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Regulation of AP-1 Activity in Glucose-Stimulated Insulinoma Cells

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ABSTRACT

The transcriptional activity of AP-1 has been analyzed in glucose-stimulated INS-1 insulinoma cells using a chromosomally embedded AP-1-responsive reporter gene. We show that AP-1 activity was significantly elevated in glucose-treated INS-1 cells. Preincubation of the cells with nifedipine or expression of the Ca^{2+} binding protein parvalbumin in the cytoplasm of INS-1 cells reduced AP-1 activity. Thus, activation of L-type Ca^{2+} channels and an elevated cytoplasmic Ca^{2+} concentration are crucial to connecting glucose stimulation with enhanced AP-1 activity. Expression of dominant negative forms of A-Raf, MKK4 or MKK6 and pharmacological inhibition of MEK and p38 revealed that extracellular signal-regulated protein kinase, p38 and c-Jun NH_2-terminal protein kinase participate in the upregulation of AP-1 activity. Expression of dominant-negative mutants of c-Jun and Elk-1 reduced AP-1 transcriptional activity in INS-1 cells indicating that c-Jun and ternary complex factors are involved in the regulation of AP-1 activity in glucose-stimulated insulinoma cells. J. Cell. Biochem. 110: 1481–1494, 2010.

KEY WORDS: L-TYPE Ca $^{2+}$ CHANNEL; ERK; Elk-1; p38 PROTEIN KINASE; MKK4; MKK6; c-Jun

Elevated levels of glucose are the main stimuli for insulin secretion in β-cells and also have a profound effect on gene transcription. A microarray study revealed that more than 90 genes are activated in MIN6 insulinoma cells as a result of glucose stimulation [Ohsugi et al., 2004]. A microarray analysis performed with glucose and cpt-cAMP-treated MIN6 cells identified 592 target genes [Glauser et al., 2006]. Several transcription factors have been identified to be activated in glucose-stimulated β-cells, including Egr-1 [Frödin et al., 1995; Josefsen et al., 1999; Bernal-Mizrachi et al., 2000; Mayer and Thiel, 2009], CREB [Wang et al., 2008; Mayer and Thiel, 2009], Elk-1 [Bernal-Mizrachi et al., 2001; Mayer and Thiel, 2009], and c-Fos [Glauser and Schlegel, 2007].

An important role for the transcription factor AP-1 in MIN6 insulinoma cells has been proposed, based on the fact that a microarray analysis revealed a significant overrepresentation of genes containing AP-1 binding sites in their regulatory regions [Glauser et al., 2006]. The activator protein-1 (AP-1) transcription factor was originally described as a heterodimer of c-Jun and c-Fos [Chiu et al., 1988; Curran and Franza, 1988]. These bZIP transcription factors dimerize via their leucine zipper domains, which in turn bring together their basic domains to bind DNA in a sequence-specific manner. In recent years several homologs of c-Jun (JunB and JunD) and c-Fos (JunB, JunD, FosB, Fra1, Fra2, and BATF) have also been shown to form heterodimers. In addition, members of the activating transcription factor family (ATF) such as ATF2, ATF3, and ATF4 can interact with members of the Fos and Jun family of proteins [Cohen et al., 1989; Hai and Curran, 1991; Vinson et al., 2002]. Thus, initially described as a heterodimer of c-Jun and c-Fos, the current view is that AP-1 is actually a group of several distinct homodimers or heterodimers composed of various members of the Fos, Jun, and ATF bZIP subfamilies.

Here, we have analyzed the signaling pathways leading to enhanced AP-1 activity in INS-1 insulinoma cells that have been stimulated with glucose. Using lentiviral gene transfer, we inserted a collagenase promoter/luciferase reporter gene into the chromatin of INS-1 cells to ensure that the reporter gene is packed into an ordered nucleosomal structure. The collagenase promoter contains the sequence motif 5′-TGAGTCA-3′, also known as phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element (TRE), a high-affinity binding site for the c-Jun:c-Fos heterodimer. We show that activation of L-type Ca^{2+} channels and an elevation of the cytoplasmic Ca^{2+} concentration are essential for the upregulation of

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AP-1 activity in glucose-treated INS-1 cells. Moreover, we show that a combinatorial activation of the protein kinases Raf, extracellular signal-regulated protein kinase (ERK), p38, and c-Jun NH_2-terminal protein kinase (JNK) contribute to enhanced AP-1 activity. We additionally present evidence that c-Jun and ternary complex factors are involved in the regulation of AP-1 activity in glucose-stimulated INS-1 cells.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Rat INS-1 insulinoma cells were cultured as described [Groot et al., 2000]. Cells were incubated for 24 h in medium containing 2 mM glucose but lacking fetal calf serum. Stimulation with glucose (11 or 25 mM) was performed as indicated for 48 h. The MAP kinase kinase inhibitor PD98059 was purchased from Axxora (Lörrach, Germany, # 385-023), dissolved in DMSO, and used at a concentration of 50 μM. The p38 protein kinase inhibitor SB203580 was purchased from Alexis (Lörrach, Germany, # 270-179-M001) and used at a final concentration of 10 μM. The L-type Ca^{2+} channel blocker nifedipine was purchased from Sigma–Aldrich (Steinheim, Germany, # N7634), dissolved in DMSO, and used at a final concentration of 50 μM. Cells were preincubated with PD98059 or nifedipine for 1 h. Human HepG2 hepatoma cells were cultured as described [Steinmüller et al., 2001]. Stimulation with TPA (50 ng/ml, dissolved in DMSO) was performed for 24 h. 4-Hydroxytamoxifen (4OHT, Sigma, # H7904) was dissolved in ethanol and used at a concentration of 0.25 μM for 24 h.

RETROVIRAL GENE TRANSFER

Plasmid pBabepuro3ΔB-Raf:ER, encoding an activated form of the protein kinase B-Raf as a fusion protein with the hormone binding domain of the murine estrogen receptor (ER^{TM}), was kindly provided by Martin McMahon, UCSF, San Francisco, USA [McMahon, 2001]. The packaging cell line Phoenix-Ampho was obtained from Gary Nolan, Stanford University, USA. INS-1 cells were transfected with retroviral vectors using the calcium coprecipitation procedure. Retroviral infection was performed as described [Rössler et al., 2004]. INS-1 cells were selected with 1.5 μg puromycin/ml. Mass pools of stable transfectants were selected and used for all experiments in order to eliminate the possibility of specific clonal effects.

LENTIVIRAL GENE TRANSFER

All lentiviral transfer vectors used in this study are based on plasmids pFUW or pFUWG [Lois et al., 2002]. The transgenes were expressed under the control of the human ubiquitin-C promoter. The lentiviral transfer vectors pFUW-FLAG-REST/Elk-1ΔC, pFUW-mycDA-Raf1, and pFUWc-JunΔN have been described elsewhere [Mayer et al., 2008, 2009]. A SEK-AL encoding plasmid [Zanke et al., 1996] was a kind gift of James Woodgett, Department of Medicine and Ontario Cancer Institute Princess Margaret Hospital, Ontario, Canada. The plasmid was digested with *Nco*I, filled in with the Klenow fragment of DNA polymerase I, and recut with *Xho*I. The fragment was isolated and cloned into plasmid p3XFLAG-CMV-7.1 (Sigma), generating plasmid pCMV-FLAG-SEK-AL.This plasmid was digested with *Nco*I and *Bam*HI and filled in with the Klenow fragment of DNA polymerase I. The insert, encoding FLAG-tagged SEK-AL, was cloned into pFUWG, replacing the EGFP open-reading frame. This plasmid was termed pFUW-FLAG-SEK-AL. Plasmid pFUW-FLAG-MKK6E, encoding a FLAG-tagged, constitutively active MKK6, was generated by cutting plasmid pcDNA3-FLAG-MKK6E [Raingeaud et al., 1996], a kind gift of Roger Davis, University of Massachusetts Medical School, with *Hin*dIII and *Bsp*120I, and filled in with the Klenow fragment of DNA polymerase I. The fragment was cloned into *Hpa*I cut pFUW. The lentiviral transfer vector pFUWmCherry was generated by replacing the EGFP coding region of plasmid pFUWG with that of mCherry. Plasmid pCMV-PV-NES-GFP was a kind gift of Anton Bennett, Yale University. We replaced the GFP coding region with that of mCherry and cloned the fragment encoding a NES-tagged parvalbumin-mCherry fusion protein into pFUWG. Cloning details can be obtained upon request. The viral particles were produced as previously described [Stefano et al., 2006] by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV glycoprotein and the transfer vector.

REPORTER ASSAYS

The lentiviral transfer vector pFWColl-luc, encoding a human collagenase promoter/luciferase reporter gene, has been described elsewhere [Rössler et al., 2008]. The vector contains the human collagenase promoter (sequence from −517 to +63) upstream of the luciferase reporter gene. Site-directed mutagenesis was performed with the Quick-change kit from Stratagene (La Jolla, # 210518), using plasmid pFWColl-luc as template and the oligonucleotides 5′-AAT CAA GAG GAT GTT ATA AAG CAT GAT AGT GAC AGC CTC TGG CTT TCT GGA AG-3′ and 5′-CTT CCA GAA AGC CAG AGG CTG TCA CTA TCA TGC TTT ATA ACA TCC TCT TGA TT-3′ as primers. Plasmid Ins-715Luc encoding an insulin promoter/luciferase reporter gene was a kind gift of Michiyo Amemiya-Kudo from the Okinawa Memorial Institute for Medical Research, Tokyo, Japan [Amemiya-Kudo et al., 2005]. The plasmid was cut with *Pme*I and *Bgl*II and cloned upstream of the luciferase gene, generating the lentiviral transfer vector pFWInsluc. Plasmid pFWEgr-1SREluc, a lentiviral transfer vector used to generate lentiviruses that express luciferase under the control of two SRE sites, has been described recently [Rössler and Thiel, 2009]. Cell extracts of stimulated cells were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

WESTERN BLOTS

Whole cell extracts and nuclear extracts were prepared as described [Kaufmann and Thiel, 2002]. Thirty micrograms of nuclear proteins were separated by SDS–PAGE and the blots were incubated with antibodies directed against c-Jun (Santa Cruz, Heidelberg, Germany, # sc-1694) or the phosphorylated form of c-Jun (Upstate Biotechnology, Lake Placid, NY, # 06-659). An antibody directed against HDAC1 (Santa Cruz, # sc-81598) was used as a loading control. An antibody directed against mCherry was purchased from Clontech (# 632496). To detect the ΔB-Raf:ER fusion protein 30 μg of protein derived from whole cell extract preparations was

separated on SDS–PAGE and transferred to nitrocellulose membranes. Blots were probed with an antibody directed against the murine ER (Santa Cruz, # sc542). To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma–Aldrich, # F3165), at 1:3,000 dilution in TBS. Antibodies against the myc epitope were prepared from CRL-1729 hybridomas, purchased from ATCC. Immunoreactive bands were detected via enhanced chemiluminescence using a 1:1 solution of solution 1 (100 mM Tris–HCl, pH 8.5, 5.4 mM H_2O_2) and solution 2 (2.5 mM luminol, 400 μM *p*-coumaric acid, 100 mM Tris–HCl, pH 8.5).

RESULTS

UPREGULATION OF INSULIN PROMOTER ACTIVITY IN GLUCOSE-STIMULATED INS-1 INSULINOMA CELLS

Elevated levels of glucose regulate gene transcription in β-cells and insulinoma cells. As a cellular model system, we used the insulin-secreting rat INS-1 pancreatic β-cell line that has been established from a radiation-induced rat insulinoma [Asfari et al., 1992] to assess the impact of high glucose concentration on the activity of the insulin promoter. An insulin promoter/luciferase reporter gene was integrated into the chromatin of INS-1 insulinoma cells by lentiviral gene transfer. A schematic depiction of the integrated provirus is seen in Figure 1A. The infected cells were serum-starved for 24 h in medium containing 2 mM glucose. Cells were stimulated with glucose (11 mM) for 48 h. The cells were harvested, cytoplasmic extracts were prepared, and luciferase activities were measured. Figure 1B shows that glucose stimulation strikingly enhanced transcription of the insulin promoter/luciferase reporter gene.

UPREGULATION OF AP-1 ACTIVITY IN GLUCOSE-STIMULATED INS-1 INSULINOMA CELLS

It has been suggested that glucose stimulation preferentially activate gene transcription of those genes that are regulated by the transcription factor AP-1 [Glauser et al., 2006]. To measure AP-1-regulated transcription, we implanted a collagenase promoter/luciferase reporter gene into the chromatin of INS-1 insulinoma cells. The human collagenase promoter contains an AP-1 binding site in the proximal promoter region and therefore frequently has been used to monitor AP-1 activity [Angel et al., 1987; Steinmüller et al., 2001]. A schematic depiction of the integrated provirus encoding the collagenase promoter/luciferase reporter gene is seen in Figure 1C, including the sequence of the wild-type and the mutated TRE. INS-1 cells that had been infected with a lentivirus encoding the collagenase promoter/luciferase reporter gene were serum-starved for 24 h in medium containing 2 mM glucose. Cells were stimulated with glucose (11 or 25 mM) for 48 h. Figure 1D shows that glucose stimulation enhanced transcription mediated by AP-1. The activation of reporter gene transcription was on the order of 10-fold (stimulation with 11 mM glucose) or 14-fold (stimulation with 25 mM glucose). Despite some experimental variations in the fold-induction of AP-1-mediated transcription, these results indicate that glucose stimulation alters the genetic program of INS-1 cells by activating genes controlled by the transcription factor AP-1. To show the importance of the TRE within the collagenase promoter, we mutated the TRE sequence 5′-TGAGTCA-3′ to 5′-TGATAGT-3′ as described [Ayala et al., 2002]. Figure 1D shows that the stimulation of reporter gene transcription by glucose was strikingly reduced when the TRE was mutated. This experiment reveals that the TRE within the collagenase promoter is responsible for the AP-1-specific activity.

As a further control HepG2 hepatoma cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. Subsequently, the cells were stimulated with the phorbol ester TPA, a polycyclic ester that mimics diacylglycerol and thereby irreversibly activate protein kinase C. TPA treatment has been shown to strongly stimulate AP-1 activity in transiently transfected HepG2 cells [Steinmüller et al., 2001]. Luciferase activities were measured 24 h after stimulation. Figure 1F shows that TPA strikingly enhanced transcription of the chromatin-embedded AP-1-responsive collagenase promoter/luciferase reporter gene. The activation was on the order of 26-fold.

ENHANCED AP-1 ACTIVITY IN INS-1 PANCREATIC β-CELLS REQUIRES ACTIVATED L-TYPE Ca^{2+} CHANNELS AND ELEVATED CYTOPLASMIC Ca^{2+} CONCENTRATIONS

The treatment of pancreatic β-cells with glucose induces a Ca^{2+} influx into the β-cells via the opening of L-type Ca^{2+} channels. The involvement of L-type Ca^{2+} channels in the context of glucose-induced upregulation of AP-1 activity was verified using INS-1 cells preincubated with nifedipine. Figure 2A shows that AP-1 activity was significantly reduced in nifedipine-treated cells, indicating that activation of L-type Ca^{2+} channels is required to connect glucose stimulation with enhanced AP-1 activity. The importance of elevated cytosolic Ca^{2+} levels for the signaling cascade in glucose-stimulated INS-1 cells was analyzed using expression of the Ca^{2+} binding protein parvalbumin (PV). Figure 2B shows the modular structure of the PVmCherry fusion protein that contains a nuclear export signal (NES) derived from MKK1 on its N-terminus, to direct expression to the cytosol [Pusl et al., 2002]. Figure 2C shows that mCherry and NES-PVmCherry are expressed in INS-1 cells following lentiviral infection with the appropriate viruses. The NES-tagged parvalbumin-mCherry fusion protein was expressed in the cytosol, while mCherry was found in the cytosol and in the nucleus (Fig. 2D). When the elevation of the cytosolic Ca^{2+} concentration was buffered in parvalbumin-expressing INS-1 cells, the glucose-induced upregulation of AP-1 was significantly reduced (Fig. 2E). Hence, a rise of cytosolic Ca^{2+} levels is essential for the upregulation of AP-1 activity in glucose-stimulated INS-1 cells.

ACTIVATION OF RAF AND ERK1/2 IS ESSENTIAL FOR THE UPREGULATION OF AP-1 ACTIVITY IN INS-1 INSULINOMA CELLS STIMULATED WITH GLUCOSE

Stimulation of pancreatic β-cells with glucose has been reported to activate ERK1/2 [Frödin et al., 1995; Arnette et al., 2003], suggesting that activated ERK1/2 may be crucial for the upregulation of AP-1 transcriptional activity. We assessed the role of ERK1/2 in glucose-induced upregulation of AP-1 activity in two ways: First, we inhibited Raf, a MAP kinase kinase kinase via expression of a dominant-negative antagonist of the Ras/ERK pathway. Second, we treated INS-1 cells with PD98059, a compound that inhibits

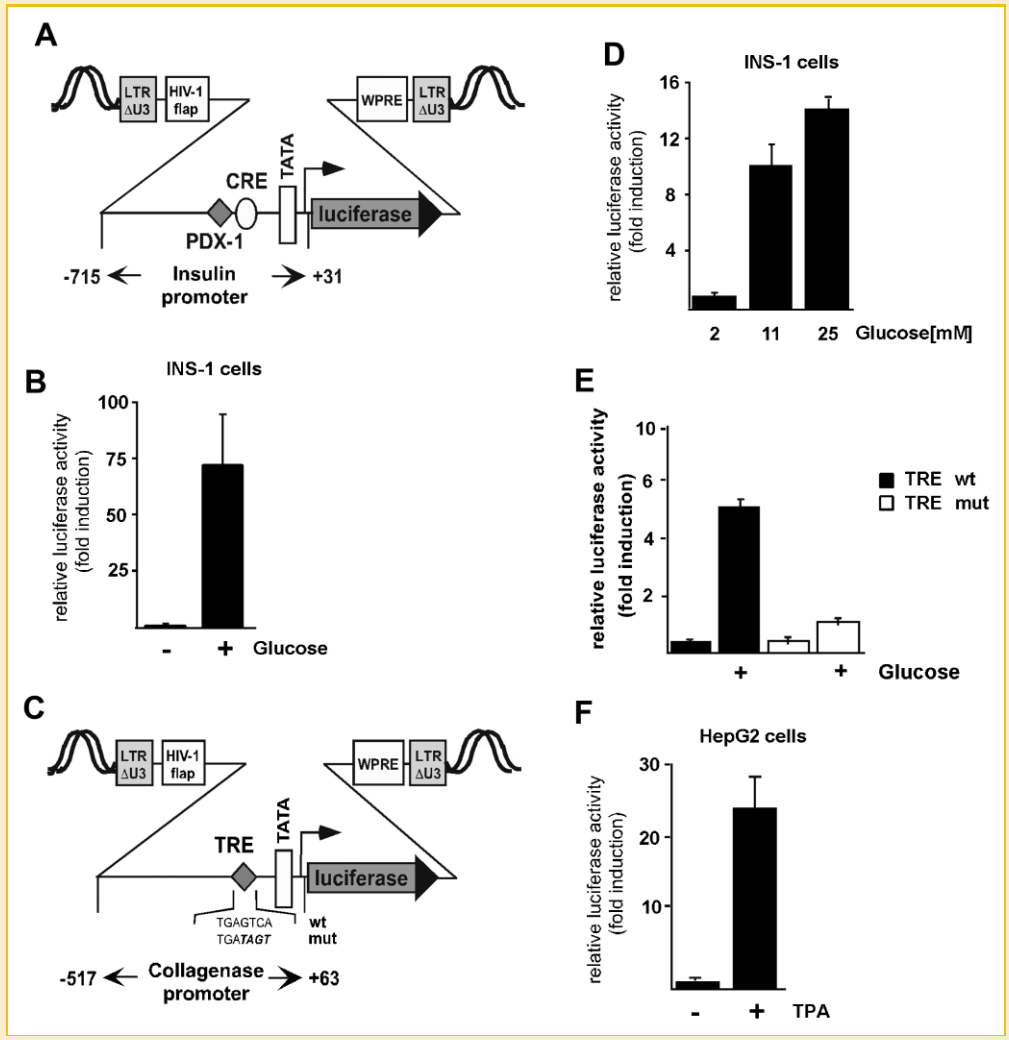


Fig. 1. Glucose stimulation upregulates the AP-1 activity in INS-1 insulinoma cells. A: Schematic representation of the integrated provirus encoding an insulin promoter/luciferase reporter gene. The cyclic AMP response element (CRE) and the binding site for Pdx-1 are depicted. The U3 region of the 5′ LTR of the transfer vector is deleted. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and the HIV flap element are indicated. B: INS-1 insulinoma cells were infected with a recombinant lentivirus encoding an insulin promoter/luciferase reporter gene. The cells were serum-starved for 24 h in medium containing 2 mM glucose and then stimulated with glucose (11 mM) for 48 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. C: Schematic representation of the integrated provirus encoding a collagenase promoter/luciferase reporter gene. The promoter fragments were inserted upstream of the luciferase reporter gene. The location and sequence of the wild-type and mutated phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element (TRE) within the collagenase promoter are depicted. D: INS-1 insulinoma cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h in medium containing 2 mM glucose and then stimulated with glucose (11 and 25 mM) for 48 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E: INS-1 insulinoma cells were infected with recombinant lentiviruses encoding collagenase promoter/luciferase reporter genes with an intact or a mutated TRE. The cells were serum-starved for 24 h in medium containing 2 mM glucose and then stimulated with glucose (11 mM) for 48 h. Cell extracts were prepared and analyzed for luciferase activities. F: HepG2 hepatoma cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h and treated with TPA (50 ng/ml) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Each experiment illustrated here and in all subsequent figures was repeated a minimum of three times with consistent results.

phosphorylation of the MAP kinase kinase by Raf. Figure 3A shows the modular structure of DA-Raf1, a splicing isoform of A-Raf that functions as an antagonist of the Ras/Raf-ERK1/2 pathway [Yokoyama et al., 2007]. Cellular proteins of mock-infected INS-1 cells or cells infected with a myc-tagged DA-Raf1 encoding lentivirus were fractionated by SDS–PAGE. The fusion protein was identified by Western blot analysis using an antibody targeting the myc epitope (Fig. 3B). Next, the functional implication of DA-Raf1 expression was assessed. The results show that expression of DA-Raf1 significantly reduced the upregulation of AP-1 activity in glucose-stimulated INS-1 cells. Moreover, preincubation of the cells with PD98059 efficiently blocked the upregulation of AP-1 activity in glucose-stimulated insulinoma cells. Together, these data indicate that ERK1/2 activation is a key event in controlling the upregulation of AP-1 activity as a result of glucose treatment.

CONDITIONAL ACTIVATION OF THE ERK1/2 SIGNALING PATHWAY IN INS-1 CELLS VIA EXPRESSION OF A ΔB-Raf/ESTROGEN RECEPTOR FUSION PROTEIN TRIGGERS AN UPREGULATION OF AP-1 ACTIVITY

In the preceding section we described experiments showing that ERK1/2 activation is a key event in the signaling cascades that connect glucose stimulation with enhanced AP-1-mediated gene transcription. To specifically activate the ERK1/2 pathway in INS-1 cells in an L-type Ca^{2+} channel-independent manner, we generated INS-1 cells expressing a ΔB-Raf/ER fusion protein, a conditionally active form of B-Raf. The modular structure of B-Raf and ΔB-Raf:ER is schematically depicted in Figure 3E. B-Raf contains three functional domains (CR1, CR2, and CR3). CR1 is a cysteine-rich region and functions as binding site for activated Ras (Ras-GTP) at the cell membrane. CR2 is rich in serine and threonine residues and negatively regulates the biological activity of the catalytic domain. CR3 encompasses the protein kinase domain. We expressed the catalytic domain of B-Raf as a fusion protein with the ligand binding domain of the murine estrogen receptor (ER) to maintain the protein kinase in an inactive state in the absence of hormone. Addition of hormone leads to an enhancement of B-Raf kinase activity [Thiel et al., 2009]. The use of the ER mutant termed $ER^{Tamoxifen\ Mutant}$ allowed us to use the synthetic ligand 4OHT for induction. We infected INS-1 cells with a recombinant retrovirus encoding ΔB-Raf:ER. The expression of the ΔB-Raf/ER fusion protein that occured under the control of the murine stem cell virus long terminal repeat was verified using an antibody specific for the ER ligand binding domain (Fig. 3F). Next, INS-1ΔB-Raf:ER cells were stimulated with 4OHT for 24 h. Figure 3G shows that treatment of INS-1ΔB-Raf:ER cells with 4OHT induced an upregulation of transcription of a chromatin-embedded AP-1-responsive reporter gene. The induction was on the order of ≈10-fold. Preincubation of the cells with PD98059 reduced the AP-1 activity.

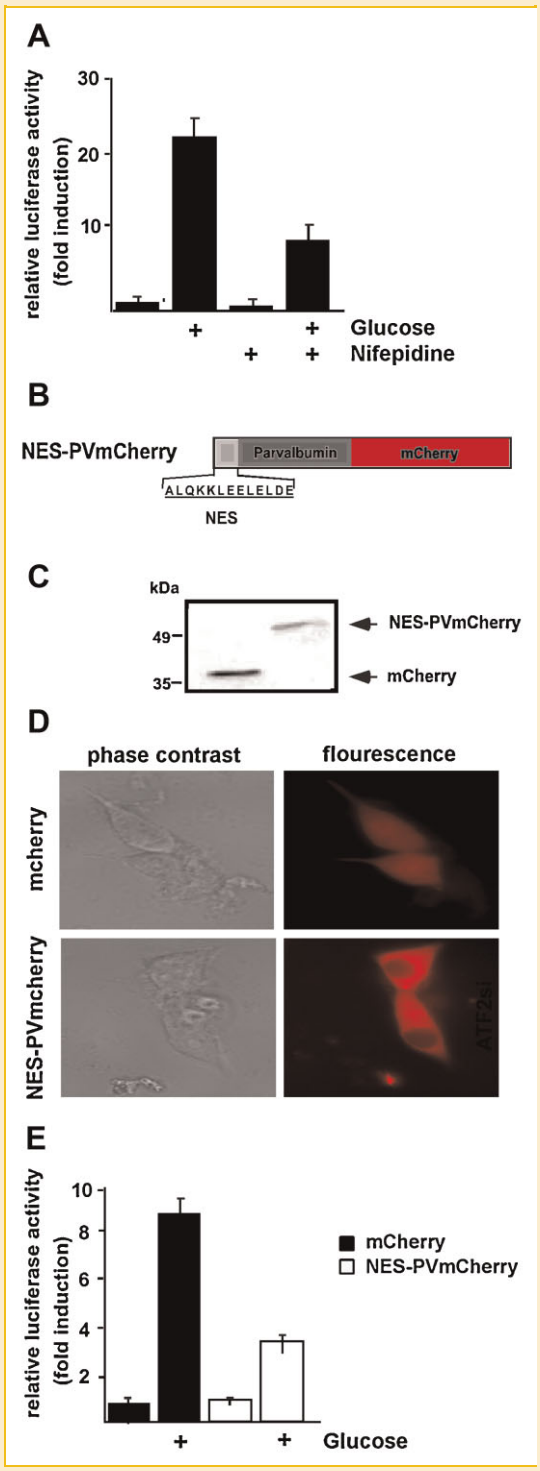


Fig. 2. Stimulation of AP-1-mediated gene transcription in glucose-treated INS-1 pancreatic β-cells requires the activation of L-type Ca^{2+} channels and an elevation of the Ca^{2+} concentration in the cytosol. A: INS-1 insulinoma cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h in medium containing 2 mM glucose. Cells cultured in the presence or absence of nifedipine (50 μM) were stimulated with glucose (11 mM) for 48 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. B: Schematic representation of the NES-PVmCherry fusion protein. C: Western blot analysis of INS-1 cells infected with a recombinant lentivirus encoding NES-PVmCherry. As a control, extracts from INS-1 cells that had been infected with a lentivirus encoding mCherry were analyzed. Western blots were probed with an antibody against mCherry. Molecular mass markers in kDa are shown on the left. D: Phase-contrast and fluorescence images of INS-1 cells that had been infected with lentiviruses encoding either mCherry (top) or NES-PVmCherry (bottom). E: INS-1 insulinoma cells were double-infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene and with lentiviruses encoding either mCherry or NES-PVmCherry. The cells were serum-starved for 24 h in medium containing 2 mM glucose. Stimulation with glucose (11 mM) was performed for 48 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

p38 PLAYS AN ESSENTIAL ROLE IN CONTROLLING AP-1 ACTIVITY IN GLUCOSE-TREATED INS-1 CELLS

In glucose-treated smooth muscle cells glucose stimulation has been shown to increase p38 protein kinase activity in a time-dependent manner [Igarashi et al., 1999]. We therefore assessed the impact of p38 on the upregulation of AP-1 activity in glucose-treated INS-1 cells. Figure 4A shows that preincubation of INS-1 cells with the

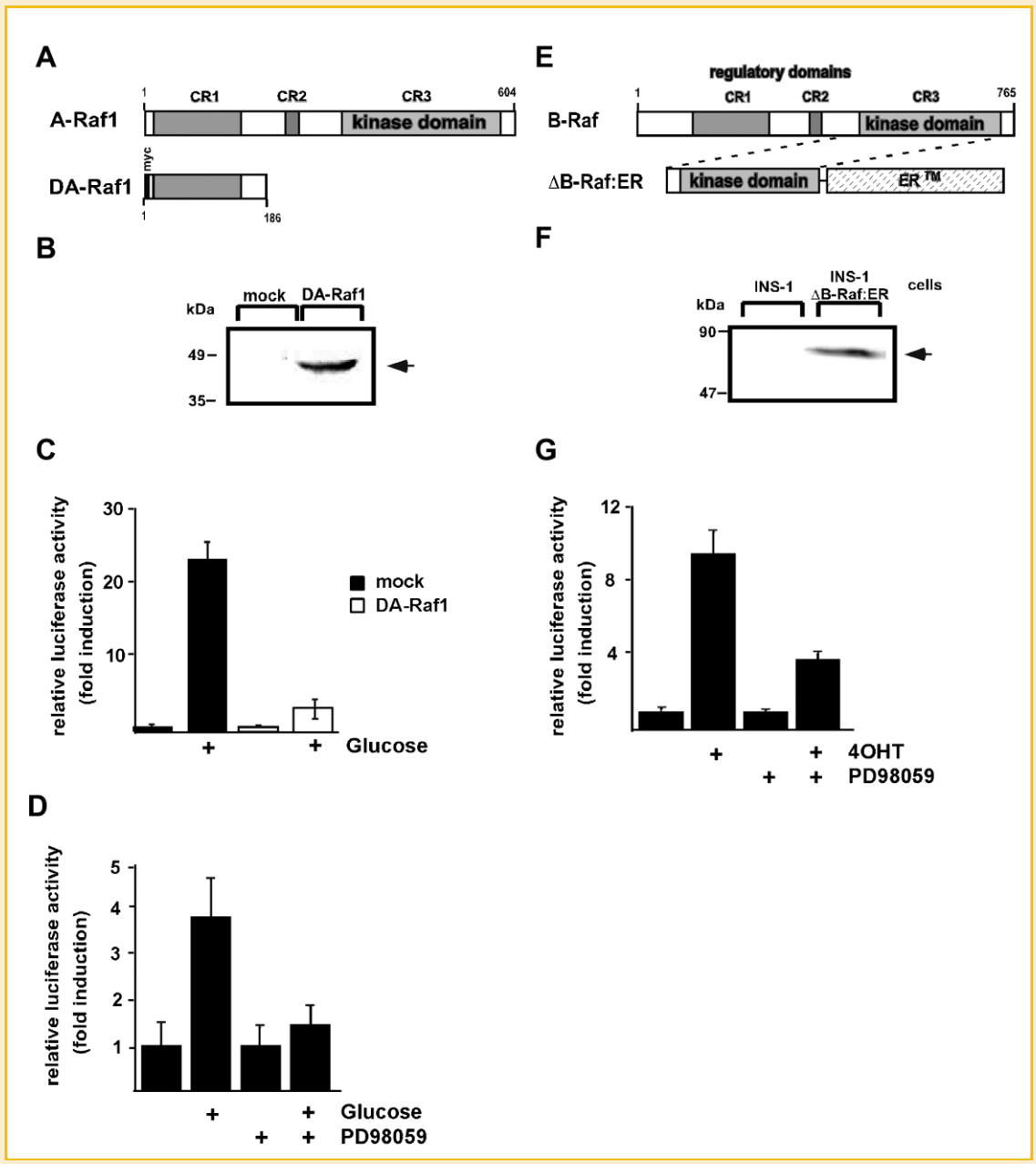
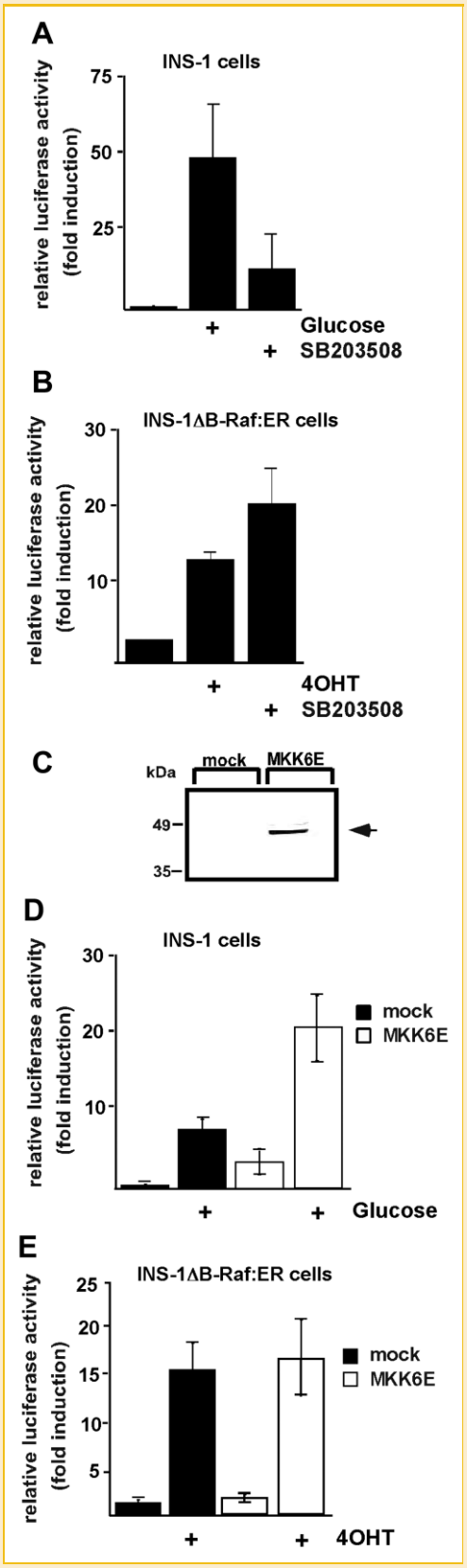


Fig. 3. Essential role of Raf and ERK for the upregulation of AP-1 activity in glucose-stimulated INS-1 pancreatic β-cells. A: Modular structure of A-Raf and the DA-Raf1. B: Expression of DA-Raf1 in lentiviral-infected INS-1 is detected using an antibody against the N-terminal myc-tag. Molecular mass markers in kDa are shown on the left. C: INS-1 insulinoma cells were double-infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene and with a lentivirus encoding DA-Raf1. As a control INS-1 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h in medium containing 2 mM glucose. Stimulation with glucose (11 mM) was performed for 48 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. D: INS-1 insulinoma cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h in medium containing 2 mM glucose. Cells cultured in the presence or absence of PD98059 (50 μM) were stimulated with glucose (11 mM) for 48 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E: Generation of INS-1 cells expressing a conditionally active form of B-Raf. Schematic representation of the modular structure of B-Raf and ΔB-Raf:ER. The functional domains of Raf-1 (CR3, CR2, and CR1) are depicted. Fusion of the catalytic CR3 domain to the hormone binding domain of the estrogen receptor generates the ΔB-Raf:ER fusion protein. F: Expression of ΔB-Raf:ER in INS-1 cells. Whole cell extracts of ΔB-Raf:ER expressing INS-1 cells were prepared and analyzed by immunoblotting using an antibody directed against the murine estrogen receptor. As a control, INS-1 cells were analyzed (mock). G: INS-1ΔB-Raf:ER cells were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h in medium containing 2 mM glucose. Cells were cultured in the presence or absence of PD98059 (50 μM, 1 h) as indicated and stimulated with 4OHT (250 nM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.



p38-specific inhibitor SB203580 reduced AP-1 activity in glucose-stimulated cells. In contrast, pretreatment with SB203580 did not impair the upregulation of AP-1 activity in INS-1ΔB-Raf:ER cells that had been stimulated with 4OHT (Fig. 4B). To corroborate these results with an alternative experimental approach, we tested whether forced activation of p38 induced AP-1 activity in INS-1 cells in the absence of glucose stimulation. We expressed a constitutively active MKK6, MKK6E, in INS-1 cells using lentiviral gene transfer. The MKK6E mutant contains two point mutations of the phosphoacceptor sites, Ser207Glu and Thr211Glu, thus generating a mutant with constitutive negative charges on these sites [Raingeaud et al., 1996]. MKK6E additionally contains a FLAG epitope on its N-terminus. Expression of MKK6E was verified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 4C). The functional analysis of MKK6E is depicted in Figure 4D. Expression of MKK6E increased AP-1 activity on the order of 3.5-fold. Stimulation with glucose led to a synergistic effect that resulted in an upregulation of AP-1 activity of about 22-fold. In contrast, no synergistic effect was observed in 4OHT-stimulated INS-1ΔB-Raf:ER cells that expressed MKK6E (Fig. 4E). Together these data indicate that p38 plays a sigificant role in the signaling cascade that connects glucose stimulation with enhanced AP-1 gene transcription in insulinoma cells. p38 protein kinase is not required for the upregulation of AP-1 transcriptional activity as a result of ΔB-Raf:ER activation.

A DOMINANT-NEGATIVE MUTANT OF SEK1/MKK4 IMPAIRS AP-1 ACTIVATION IN GLUCOSE-STIMULATED INS-1 CELLS

SEK1/MKK4 is a MAP kinase kinase that is activated by MEKK1 via phosphorylation of serine residues 204 and 207 and directly phosphorylates and activates JNK. Gene disruption of the SEK1/MKK4-encoding gene causes a selective defect in AP-1 transcriptional activity [Yang et al., 1997]. Mutation of serine residues 204 and 207 to alanine or leucine, respectively, generated a dominant-negative mutant, SEK-AL. Expression of SEK-AL in murine

Fig. 4. AP-1 activity in glucose-stimulated INS-1 cells is controlled by p38. INS-1 insulinoma cells (A) or INS-1ΔB-Raf:ER cells (B) were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h in medium containing 2 mM glucose. Cells were cultured in the presence or absence of SB203580 (10 μM) as indicated. The cells were stimulated either with glucose (11 mM) for 48 h (A) or with 4OHT (250 nM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. C: Western blot analysis of INS-1 cells infected with a recombinant lentivirus encoding FLAG-tagged MKK6E. As a control INS-1 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). Western blots were probed with an antibody against the FLAG-tag. Molecular mass markers in kDa are shown on the left. INS-1 (D) and INS-1ΔB-Raf:ER cells (E) were double-infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene and a FLAG-tagged MKK6E. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h in medium containing 2 mM glucose. Stimulation of INS-1 cells with glucose (11 mM) was performed for 48 h. Stimulation with of INS-1ΔB-Raf:ER cells with 4OHT (250 nM) was performed for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

fibroblasts showed that JNK but not p38 activation was specifically impaired [Zanke et al., 1996]. We generated a lentiviral transfer vector encoding FLAG-tagged SEK-AL. Western Blot analysis showed that the SEK-AL mutant was correctly expressed in INS-1 cells infected with a lentivirus encoding FLAG-SEK-AL (Fig. 5A). An analysis of AP-1 activity in infected cells revealed that expression of SEK-AL blocked the upregulation of AP-1 activity as a result of glucose stimulation (Fig. 5B). Likewise, AP-1 activity was significantly reduced in 4OHT-stimulated INS-1-ΔB-Raf:ER cells (Fig. 5C) and in TPA-treated HepG2 cells (Fig. 5D). Hence, JNK activation is part of the signaling cascade that leads to an upregulation of AP-1 activity in glucose-stimulated INS-1 cells.

EXPRESSION AND PHOSPHORYLATION OF c-Jun ARE ENHANCED IN GLUCOSE-STIMULATED INS-1 CELLS

The preceding results showed that the activation of ERK1/2, p38, and JNK contribute to an enhanced AP-1 activity in INS-1 cells that have been stimulated with glucose. Interestingly, activation of these kinases has been connected with phosphorylation and activation of c-Jun, suggesting that c-Jun functions as a point of convergence for intracellular signaling cascades involving MAP kinases. Figure 6A (upper panel) shows that expression and phosphorylation of c-Jun are enhanced in glucose-treated INS-1 cells. Likewise, c-Jun expression and phosphorylation were upregulated in 4OHT-stimulated INS-1ΔB-Raf:ER cells and in TPA-treated HepG2 cells (Fig. 6A, middle and lower panels).

c-Jun REGULATES AP-1 ACTIVITY IN GLUCOSE-STIMULATED INS-1 CELLS

To assess the role of c-Jun in the regulation of AP-1 activity in INS-1 insulinoma cells, we chose a dominant-negative approach. The modular structure of c-Jun and a dominant-negative mutant of c-Jun, c-JunΔN, lacking the N-terminal regulatory region and transcriptional activation domain, is depicted in Figure 6B. c-JunΔN encompasses amino acid residues from amino acid 188 to amino acid 331 of c-Jun. The dominant-negative mutant fails to activate transcription because it lacks the transcriptional activation domain. Rather, the mutant inhibits DNA binding of its wild-type bZIP counterpart by blocking the cognate sites for DNA binding. The biological activity of these mutants has been demonstrated [Steinmüller and Thiel, 2003]. c-JunΔN was expressed in INS-1 cells following infection with a recombinant lentivirus. Proteins derived from nuclear extracts of INS-1 cells, either "mock"-infected cells or cells infected with a lentivirus encoding c-JunΔN, were fractionated by SDS–PAGE. The proteins were identified by Western

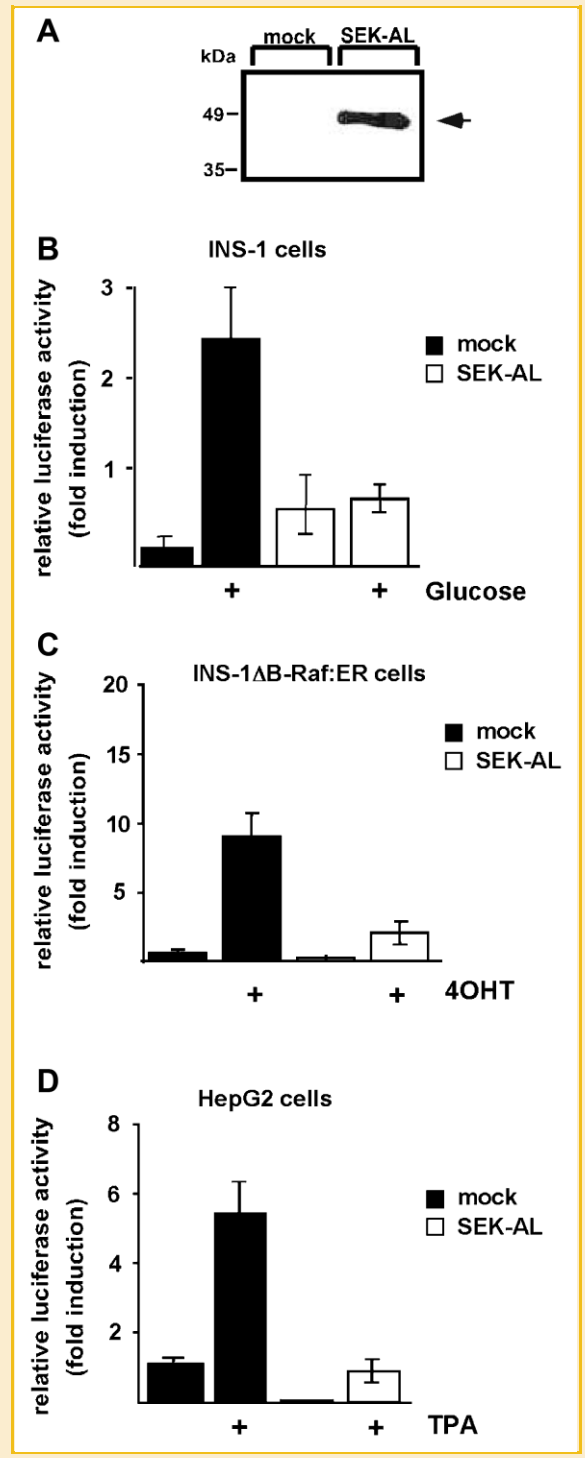


Fig. 5. A dominant-negative form of SEK1/MKK4 impairs glucose-induced upregulation of AP-1 activity in INS-1 cells. A: Western blot analysis of INS-1 cells infected with a recombinant lentivirus encoding FLAG-tagged SEK-AL. As a control INS-1 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). Western blots were probed with an antibody against the FLAG-tag. Molecular mass markers in kDa are shown on the left. INS-1 insulinoma cells (B), INS-1ΔB-Raf:ER cells (C), or HepG2 cells (D) were double-infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene and a lentivirus encoding FLAG-tagged SEK-AL. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). INS-1 and INS-1ΔB-Raf:ER cells were serum-starved for 24 h in medium containing 2 mM glucose. Stimulation of infected INS-1 cells with glucose (11 mM) was performed for 48 h (B). Stimulation of infected INS-1ΔB-Raf:ER cells with 4OHT (250 nM) was performed for 24 h (C). Stimulation of infected HepG2 cells with TPA (50 ng/ml) was performed for 24 h (D). Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

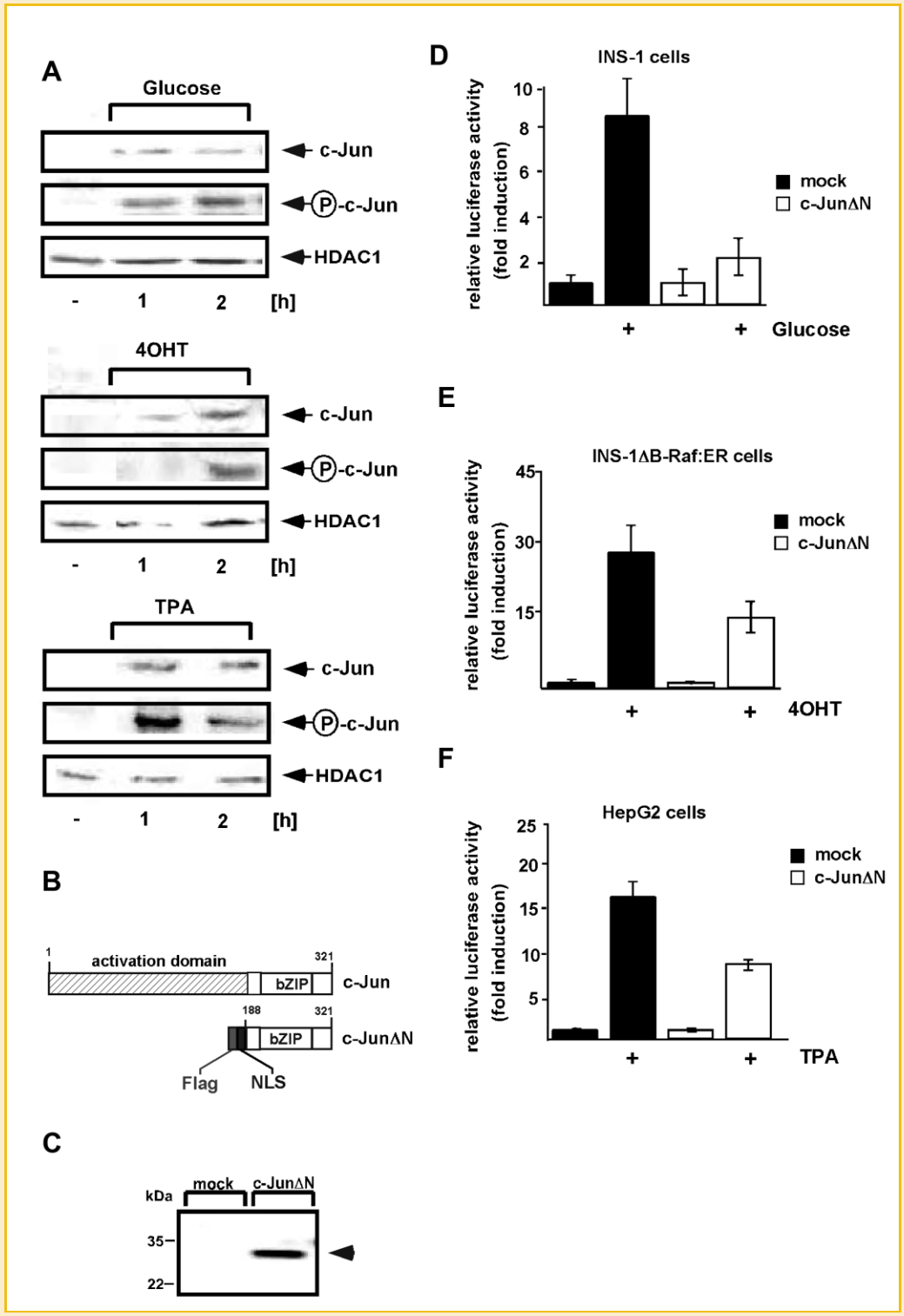


Fig. 6. Glucose-induced upregulation of AP-1 transcriptional activity requires c-Jun. A: Upper panel: INS-1 insulinoma cells were serum-starved for 24 h in medium containing 2 mM glucose and stimulated with glucose (11 mM) for the indicated periods. Middle panel: INS-1ΔB-Raf:ER cells were serum-starved for 24 h in medium containing 2 mM glucose and stimulated with 4OHT (250 nM) for the indicated periods. Lower panel: HepG2 hepatoma cells were serum-starved for 24 h and treated with TPA (50 ng/ml) for 1 or 2 h. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with antibodies directed against either c-Jun, phosphorylated c-Jun, or HDAC1 as indicated. Each experiment illustrated here was repeated a minimum of three times with consistent results. B: Modular structure of c-Jun and the dominant-negative form c-JunΔN. The dominant-negative mutant retains the basic region leucine zipper domain (bZIP) responsible for dimerization and DNA binding but lacks the NH_2-terminal transcriptional activation domain. C: Western blot analysis of INS-1 cells infected with a lentivirus encoding c-JunΔN. As a control, mock-infected cells were analyzed. Western blots were probed with the antibody directed against the FLAG epitope. Molecular mass markers in kDa are shown on the left. D: INS-1 insulinoma cells were double-infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene and FLAG-tagged c-JunΔN. As a control INS-1 cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h in medium containing 2 mM glucose. Stimulation with glucose (11 mM) was performed for 48 h. E: INS-1ΔB-Raf:ER cells were double-infected with lentiviruses encoding a collagenase promoter/luciferase reporter gene and FLAG-tagged c-JunΔN. As a control INS-1ΔB-Raf:ER cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h in medium containing 2 mM glucose. Cells were stimulated with 4OHT (250 nM) for 24 h. F: HepG2 hepatoma cells were double-infected with lentiviruses encoding a collagenase promoter/luciferase reporter gene and FLAG-tagged c-JunΔN. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h and stimulated with TPA (50 ng/ml) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

blot analysis using antibodies targeting the FLAG epitope (Fig. 6C). Expression of c-JunΔN blocked the upregulation of AP-1 activity in glucose-stimulated INS-1 cells (Fig. 6D), indicating that c-Jun is involved in the upregulation of AP-1-mediated gene transcription in INS-1 cells that had been stimulated with glucose. Likewise, AP-1 activity was significantly reduced in 4OHT-stimulated INS-1ΔB-Raf:ER cells (Fig. 6E) and in TPA-treated HepG2 cells (Fig. 6F) that expressed c-JunΔN.

TERNARY COMPLEX FACTOR ACTIVATION IS REQUIRED FOR THE UPREGULATION OF AP-1 ACTIVITY IN GLUCOSE-STIMULATED INS-1 CELLS

It has been shown that glucose stimulation induces the expression and phosphorylation of c-Fos, involving the ERK signaling pathway [Glauser and Schlegel, 2007]. ERK1/2 translocates to the nucleus and phosphorylates and activates ternary complex factors, that in turn stimulate c-Fos gene transcription. We assessed the impact of ternary complex factor activation on the regulation of AP-1 activity in INS-1 cells using a dominant-negative mutant of the ternary complex factor Elk-1, termed REST/Elk-1ΔC (Fig. 7A). This mutant retains the DNA binding and SRF interaction domains but lacks the C-terminal activation domain of Elk-1. REST/Elk-1ΔC additionally contains the N-terminal repression domain of the transcriptional repressor REST [Thiel et al., 1998], a FLAG epitope for immunological detection and a nuclear localization signal (NLS). Nuclear proteins of mock-infected INS-1 cells or INS-1 cells infected with a REST/Elk-1ΔC encoding lentivirus were fractionated by SDS–PAGE and the fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 7B). To demonstrate the activity of REST/Elk-1ΔC, we expressed this fusion protein in INS-1 cells together with the reporter SREluc, that contained the proximal SREs # 1 and 2 of the Egr-1 promoter upstream of a minimal promoter [Rössler and Thiel, 2009]. A schematic representation of the integrated proviruses encoding the SRE-controlled luciferase reporter gene is depicted in Figure 7C. Figure 7D (upper panel) shows that glucose stimulation of INS-1 cells induced a striking increase in SRE-regulated transcription that could be blocked by REST/Elk-1ΔC. Likewise, SRE-mediated transcription was upregulated in 4OHT-stimulated INS-1ΔB-Raf:ER cells and in TPA-treated HepG2 cells (Fig. 7D, middle and lower panels). Expression of REST/Elk-1ΔC reduced or abolished transcription of the SRE-controlled reporter gene, illustrating that the fusion protein functions as a dominant negative in this context. Next, we assessed the biological role of ternary complex factors on the AP-1 activity in glucose-treated INS-1 cells. Figure 7E (upper panel) reveals that expression of REST/Elk-1ΔC blocked the upregulation of AP-1 activity in glucose-stimulated INS-1 cells. Likewise, AP-1 activity was significantly reduced in 4OHT-stimulated INS-1ΔB-Raf:ER cells (Fig. 7E, middle panel) and in TPA-treated HepG2 cells (Fig. 7E, lower panel).

DISCUSSION

Elevated levels of glucose are not only the main stimuli for insulin biosynthesis and secretion in β-cells. Glucose, the most abundant monosaccharide in nature, has additionally a profound effect on gene transcription in β-cells and other cell types [Vaulont et al., 2000]. A microarray study suggested that the transcription factor AP-1 may play an important role in the upregulation of transcription in MIN6 insulinoma cells that had been stimulated with glucose and cpt-cAMP. More than 200 glucose-regulated genes of 755 encode proteins involved in signaling and transcriptional regulation. In addition, glucose regulates the expression of genes involved in the secretory pathway and the control of β-cell mass [Glauser et al., 2006]. Interestingly, expression of synaptotagmin-4 is upregulated in glucose and cpt-cAMP-treated insulinoma cells. Synaptotagmin-4 has been described as a key mediator of NGF enhancement of Ca^{2+}-evoked release in PC12 cells [Mori et al., 2008].

We are interested in understanding the mechanisms of selective gene transcription following cellular stimulation. The objective of this study was to investigate the regulation of AP-1 activity in INS-1 insulinoma cells. To measure AP-1 transcriptional activity, we used an AP-1 responsive collagenase promoter/luciferase reporter gene. Frequently, reporter genes are introduced into cultured cells via transient transfection of plasmids. This approach has the disadvantage that the structure of these plasmids may be incompletely organized in comparison to cellular chromatin [Smith and Hager, 1997], and may thus resemble a prokaryotic gene organization including a nonrestrictive transcriptional ground state. In contrast, the chromatin structure in eukaryotes causes a restrictive ground state, occluding proteins such as RNA polymerases and transcriptional regulators from binding to DNA. Hence, promoter/reporter genes should be integrated into the chromatin to investigate transcriptional regulatory mechanisms. We therefore used a lentivirus-based technique to implant the reporter gene into the chromatin of hepatoma cells. This strategy enabled us to analyze AP-1-mediated gene transcription of the collagenase promoter/luciferase reporter gene that was packed into an ordered chromatin structure. Using this strategy we showed that glucose stimulation triggers an upregulation of AP-1 activity in INS-1 insulinoma cells.

There is no dispute about the essential role of increased Ca^{2+} concentration in glucose-treated β-cells. The results presented here show that upregulation of AP-1 transcriptional activity relies on the activation of L-type Ca^{2+} channels and the subsequent increase of the intracellular Ca^{2+} concentration. The involvement of L-type Ca^{2+} channels in stimulation–transcription coupling was verified using a pharmacological approach. We additionally used an alternate approach to assess the role of cytoplasmic Ca^{2+} ions for the stimulus-induced upregulation of AP-1 activity. Expression of a parvalbumin-mCherry fusion protein in the cytosol was used to buffer Ca^{2+} in this compartment. Accordingly, activation of AP-1 transcriptional activity was reduced when the glucose-induced upregulation of Ca^{2+} was buffered. Together, these data show that Ca^{2+} influx and the subsequent rise of the cytosolic Ca^{2+} concentration are essential for the activation of AP-1 in glucose-stimulated INS-1 insulinoma cells.

Influx of Ca^{2+} ions via L-type voltage-dependent Ca^{2+} channels has been described to be the essential mediator of ERK1/2 activation by glucose in INS-1 cells [Arnette et al., 2003]. The results presented here reveal that—in addition to an influx of Ca^{2+} ions into the

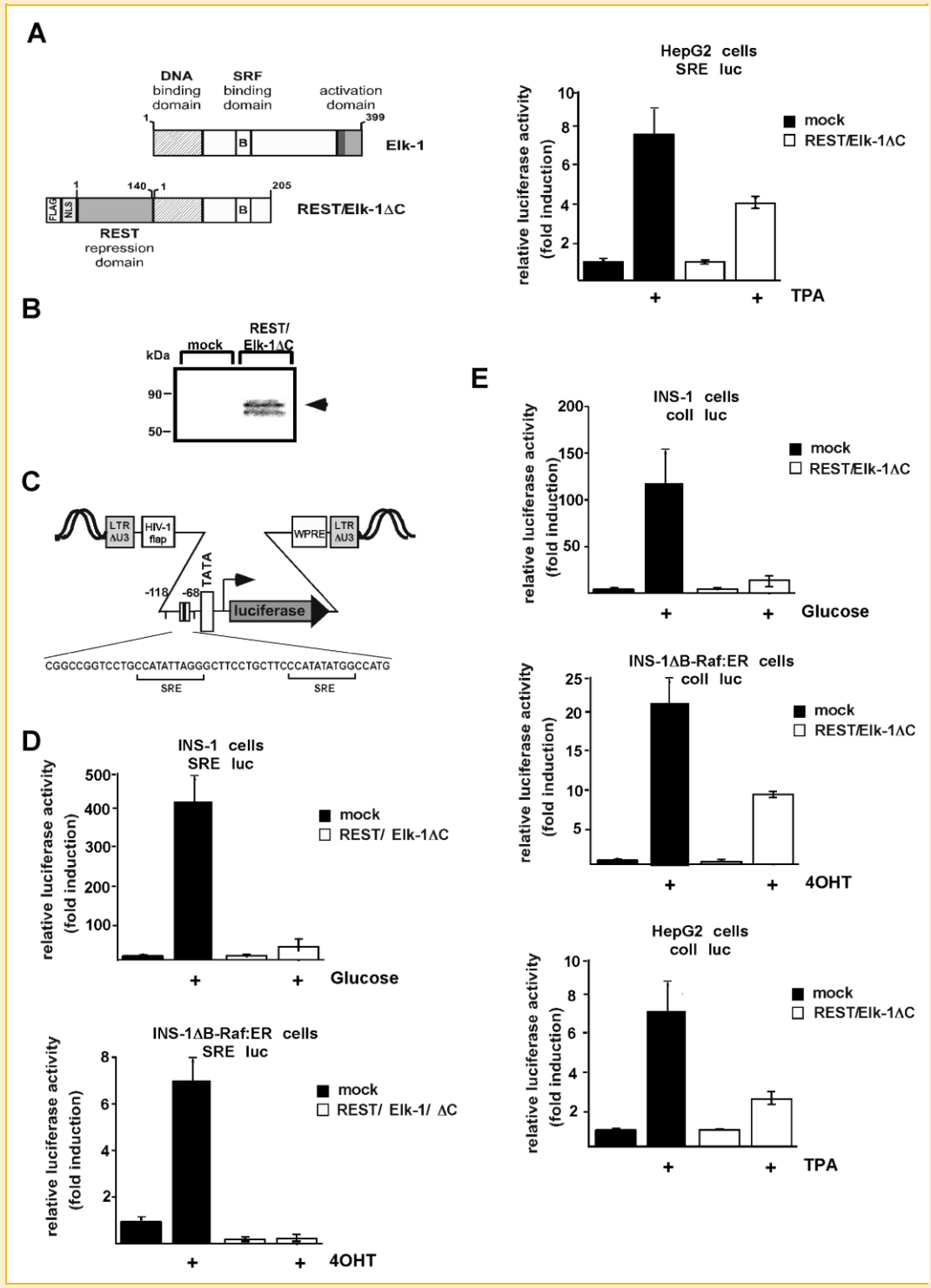


Fig. 7. Suppression of ternary complex factor activity prevents glucose-induced upregulation of AP-1 activity. A: Schematic representation of wild-type Elk-1 and the dominant-negative mutant REST/Elk-1ΔC. The DNA binding domain is located on the N-terminus. The transcriptional activation domain is located on the C-terminus. A regulatory domain lies within this transcriptional activation domain encompassing the key phosphoacceptor sites S383 and S389. Elk-1 binds with its B-domain to SRF, allowing the formation of the ternary Elk-1–SRF complex. The B-domain also couples the C-terminal phosphorylation of Elk-1 with enhanced DNA binding via the ETS domain. The dominant-negative mutant REST/Elk-1ΔC lacks the phosphorylation-regulated activation domain but retains the DNA and SRF binding domains. The truncated Elk-1 is expressed as a fusion protein together with a transcriptional repression domain derived from the transcriptional repressor REST. B: Western blot analysis of INS-1 cells either mock infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC. Western blots were probed with an antibody against the FLAG-tag. Molecular mass markers in kDa are shown on the left. C: Schematic representation of an integrated provirus encoding a SRE-controlled reporter gene. The transcription unit contains the two proximal SREs # 1 and 2 of the Egr-1 promoter upstream of a minimal promoter. D: INS-1 cells (upper panel), INS-1ΔB-Raf:ER cells (middle panel), and HepG2 hepatoma cells (lower panel) were double-infected with lentiviruses encoding a SRE-controlled luciferase reporter gene and FLAG-tagged REST/Elk-1ΔC. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h and stimulated with either glucose (11 mM, upper panel) for 48 h, or 4OHT (250 nM, middle panel) or TPA (50 ng/ml, lower panel) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E: INS-1 cells (upper panel), INS-1ΔB-Raf:ER cells (middle panel), and HepG2 hepatoma cells (lower panel) were double-infected with lentiviruses encoding a collagenase promoter/luciferase reporter gene and FLAG-tagged REST/Elk-1ΔC. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 h and stimulated with glucose (11 mM, upper panel) for 48 h, and 4OHT (250 nM, middle panel) or TPA (50 ng/ml, lower panel) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

cytoplasm—an activation of ERK1/2 is essential for the induction of AP-1 transcriptional activity in glucose-stimulated INS-1 cells. We generated INS-1 cells expressing a ΔB-Raf/ER fusion protein that functions as a conditionally active form of B-Raf. In these cells we were able to activate the ERK1/2 signaling pathway in a ligand-independent manner. The results showed that activation of ERK1/2 is sufficient to increase the AP-1 transcriptional activity in INS-1 cells.

Using the p38-specific inhibitor SB203580, we have clearly shown that p38 is involved in the regulation of AP-1 activity in glucose-stimulated insulinoma cells. These results were corroborated by expression experiments involving constitutively active MKK6E that alone induced an upregulation of AP-1 transcriptional activity. Together with glucose stimulation, a synergistic effect on AP-1-mediated reporter gene transcription was observed. In contrast, the upregulation of AP-1 transcriptional activity in 4OHT-treated INS-1ΔB-Raf:ER cells was not blocked by pretreatment with SB203580. These data indicate that upregulation of AP-1 as a result of B-Raf activation is independent of p38.

The involvement of JNK in the stimulus-induced upregulation of AP-1 activity in INS-1 cells has been assessed using INS-1 cells expressing a dominant-negative mutant of SEK1/MKK4, SEK-AL, that inhibits the phosphorylation and activation of JNK. Although SEK1/MKK4 has been shown to phosphorylate both JNK and p38 when overexpressed in mammalian cell lines [Lin et al., 1995], three lines of evidence indicate that this kinase functions as a specific activator of JNK and not p38: Firstly, SEK1/MKK4 coprecipitates with JNK but not with p38; secondly, the dominant-negative mutant SEK-AL impaires JNK but not p38 activation in mouse fibroblasts; thirdly, the activation of JNK, but not the activation of p38 was impaired in SEK1/MKK4-deficient cells [Zanke et al., 1996; Yang et al., 1997]. Thus, the fact that SEK-AL expression completely inhibits the upregulation of AP-1-mediated gene transcription indicates that activation of JNK is part of the signaling cascade that leads to enhanced AP-1 activity in glucose-stimulated INS-1 insulinoma cells. SEK-AL expression also inhibited the upregulation of AP-1 activity as a result of ERK1/2 activation in INS-1ΔB-Raf:ER cells, suggesting that ERK1/2 activation may also trigger an activation of JNK in these cells.

Initially described as a heterodimer of c-Jun and c-Fos, the current view is that the AP-1 actually comprises several distinct homodimers or heterodimers composed of various members of the Fos, Jun, and ATF bZIP subfamilies. The fact that c-Jun is activated by several distinct MAP kinases indicates that c-Jun is in the center of intracellular signaling pathways leading to changes in gene transcription. JNK directly phosphorylates serine residues 63 and 73 in the NH_2-terminal activation domain of c-Jun. Activated c-Jun increases c-Jun expression through binding to two AP-1 sites located in the proximal c-Jun promoter [Angel et al., 1988]. Stimulation of the ERK1/2 signaling pathway induces c-Jun expression in HeLa and PC12 cells [Leppä et al., 1998; Gupta and Prywes, 2002]. Expression of p38 protein kinase together with a constitutively active MKK6 strongly induced c-Jun promoter activity in HeLa cells [Gupta and Prywes, 2002]. p38 has also been shown to induce c-Jun phosphorylation in cultured cerebellar granule neurons [Yamagishi et al., 2001]. Here, we have assessed the involvement of c-Jun in glucose-regulated AP-1 activity in insulinoma cells using a dominant-negative mutant of c-Jun, c-JunΔN. Induction of AP-1 activity was almost completely blocked in INS-1 cells expressing c-JunΔN, indicating that c-Jun is one of the bZIP proteins constituting the AP-1 complex in glucose-stimulated INS-1 cells. The reduction of AP-1 activity in 4OHT-treated INS-1ΔB-Raf:ER cells was less pronounced. Recently, it has been shown that activation of the ERK1/2 pathway is sufficient to induce c-Fos phosphorylation and transcriptional activation in 293 cells [Gilley et al., 2009] and an upregulation of c-Fos expression in glucose-stimulated insulinoma cells has been shown [Glauser and Schlegel, 2007]. Stimulation of c-Fos gene transcription as a result of ERK1/2 activation is mainly regulated by phosphorylation and activation of ternary complex factors such as Elk-1 or Sap1. These proteins are also substrates for p38, and JNK [Raingeaud et al., 1996; Whitmarsh et al., 1997], indicating that they function as convergence point of cellular signaling cascades. We have examined the necessity of ternary complex factor activation for glucose-induced upregulation of AP-1 activity using a dominant negative version of Elk-1 in loss-of-function experiments. Expression of this Elk-1 mutant revealed that ternary complex factor activation is a key step that connects glucose stimulation with enhanced transcriptional activity of AP-1.

In summary, we have shown that Ca^{2+} influx into INS-1 insulinoma cells as a result of glucose stimulation promotes an upregulation of AP-1 activity. The signaling cascade includes an influx of Ca^{2+} ions and the activation of the the three major MAP kinases (ERK1/2, p38 and JNK). On the transcriptional level, c-Jun and Elk-1 activity is required to connect the glucose-induced signaling cascade with AP-1.

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