhibitor correlates with the fact that in these cells this inhibitor produced only a transient and partial inhibition of MAP kinase activity (Table 2). The transient and partial inhibition suggests that MAP kinase activation in CHO cells might utilize family members other than MEK1 and MEK2 or that the compound has a short half-life under the conditions used.

DISCUSSION

The activation of MAP kinase and CKII by growth factors has been implicated in the phosphorylation of a number of transcription factors, as well as the regulation of their DNA binding activity and the transactivation process [43]. The aim of the present study was to test whether insulin induces nuclear translocation of MEK, MAP kinase and CKII or stimulates the activities of these enzymes in the nucleus, and whether this might play a role in the stimulation of AP-1 DNA-binding activity by insulin. The results show that in CHO(Hirc) cells insulin induces the translocation of MEK from the cytoplasm to the nucleus and stimulates nuclear MAP kinase and CKII activity. In addition, insulin stimulates nuclear protein binding to the AP-1 site, and this is inhibited by pretreatment with DRB, a cell-permeable CKII inhibitor.

The identification of MAP kinase in the nucleus is consistent with previous reports by several investigators. The more than 95% purity of nuclei preparation by sucrose density gradient centrifugation rules out significant contamination of nuclei by these enzymes in the cytosol. Studies have shown that stimulation of Hela cells, chicken embryo fibroblasts and mouse 3T3 fibroblasts with serum or phorbol 12-myristate 13-acetate results in a time-dependent translocation of MAP kinase from cytoplasm to the nucleus [15,44]. In the present study with CHO(Hirc), Fao hepatoma and NIH-3T3(IR) cells, as well as 3T3-F442A adipo-

cytes, we find large amounts of MAP kinase localized in the nucleus under basal conditions, and in these cells insulin does not induce any nuclear translocation of MAP kinase over a 30 min incubation as determined by direct blotting. Thus the nuclear translocation of MAP kinase reported by other investigators might be cell-type-specific or ligand-specific, or might simply require longer than 30 min to be observed. Despite the lack of translocation, however, insulin rapidly stimulates resident nuclear MAP kinase activity as evidenced by an MBP-containing gel kinase assay *in situ* after immunoprecipitation with anti-MAP kinase antibody. These results imply that there must be some mechanism other than translocation of activated cytosolic MAP kinase that can lead to the activation of nuclear MAP kinase.

Taking into consideration the fact that MEK can phosphorylate and activate MAP kinase and that MEK contains a potential nuclear localization signal in the N-terminal sequence [45], as well as a bipartite motif located between Lys-34 and Arg-49, which has been suggested to play a role in the transport of proteins into the nucleus [6], MEK is a candidate for the activation of nuclear MAP kinase, possibly via nuclear translocation. Indeed, in contrast with MAP kinase, MEK is rapidly translocated from the cytoplasm to the nucleus in response to insulin in CHO(Hirc) cells. However, the time course of MEK translocation to the nucleus is somewhat slower than that for activation of the nuclear MAP kinase by insulin. Thus it is possible that the nuclear translocation of MEK in response to insulin and the rapid activation of the nuclear MAP kinase are independent events. Furthermore nuclear localization of MEK is cell-type-specific. For example, CHO(Hirc) and Fao cells contain MEK in the nucleus, whereas 3T3-F442A adipocytes and NIH-3T3(IR) cells contain no detectable MEK in the nucleus. Likewise, Zheng and Guan [31] have reported that MEK is exclusively localized in the cytoplasm in Swiss 3T3 cells and is not translocated into the nucleus in response to epidermal growth factor or phorbol 12-myristate 13-acetate. These results suggest that the nuclear translocation of MEK might occur in only specific cell types or in response to specific growth factors.

In terms of understanding the functional consequences of the stimulation by insulin of nuclear MAP kinase and CKII, which are known to phosphorylate AP-1 transcription factors, we explored the possibility that the interaction between AP-1 and the specific DNA element (AP-1 binding site) could be modulated in response to insulin. Our results clearly show that the DNA-binding activity of AP-1 (c-Jun/c-Fos) increases significantly in response to insulin in a time-dependent manner. Identification of the nuclear factor as AP-1 was confirmed by a gel-shift assay in the presence of either anti-c-Fos or anti-c-Jun antibody. This is consistent with our finding that insulin rapidly stimulates the serine phosphorylation of c-Jun and c-Fos and the tyrosine phosphorylation of a Fos-related protein, pp82, suggesting the involvement of insulin-stimulated serine/threonine, as well as tyrosine, kinases in the nucleus in the regulation of AP-1 [11,13].

Previous studies have shown that c-Jun contains several phosphorylation sites. *In vitro*, MAP kinase phosphorylates the N-terminal *trans*-activation domain (Ser-63 and Ser-73) resulting in the stimulation of *trans*-acting activity [46], whereas glycogen synthase kinase 3 and CKII have been shown to phosphorylate the C-terminal DNA-binding domain (Thr-239, Ser-243 and Ser-249), resulting in an inhibition of DNA binding *in vitro* [47,48]. Because the pretreatment of cells with a MEK inhibitor had little effect on the DNA-binding activity of AP-1 stimulated by insulin, it is possible that the MAP kinase activated in the nucleus by insulin might be involved in the *trans*-activation process rather than in the DNA-binding activity. In contrast, DRB, a cell-

permeable CKII inhibitor, inhibited the insulin-stimulated DNA-binding activity of AP-1 as well as nuclear insulin-stimulated CKII activity, suggesting that in CHO cells CKII-mediated phosphorylation might be positively involved in the AP-1 protein-DNA interaction. The involvement of phosphorylation in the insulin-stimulated DNA-binding activity of AP-1 was also supported by the fact that incubation of nuclear extracts with PP2A resulted in the inhibition of the insulin-induced DNA-binding activity of AP-1, whereas okadaic acid prevented the action of PP2A. In support of this hypothesis is the fact that CKII is present in the nucleus and is activated by insulin within 1–30 min. The fact that the activation of nuclear CKII by insulin is transient, however, suggests that this phosphorylation might trigger the DNA binding of AP-1, after which some other nuclear factors participate in maintaining the bound state of AP-1 with DNA.

In summary, insulin induces a rapid translocation of MEK from cytoplasm to the nucleus and activates resident nuclear MAP kinase (ERK I) and CKII. The DNA-binding activity of AP-1 is also stimulated by insulin with kinetics similar to those of MEK translocation and the activation of MAP kinase and CKII in response to insulin. DNA binding by AP-1 is diminished by preincubation with a cell-permeable CKII inhibitor, suggesting that CKII serves as a key positive regulator for the DNA-binding activity of AP-1. The insulin-induced DNA-binding activity of AP-1 is mediated by serine phosphorylation. These events seem to be dependent on cell type, which might account in part for differing nuclear actions of insulin in different cell types.

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