Activating Transcription Factor-2 Is a Positive Regulator in CaM Kinase IV–Induced Human Insulin Gene Expression

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Insulin plays a crucial role in the regulation of glucose-homeostasis, and its synthesis is regulated by several stimuli. The transcription of the human insulin gene, enhanced by an elevated intracellular concentration of calcium ions, was completely blocked by Ca^{2+}/calmodulin-dependent protein kinase inhibitor. The activity of the transcription factor activating transcription factor-2 (ATF-2), which binds to the cAMP responsive elements of the human insulin gene, was enhanced by Ca^{2+}/ calmodulin-dependent protein kinase IV (CaMKIV). Mutagenesis studies showed that Thr^{69}, Thr^{71}, and Thr^{73} of ATF-2 are all required for activation by CaMKIV. CaMKIV-induced ATF-2 transcriptional activity was not altered by activation of c-Jun NH_2-terminal protein kinase (JNK) or p38 mitogen-activated protein (MAP) kinase. Furthermore, when transfected into rat primary cultured islets, ATF-2 enhanced glucose-induced insulin promoter activity, whereas cAMP response element–binding protein (CREB) repressed it. These results suggest a mechanism in which ATF-2 regulates insulin gene expression in pancreatic β-cells, with the transcriptional activity of ATF-2 being increased by an elevated concentration of calcium ions. *Diabetes* 49:1142–1148, 2000

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ATF-2, activating transcription factor-2; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; CRE, cyclic AMP responsive element; CREB, cyclic AMP response element–binding protein; FCS, fetal calf serum; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MEK, MAP kinase kinase; MEKK, MEK kinase; PCR, polymerase chain reaction; PKA, protein kinase A; RT, reverse transcriptase; TBS-T, Tris-buffered saline containing 0.1% Tween-20.

The intracellular calcium ion concentration influences a variety of biochemical signaling pathways, and the convergence and divergence of the intracellular signaling pathways modulate the activity of transcription factors. Cyclic AMP response element–binding protein (CREB) is a well-characterized transcription factor (1–5). Ser^{133} of CREB is phosphorylated by protein kinase A (PKA) and phosphorylation of this residue is essential for PKA-mediated activation of CREB (1). Both Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) and IV (CaMKIV) can phosphorylate Ser^{133} of CREB, but CaMKII also phosphorylates the second site (Ser^{142}) within the transcriptional activation domain of CREB, blocking the activation that would otherwise occur following phosphorylation of Ser^{133} (4). The phosphorylated CREB then interacts with a basal transcription unit through binding to the coactivators, such as CREB-binding protein (CBP) or p300 (2,3,5).

The E1A-inducible adenoviral gene and the cAMP-inducible cellular gene contain cAMP responsive elements (CREs). Activating transcription factor-2 (ATF-2, also called CRE-BP1), is a member of CREB/ATF family, characterized by their kination domains and basic leucine zipper domains (b-ZIP domains) (6–8). ATF-2 is capable of forming homodimers or heterodimers with c-Jun and of binding to CREs, including the promoter region in the human insulin gene (9). ATF-2 is characterized by its role in c-Jun NH_2-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase signal transduction pathways (10). JNK and p38 MAP kinase phosphorylate ATF-2 at Thr^{69}, Thr^{71}, and Ser^{90}, which lie close to the NH_2-terminal transcriptional activation domain and stimulate its transactivating capacity.

CREB and ATF-2 are linked to distinct signaling cascades, but it is not known if other intracellular signals are involved. In this study, we demonstrate that the transcriptional activity of ATF-2 is increased by elevation of the intracellular calcium ion concentration through the activation of CaMKIV and that the activated ATF-2 increases insulin gene transcription (while CREB represses it), suggesting a mechanism in which divergence in calcium signaling pathways regulates gene expression.

RESEARCH DESIGN AND METHODS

Plasmids. Plasmids for 5×GAL4-TATA-luciferase reporter gene, expression of p300, full-length ATF-2 and CREB, and expression of PKA catalytic domain were provided by Dr. Maurer (Oregon Health Sciences University, Portland,

OR), Dr. Goodman (Oregon Health Sciences University, Portland, OR), Dr. Ishii (Riken, Tsukuba, Japan), and Dr. Arai (Dnax Research Institute, Palo Alto, CA), respectively.

The human insulin promoter-luciferase or chloramphenicol acetyltransferase (CAT) reporter gene contains the human insulin promoter (–339 to +112) upstream of the coding sequence of the firefly luciferase or CAT.

GAL4-ATF-2 and GAL4-c-Jun expression plasmids containing the DNA-binding domain of GAL4 and the transcriptional activation domain of ATF-2 or c-Jun were constructed as follows. The control pCG4 was generated by introducing the coding sequence for the GAL4 DNA-binding domain (amino acids 1–147) from pGBT9 (Clonetech, Palo Alto, CA) downstream of the cytomegalovirus promoter in plasmid pCMV6c. The NH_2-terminal region of human ATF-2 (amino acids 1–341) and mouse c-Jun (amino acids 1–177) were then fused in-frame to the 3′ end of the GAL4 DNA-binding domain in plasmid pCG4. The mutagenesis studies were performed using in vitro Mutagenesis Systems (Promega, Madison, WI).

Expression vectors for the catalytic domains of rat CaMKII, human CaMKIV, and mouse MAP kinase kinase (MEK) kinase (MEKK) and the dominant negative forms of JNK and p38 MAP kinase were constructed by polymerase chain reaction. The sequences of the cloning fragments were confirmed.

Cell culture and transfections. HIT-T15 cells, a hamster insulin-producing cell line, were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% FCS.

Plasmid DNAs were transfected into HIT-T15 and HeLa cells through a standard calcium phosphate precipitation procedure (11). If indicated, 3.75 mmol/l EGTA and 1 μmol/l glibenclamide or 30 μmol/l KN-93 (Ca^{2+}/calmodulin-dependent protein kinase inhibitor) (Seikagaku, Tokyo) was added to the medium 6 h before harvest. These cells were harvested 48 h after transfection, and luciferase activity was determined according to the manufacturer's manual (Nippon Gene, Tokyo). Luciferase activity values were normalized to β-gal activity.

Reverse transcriptase–polymerase chain reaction. The reverse transcriptase (RT)–polymerase chain reaction (PCR) analyses were carried out using cDNAs derived from total RNA of rat brain, spleen, liver, skeletal muscle, pancreatic islets or rat insulin-producing RINm5F cells with oligonucleotide primers as follows: for rat CaMKIV, sense, 5′-TGCACCTGAGATTCT CCGAG-3′ and antisense, 5′-GATCTGTCTTGTCCTTGCCG-3′; for rat β-actin, sense, 5′-ATCCGTAAAGACCTCTATGC-3′ and antisense, 5′-AACGCAGCTCA GTAACAGTC-3′. Thirty-three or 25 PCR cycles were conducted at 94°C/15 s, 55°C/15 s, and 72°C/30 s with the indicated primers for rat CaMKIV and β-actin, respectively. The PCR products were electrophoresed on a 2% agarose gel. To exclude any amplification product derived from genomic DNA that could contaminate the RNA preparation, total RNA without RT was amplified as a negative control.

Immunoblotting analysis. HIT-T15 cells were harvested and whole cell extracts were prepared for immunoblotting analysis. These cells were lysed in 1 ml cell lysis buffer, which contained 10 mmol/l Tris-HCl (pH 7.4), 2 mmol/l EDTA, 100 mmol/l NaCl, 20% glycerol (wt/vol), and protease inhibitors. Proteins were resolved on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) by electroblotting. The membranes were blocked in 5% low-fat dried milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T, pH 8.0) overnight at 4°C. After extensive washing in TBS-T, the membranes were incubated for 1 h at 37°C with the primary antibody.

The primary antibodies—rabbit anti–ATF-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-CaMKIV polyclonal antibody (M-20; Santa Cruz Biotechnology), and rabbit phospho-ATF-2 (Thr^{71}) antibody (New England Biolabs, Beverly, MA)—were used at a 1:2,000, 1:5,000, and 1:2,000 dilution with TBS-T, respectively. Detection was performed using the ECL kit (Amersham, Buckinghamshire, U.K.) and required a secondary horseradish peroxidase-conjugated antibody (diluted to 1:2,000, except for detection of ATF-2, 1:5,000). The expression levels of ATF-2, CaMKIV, and phospho-ATF-2 (Thr^{71}) were determined using NIH Image (12).

Immunohistochemistry. The pancreases of 8-week-old Wistar rats were removed under pentobarbital anesthesia (40 mg/kg body weight) and fixed in Bouin's solution. Pancreatic specimens were embedded in paraffin and sectioned at 3.5 μm. The avidin-biotin complex method with alkaline phosphatase was used as previously described (13) with a slight modification. After deparaffinization, normal goat serum (diluted to 1:75) (Dako, Kyoto, Japan) for the inhibition of nonspecific binding of secondary antibody, rabbit anti–ATF-2 polyclonal antibody (diluted to 1:500) or rabbit anti-insulin polyclonal antibody (diluted to 1:500) (Dako), the biotin-labeled goat anti-rabbit IgG serum (diluted to 1:300) (Dako), and avidin-biotin-alkaline phosphatase complex (diluted to 1:100) (Vector Laboratories, Burlingame, CA) were sequentially applied on consecutive sections, followed by hematoxylin nuclear counterstaining. Staining was visualized in black and red by alkaline phosphatase substrate (Vector Laboratories) for ATF-2 and insulin, respectively.

Islet cell preparation and transfection. Pancreatic islets were isolated from male Wistar rats weighting 200–220 g by collagenase digestion (14), followed by purification on Ficoll gradients. After isolation, dispersion of the islet cells was accomplished as described (15). The dispersed cells were then transferred into RPMI 1640 medium supplemented with 5% FCS.

Soon after preparation, islet cells were transfected with 7 μg plasmid DNA using DOTAP liposomal transfection reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Six hours after transfection, the medium was replaced by RPMI 1640 with the indicated glucose concentrations and 20% FCS. Forty-eight hours after transfection, CAT activities were measured as described previously (11). Radioactivity was estimated with a FUJIX Bio-Imaging Analyzer BAS-2000 system (FUJI Photo Film, Tokyo).

Statistical analysis. Statistical analyses were performed by an unpaired *t* test.

RESULTS

Expression of ATF-2 in pancreatic β-cells. To determine if ATF-2 is expressed in pancreatic β-cells, immunohistochemical

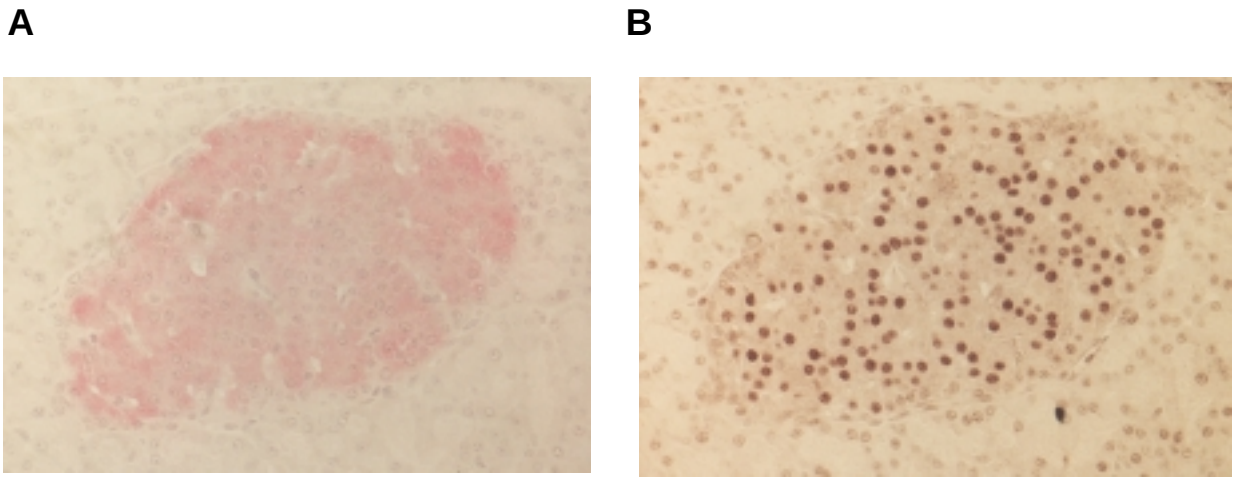

FIG. 1. ATF-2 expression in pancreatic β-cells. Expression of insulin and ATF-2 in rat pancreatic islets was examined on consecutive 3.5-μm sections. *A*: Immunohistochemical analysis of insulin. The pancreatic β-cells were confirmed by staining the cytoplasm with anti-insulin antibody. Hematoxylin nuclear counterstaining, original magnification ×400. *B*: Immunohistochemical analysis of ATF-2. Immunohistochemical localization of the ATF-2 protein in pancreatic islets of 8-week-old rats was shown using anti–ATF-2 antibody. Intense nuclear staining for ATF-2 was observed in pancreatic β-cells. Hematoxylin nuclear counterstaining, original magnification ×400.

analyses were performed in pancreatic islets of 8-week-old rats using anti–ATF-2 antibody and anti-insulin antibody. Pancreatic β-cells were detected by cytoplasmic staining with the anti-insulin antibody (Fig. 1*A*). More than 90% of pancreatic β-cells showed intense nuclear staining by anti–ATF-2 antibody (Fig. 1*B*).

Calcium influx and CaMKIV activation increases insulin gene expression. To examine the effects of calcium on insulin gene expression, hamster insulin-producing HIT-T15 cells were transfected with the ATF-2 expression plasmid and the human insulin promoter-luciferase reporter gene. Forty-two hours after transfection, these cells were further incubated with 1 µmol/l glibenclamide in the presence or absence of 3.75 mmol/l EGTA for 6 h. In the absence of ATF-2, glibenclamide increased insulin promoter activity by 23% (Fig. 2*A*). In the presence of ATF-2, glibenclamide increased insulin promoter activity by 75% ($P < 0.05$), but this increment was abolished in the presence of EGTA, suggesting that influx of extracellular calcium ions is important for the increase in insulin promoter activity induced by ATF-2 (Fig. 2*B*). A CaM kinase inhibitor (KN-93) (16) has been shown to significantly reduce the glibenclamide-induced insulin gene promoter activity, indicating that ATF-2 increases insulin gene expression through activation of CaM kinase.

Transcriptional activation of ATF-2 by CaMKIV. To examine the signals that control ATF-2 transcriptional activity, we co-transfected the activated forms of PKA, CaMKII, or CaMKIV with the GAL4-ATF-2 expression plasmid and the 5×GAL4-TATA-luciferase reporter gene (4) in HeLa cells where little ATF-2 is expressed so as to avoid the interference by endogenous ATF-2. We found that the activated form of CaMKIV increased ATF-2 transcriptional activity 12.9-fold ($P < 0.01$, Fig. 3*A*). On the contrary, neither the activated form of PKA nor CaMKII was effective in activating ATF-2

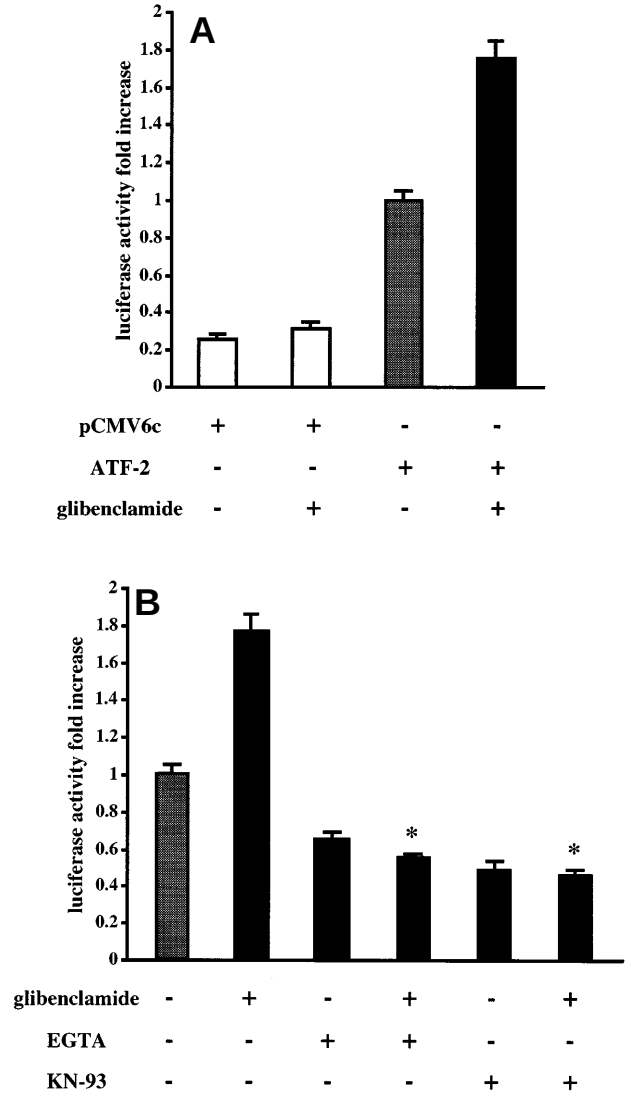

FIG. 2. Effects of calcium influx and CaMK on insulin gene expression. *A*: HIT-T15 cells were cotransfected with 5 µg human insulin promoter-luciferase reporter gene and 5 µg ATF-2 expression plasmid or the empty expression vector by the calcium phosphate precipitation method. Six hours before harvest of the cells, 1 µmol/l glibenclamide was added. Luciferase activity was determined 48 h after transfection. *B*: HIT-T15 cells were cotransfected with 5 µg ATF-2 expression plasmid and 5 µg human insulin promoter-luciferase reporter gene by the calcium phosphate precipitation method. Six hours before harvest of the cells, 3.75 mmol/l EGTA, 1 µmol/l glibenclamide, or 30 µmol/l KN-93 was added. Luciferase activity was determined 48 h after transfection. Data are means ± SE. *$P < 0.05$.

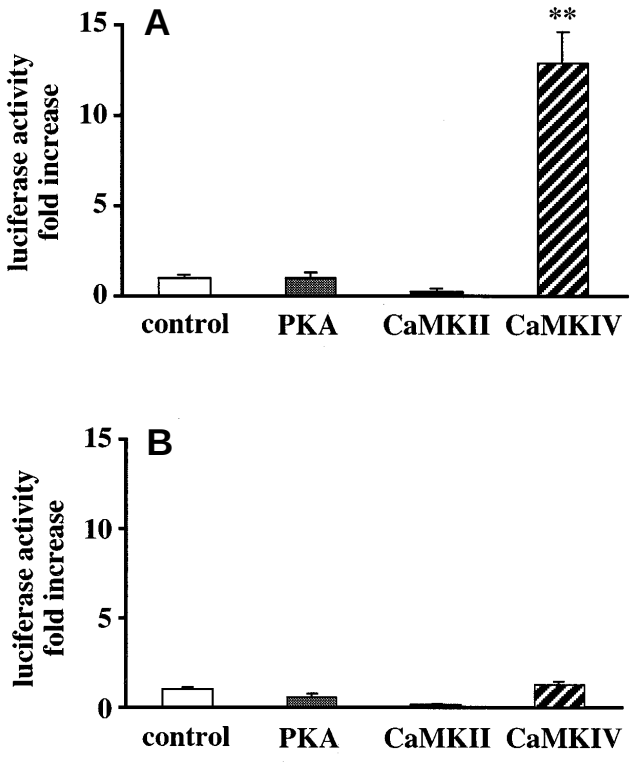

FIG. 3. ATF-2 activation by CaMKIV. *A*: Effects of PKA, CaMKII, and CaMKIV on ATF-2 transactivity. HeLa cells were cotransfected with 4 µg of 5×GAL4-TATA-luciferase reporter gene, 4 µg GAL4-ATF-2, the fusion protein for GAL4 DNA-binding domain and the NH_2-terminal region of ATF-2 (amino acids 1–341), and 4 µg of an expression plasmid for PKA, CaMKII, or CaMKIV by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection. *B*: Effects of PKA, CaMKII, and CaMKIV on c-Jun transactivity. HeLa cells were cotransfected with 4 µg of 5×GAL4-TATA-luciferase reporter gene, 4 µg of an expression plasmid for GAL4 DNA-binding domain and the NH_2-terminal region of c-Jun (amino acids 1–177), and 4 µg of an expression plasmid for PKA, CaMKII, or CaMKIV by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection. Data are means ± SE. **$P < 0.01$.

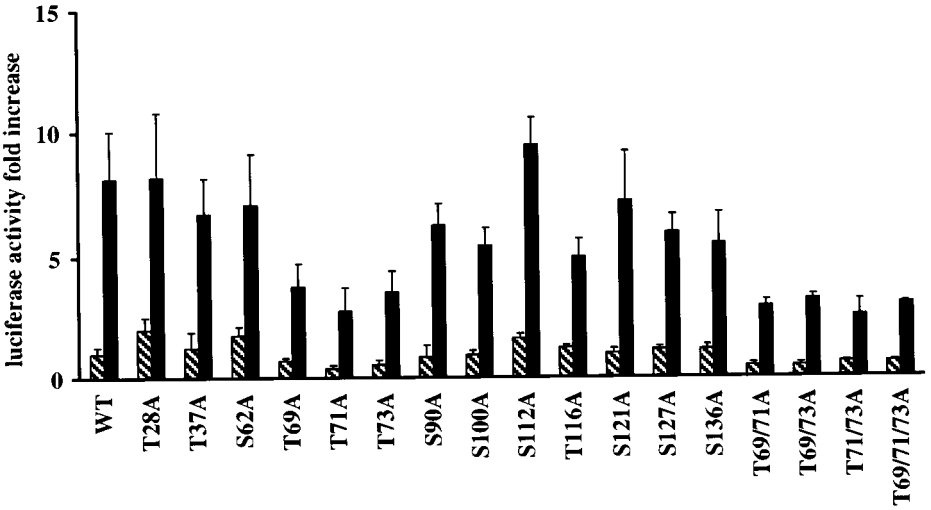

FIG. 4. Identification of amino acid residues responsible for CaMKIV-induced ATF-2 transactivity. HeLa cells were transfected with 4 µg of 5×GAL4-TATA-luciferase reporter gene, 4 µg of an expression vector for GAL4-ATF-2 fusion protein (wild type [WT] or mutants as indicated), and 4 µg of an expression plasmid for p300 in the absence (▨) or presence (■) of 4 µg of an expression vector for CaMKIV. Luciferase activity was determined 48 h after transfection. The degree of transactivation (compared with the wild-type ATF-2 without the CaMKIV plasmid) is indicated as means ± SE.

(Fig. 3*A*). CaMKIV had no effect on the GAL4-c-Jun expression plasmid containing the GAL4 DNA-binding domain and the transcriptional activation domain of c-Jun (Fig. 3*B*).

Thr^{69}, Thr^{71}, and Thr^{73} of ATF-2 are necessary for the activation of ATF-2 by CaMKIV. To determine the CaMKIV-phosphorylation sites in ATF-2, we carried out transfection studies of mutant GAL4-ATF-2 fusion proteins. Each of the serine and threonine residues in ATF-2 was mutated to an alanine residue. The mutated residues included Thr^{28}, Thr^{37}, Ser^{62}, Thr^{69}, Thr^{71}, Thr^{73}, Ser^{90}, Ser^{100}, Ser^{112}, Thr^{116}, Ser^{121}, Ser^{127}, and Ser^{136}. When Thr^{69}, Thr^{71}, and Thr^{73} of ATF-2 were mutated to alanine, the stimulating effects of CaMKIV were reduced compared with wild-type ATF-2 (Fig. 4). Replacement of other serine and threonine residues allowed CaMKIV to increase the ATF-2 transcriptional activity in HeLa cells.

CaMKIV is expressed in rat pancreatic islets and insulin-producing cell lines, and Thr^{71} of ATF-2 is phosphorylated by the elevated intracellular calcium ion concentration. Gene expression of CaMKIV in rat islets and rat insulin-producing RINm5F cells was examined using RT-PCR. CaMKIV was expressed not only in rat brain and spleen but also in rat pancreatic islets and RINm5F cells (Fig. 5*A*). CaMKIV transcripts were barely detected in rat liver and skeletal muscle, consistent with that previously reported (17). CaMKIV expression was also detected in hamster insulin-producing HIT-T15 cells by immunoblotting using an anti-CaMKIV antibody (Fig. 5*B*, top panel).

To determine if ATF-2 is phosphorylated in response to an elevated calcium ion concentration, we performed the immunoblot

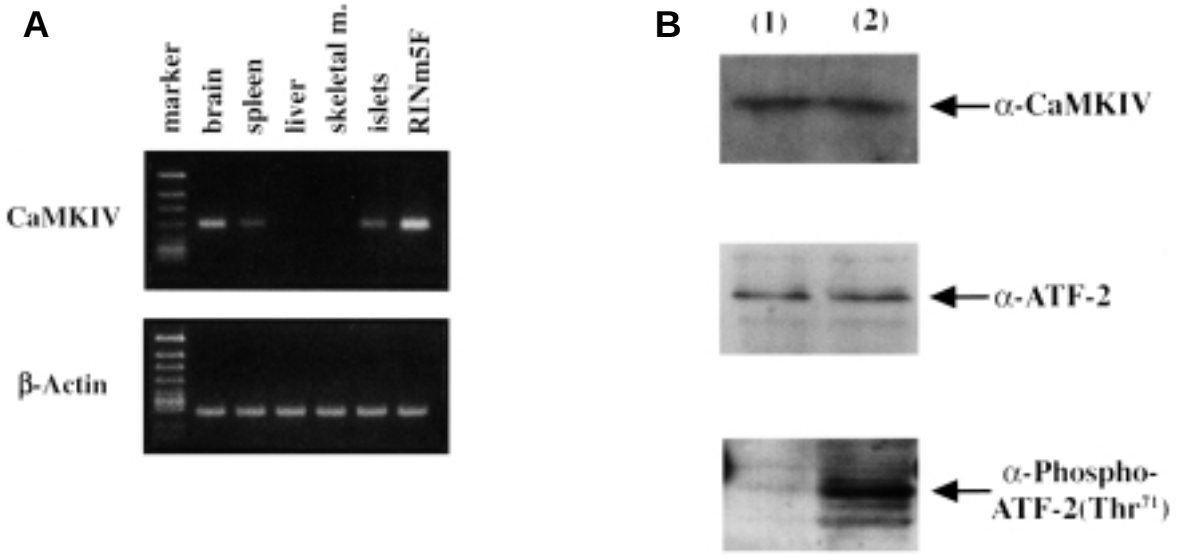

FIG. 5. RT-PCR analyses of CaMKIV and immunoblotting analyses of CaMKIV, ATF-2, and phospho–ATF-2 (Thr^{71}). *A*: RT-PCR analysis. Complementary DNA derived from rat brain, spleen, liver, skeletal muscle, pancreatic islet, and RINm5F cell cDNAs were amplified with specific primers for rat CaMKIV and rat β-actin. Marker: *Hinc*II-digested ϕX174. *B*: Immunoblotting analysis. HIT-T15 cells were cultured in the absence (lane 1) or presence (lane 2) of 1 µmol/l glibenclamide for 6 h and used for immunoblotting analyses with the anti-CaMKIV (top), anti–ATF-2 (middle), or anti–phospho-ATF-2 (Thr^{71}) (bottom) antibodies.

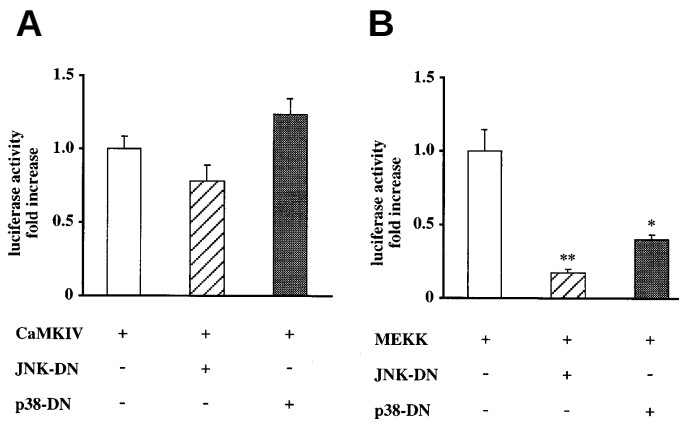

FIG. 6. The CaMKIV-induced signal transduction pathway is independent of JNK-induced or p38 MAP kinase–induced signal transduction pathways. HeLa cells were transfected with 4 µg of 5×GAL4-TATA-luciferase reporter gene, 4 µg of an expression vector for GAL4-ATF-2 fusion protein, 4 µg of an expression vector for CaMKIV (*A*) or 1 µg of an expression vector for MEKK (*B*), and 4 µg of an expression vector for a dominant negative form of JNK (JNK-DN) or p38 MAP kinase (p38-DN) by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection. Data are means ± SE. **P* < 0.05; ***P* < 0.01.

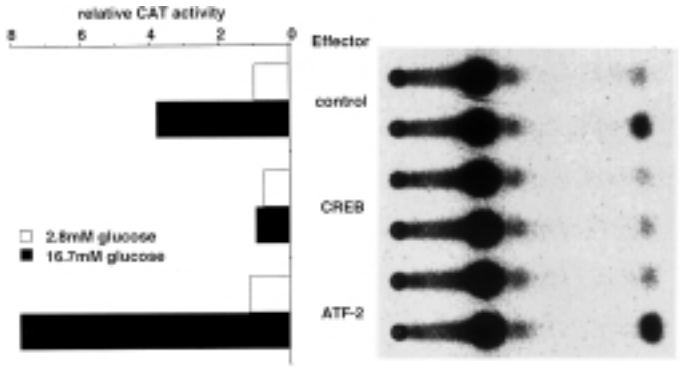

FIG. 7. Effects of coexpression of ATF-2 and CREB on glucose-induced insulin gene promoter activity. Rat primary cultured islets were cotransfected with 6.6 µg human insulin promoter-CAT reporter gene, 0.15 µg pCMV-Luciferase plasmid, and 0.25 µg CREB or ATF-2 expression plasmids or the control effector plasmid. CAT activities were measured and are shown on the right. Relative CAT activities are shown by the graph on the left. CAT activity values were normalized to luciferase activity. The experiments are representative of at least 3 similar experiments.

analysis to examine the phosphorylated Thr[71] of ATF-2 in HIT-T15 cells treated with glibenclamide and measured their expression levels by NIH Image. The expression levels of CaMKIV (Fig. 5*B*, top panel) and ATF-2 (Fig. 5*B*, middle panel) in HIT-T15 cells were not altered by treatment with 1 µmol/l glibenclamide. On the other hand, the level of phosphorylated ATF-2 was increased 82-fold in HIT-T15 cells treated with 1 µmol/l glibenclamide (Fig. 5*B*, bottom panel).

Signal transduction induced by CaMKIV is independent of JNK or p38 MAP kinase. Because Thr[69] and Thr[71] of ATF-2 are the phosphorylation sites of JNK and p38 MAP kinase, we investigated whether ATF-2 activation by CaMKIV is mediated by JNK or p38 MAP kinase. HeLa cells were transfected with expression plasmids for GAL4-ATF-2 fusion protein, the 5×GAL4-TATA-luciferase reporter gene, a constitutively active form of CaMKIV (Fig. 6*A*), or MEKK (Fig. 6*B*), together with a dominant negative form of JNK or p38 MAP kinase. The ATF-2 transcriptional activity induced by CaMKIV remained unchanged with the dominant negative forms of JNK or p38 MAP kinase (Fig. 6*A*). On the other hand, MEKK-induced activation of ATF-2 was significantly inhibited in the presence of dominant negative forms of JNK ($P < 0.01$) or p38 MAP kinase ($P < 0.05$) (Fig. 6*B*), suggesting that neither JNK nor p38 MAP kinase is involved in the CaMKIV-induced signal transduction pathway. We have also observed that this CaMKIV-induced signal transduction was not inhibited by dominant negative JNK or p38 MAP kinase in HIT-T15 cells.

Contrary effects of ATF-2 and CREB on glucose-induced insulin gene promoter activity. Although both ATF-2 and CREB bound to CREs of the insulin promoter, it is not known how these proteins affect the promoter. The human insulin promoter-CAT reporter gene and the ATF-2 or CREB expression plasmids were co-transfected into rat primary cultured islets. As shown in Fig. 7, a high glucose concentration also activated insulin promoter activity, whereas CREB repressed glucose-induced insulin promoter activity. On the other hand, ATF-2 enhanced the glucose-induced promoter activity twofold, but did not affect it at low glucose levels. Thus, ATF-2 and CREB have contrary effects on insulin promoter activity.

DISCUSSION

We have demonstrated in the present study that ATF-2 activated by CaMKIV mediates calcium-induced insulin gene expression.

Both convergence and divergence of certain signaling pathways in transcriptional regulation are found. CREB is a well-characterized transcription factor, and the phosphorylation of Ser[133] of CREB is critical for the activation of CREB by PKA and CaMKIV (1,4). ATF-2 was originally isolated as a protein binding to cAMP response elements (6) and belongs to the CREB/ATF family characterized by kination and basic leucine zipper DNA-binding domains. MEKK increased transcriptional activity of ATF-2, consistent with the previous report that ATF-2 is a target of JNK and p38 MAP kinase (10), but it is not known if other signals are involved in the control of its transcriptional activity. We examined the effect of PKA on ATF-2 transcriptional activity and found that PKA had no stimulatory effect, confirming its insensitivity to cAMP stimulation (18). CaMKII also had no effect on ATF-2 activity, but CaMKIV clearly increased ATF-2 activity.

The regulation of cellular function by growth factors, hormones, and other ligands that bind to receptors of cell surface is initiated by signal transduction mechanisms at the plasma membrane that lead to the production of intracellular signal, or second messenger. These second messengers associate with specific target molecules to initiate a cascade of biochemical events leading to the change in cellular function. Calcium acts as an intracellular second messenger and is involved in cellular processes ranging from contraction and secretion to gene expression. CaMKIV is known to play a critical role in controlling gene expression by calcium signaling (19). The activation of CaMKIV depends on Ca^{2+} entry systems (20). Some Ca^{2+} entry systems, including L-type voltage-dependent calcium channels, mobilize calmodulin from the cytoplasm into the nucleus, where CaMKIV is activated. Although ATF-2 is ubiquitously expressed, at the highest level

in the brain, expression of CaMKIV is relatively restricted to the brain, spleen, and thymus (17). We have found that CaMKIV is also expressed in the pancreatic islets and β-cell derived cell line. These results show that calcium signaling controls the activity of ATF-2 in some cells, including neurons and pancreatic β-cells.

The CaMKIV-phosphorylated amino acid residues Thr^{69}, Thr^{71}, and Thr^{73} of ATF-2 were overlapped and were not identical with the sites ($Thr^{69}$and Thr^{71}) phosphorylated by JNK, a member of the MAP kinase superfamily (10). The specificity of substrates varies with different members of the MAP kinase superfamily. ATF-2 is phosphorylated more easily by JNK and p38 MAP kinases than by p42/p44 MAP kinases (21). To find if CaMKIV activates ATF-2 via JNK, we examined the effects of CaMKIV on c-Jun, the substrate of JNK, and found that CaMKIV does not affect the transcription activity of c-Jun at all. Furthermore, the fact that the dominant negative forms of JNK or p38 MAP kinase had no effect on CaMKIV-induced ATF-2 transcriptional activity indicates that neither JNK nor p38 MAP kinase is involved in CaMKIV-induced signal transduction. We have confirmed that CaMKIV-induced signal transduction was not inhibited by dominant negative JNK or p38 MAP kinase in HIT-T15 cells. These results suggest that JNK or p38 MAP kinase is not involved or is little involved in CaMKIV-induced signal transduction in insulin-producing cells. Although a CaMKIV-dependent mechanism for regulating MAP kinase pathways recently has been proposed (22), the reason for the difference between these results and ours is not clear at present but could be due to the use of different cell lines.

We have previously shown that ATF-2 binds to multiple elements on the human insulin gene promoter (9). To determine the role of ATF-2 in insulin gene expression regulated by the calcium ion concentration, we used glibenclamide, which activates the L-type voltage-dependent calcium channels by depolarization of the plasma membrane. Glibenclamide increased the phosphorylation of ATF-2 and ATF-2–induced gene expression. Glibenclamide-induced gene expression was abolished in the presence of EGTA or the Ca^{2+}/calmodulin-dependent protein kinase inhibitor, suggesting that Ca^{2+}-activated ATF-2 plays an important role in Ca^{2+}-induced gene transcription.

It is well known that glucose increases the concentration of both cAMP and Ca^{2+} in pancreatic β-cells (23,24) and triggers insulin secretion from pancreatic β-cells. In rat primary cultured islets, ATF-2 increased the glucose-induced activities of the human insulin promoter, while CREB repressed them, indicating that ATF-2 is especially important in increased insulin gene expression at higher intracellular calcium ion concentrations. It has been reported that recombinant CREB can bind to rat insulin CRE, whereas cellular CREB binds only very poorly to insulin CRE (25), probably due to unique CRE sequences in the insulin gene. Therefore, the overexpressed CREB might compete with endogenous ATF-2 for coactivator p300 or CBP, as in the case of CREB and certain nuclear receptors (26), resulting in decreasing insulin gene expression.

In summary, we have demonstrated that transcription factor ATF-2 is activated by CaMKIV, probably through its phosphorylation at Thr^{69}, Thr^{71}, and Thr^{73} of ATF-2. We have also shown that both an elevated intracellular calcium ion concentration and the activation of Ca^{2+}/calmodulin-dependent protein kinase are critical in ATF-2–induced insulin gene expression in pancreatic β-cells, suggesting a mechanism in which the calcium signaling pathway diverges at the level of transcriptional factors to control the gene expression.

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