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The p38/Reactivating Kinase Mitogen-activated Protein Kinase Cascade Mediates the Activation of the Transcription Factor Insulin Upstream Factor 1 and Insulin Gene Transcription by High Glucose in Pancreatic β-Cells*

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Insulin upstream factor 1 (IUF1), a transcription factor present in pancreatic β-cells, binds to the sequence C(C/T)TAATG present at several sites within the human insulin promoter. Here we isolated and sequenced cDNA encoding human IUF1 and exploited it to identify the signal transduction pathway by which glucose triggers its activation. In human islets, or in the mouse β-cell line MIN6, high glucose induced the binding of IUF1 to DNA, an effect mimicked by serine/threonine phosphatase inhibitors, indicating that DNA binding was induced by a phosphorylation mechanism. The glucose-stimulated binding of IUF1 to DNA and IUF1-dependent gene transcription were both prevented by SB 203580, a specific inhibitor of stress-activated protein kinase 2 (SAPK2, also termed p38 mitogen-activated protein kinase, reactivating kinase, CSBP, and Mxi2) but not by several other protein kinase inhibitors. Consistent with this finding, high glucose activated mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase-2) (a downstream target of SAPK2) in MIN6 cells, an effect that was also blocked by SB 203580. Cellular stresses that trigger the activation of SAPK2 and MAPKAP kinase-2 (arsenite, heat shock) also stimulated IUF1 binding to DNA and IUF1-dependent gene transcription, and these effects were also prevented by SB 203580.

IUF1 expressed in *Escherichia coli* was unable to bind to DNA, but binding was induced by incubation with MgATP, SAPK2, and a MIN6 cell extract, which resulted in the conversion of IUF1 to a slower migrating form. SAPK2 could not be replaced by p42 MAP kinase, MAPKAP kinase-2, or MAPKAP kinase-3. The glucose-stimulated activation of IUF1 DNA binding and MAPKAP kinase-2 (but not the arsenite-induced activation of these proteins) was prevented by wortmannin and LY 294002 at concentrations similar to those that inhibit phosphatidylinositide 3-kinase. Our results indicate that high glucose (a cellular stress) activates SAPK2 by a novel mechanism in which a wortmannin/LY 294002-sensitive component plays an essential role. SAPK2 then activates IUF1 indirectly by activating a novel IUF1-activating enzyme.

The β-cells of the pancreas respond to increases in blood glucose by secreting insulin, which then restores homeostatic equilibrium by stimulating the uptake of glucose into peripheral tissues (principally skeletal muscle). However, the insulin lost via secretion must be replenished by resynthesis. In the short term, this is achieved by the glucose-stimulated translation of pre-existing insulin mRNA molecules, but in the longer term, it depends on the stimulation of insulin gene transcription.

Insulin upstream factor-1 (IUF1)[1] is a β-cell-specific transcription factor that binds to four sites within the human insulin gene promoter, termed A1, A2, A3, and A5 (Fig. 1). In the rat insulin I gene, a glucose response element has been mapped to a location between −193 and −247 upstream of the transcriptional start site (1–3), which (in the human gene) contains the A3 site. Moreover, the binding of IUF1 to the A3 site is triggered by exposure of rat islets of Langerhans to high glucose (4) and abolished by exposure to low glucose. The loss of DNA binding at low glucose is prevented by incubation of the islets with nonspecific phosphatase inhibitors (fluoride, molybdate, and glycerophosphate), suggesting that glucose-induced DNA binding might involve the phosphorylation of IUF1. However, the effects of high glucose are not mimicked by cyclic AMP-elevating agents or tumor-promoting phorbol esters, suggesting that neither cyclic AMP-dependent protein kinase nor protein kinase C mediates the glucose-induced activation of IUF1 (4).

In the present study, we have identified the signal transduction pathway that mediates the glucose-induced binding of IUF1 to DNA. We show that the glucose-induced activation of IUF1 DNA binding and the glucose responsiveness of the −50

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[1] The abbreviations used are: IUF1, insulin upstream factor 1; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase; MAPKAP-K2 and MAPKAP-K3, mitogen activated protein kinase-activated protein kinases 2 and 3, respectively; SAPK, stress-activated protein kinase; NTA, nitrolotriacetic acid; PI, phosphatidylinositide; CRE, cAMP-responsive element; CREB, CRE-binding protein; USF, upstream stimulatory factor.

to −250 base pair region of the human insulin gene promoter are both prevented by SB 203580, a specific inhibitor of stress-activated protein kinase-2 (SAPK2, also called p38 MAP kinase, reactivating kinase, CSBP, and Mxi2) (5, 6). We also show that SAPK2 triggers the activation of IUF1 indirectly by turning on a novel IUF1-kinase and that glucose activates the SAPK2 pathway in a β-cell line via a phosphoinositide 3-kinase.

MATERIALS AND METHODS

Chemicals and Reagents—Radiochemicals were purchased from Amersham International (Slough, Berks, UK), sodium arsenite from Fisons (Loughborough, UK), wortmannin from Sigma (Poole, UK), and okadaic acid, calyculin A, and KN62 from Calbiochem (Nottingham, UK). SB 203580 was a generous gift from Dr. J. Lee and Dr. P. Young (Smith-Kline Beecham, King of Prussia, PA), and PD 098059 was kindly provided by Dr. A. Saltiel (Parke-Davis, Ann Arbor, MI). Activated SAPK2 was obtained by expression of the *Xenopus* homologue (Mpk2) in *Escherichia coli* (7) followed by activation with MAP kinase kinase-6 (5). Mitogen-activated protein kinase-activated protein kinase-2 and -3 (MAPKAP-K2 and MAPKAP-K3) were expressed in *E. coli* as glutathione *S*-transferase fusion proteins and activated with SAPK2 (8).

Oligonucleotides—Oligonucleotides were purchased from Alta Bioscience (University of Birmingham, Birmingham, UK). Single-stranded complementary oligonucleotides were annealed as described previously (9) and labeled with [γ^{32}P]ATP using T4 polynucleotide kinase. The sequences of oligonucleotides B, Bm1, and Bm2 (10), G (9), P and H (11), and D, P, H, Jr1, and USF (12) were as previously published. The PCR primers IPF1 and IPF3, used in the cloning of IUF1, correspond to the mouse IPF1 sequences 5′-ACCATGAATAGTGAGGAGCA-3′ and 5′-TCACCGGGGTTCCTGCGGTCGCAGTGGGATCGC-3′, respectively (13).

Cloning of a cDNA Encoding Human IUF1—IUF1 was cloned from human islet mRNA by reverse transcriptase PCR using primers based on the sequence of mouse IPF1 (13). Total RNA was isolated from human islets of Langerhans following lysis in 4 M guanidinium isothiocyanate, 0.25 M sodium citrate, 5% (w/v) sodium sarcosyl, 0.2 M sodium acetate, pH 5.0. The lysed cells were extracted sequentially with water-saturated phenol and isopropyl alcohol and the resultant RNA precipitated with ethanol. Following centrifugation, the RNA pellets were resuspended in 200 μl of TE (10 mM Tris-HCl, 1 mM EDTA), pH 8.0, and reprecipitated with 0.1 volumes of 7 M ammonium acetate and 2.5 volumes of absolute ethanol. Final pellets were resuspended in 50 μl of diethyl pyrocarbonate-treated water. For cDNA synthesis, the RNA sample was heated to 65 °C for 10 min, and the transcription reaction was performed at 37 °C for 60 min containing the following: 1 × reverse transcriptase reaction buffer (Promega, Southampton, UK), 20 units RNasin (Promega), 40 pmol of driving primer IPF3, 0.25 mM dATP, dCTP, dTTP, and dGTP, 25 mM dithiothreitol, 0.5 μg of human islet RNA, and 50 units of avian myloblastosis virus reverse transcriptase (Promega). Polymerase chain reaction amplification was performed on a Hybaid II Thermocycler, using 5 units of thermostable *Taq* DNA polymerase (Promega), 5 pmol of primers IPF1 and IPF3, 0.2 mM dNTPs, 0.125 mM $MgCl_2$, and 1 μl of cDNA template prepared as described above. Polymerase chain reaction products were cloned into the TA-Cloning vector (Invitrogen, San Diego, CA) and sequenced by standard dideoxy sequencing methodology (U.S. Biochemical Corp.) using M13 primers.

Expression of Recombinant IUF1 in E. coli—IUF1 was produced in *E. coli* using the Qiagen histidine tag expression system. The IUF1 cDNA was cloned as a *Bam*HI/*Xho*I fragment into the pQE expression vector. Following transformation into JM109, a 10-ml overnight culture was added to 1 liter of L-broth and grown to an A_{595} of 0.5. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the culture was grown for a further 3–5 h. The bacterial cells were centrifuged and resuspended in 50 ml of solution A (6 M guanidine hydrochloride, 200 mM NaCl, 300 mM sodium phosphate, 0.05 M Tris-HCl, pH 7). Insoluble matter was removed by centrifugation at 12,000 rpm for 20 min, and the supernatant was mixed with 8 ml of a 50% slurry of nickel-chelated nitrolotriacetic acid (Ni-NTA) resin equilibrated in solution A. After stirring for 45 min, the resin was pelleted by centrifugation at 3000 rpm for 5 min. The resin was then resuspended in 10 ml of solution A, packed into a 10 × 1-cm column, and washed with 100 ml of solution B (300 mM sodium phosphate, 0.05 M Tris-HCl, pH 6.0) containing 8 M urea. The resin-bound protein was renatured at ambient temperature by passing 200 ml (at 1.5 ml/min) of a decreasing 6–0.5 M urea gradient in buffer B at pH 7.0 through the column. The resin-bound IUF1 was then eluted using a 50-ml gradient of imidazole (100–500 mM). Fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Fractions containing recombinant IUF1 were pooled and dialyzed against 200 mM sodium phosphate, 200 mM NaCl, 0.05 M Tris-HCl, 200 mM imidazole, 5% (v/v) glycerol, pH 7.5.

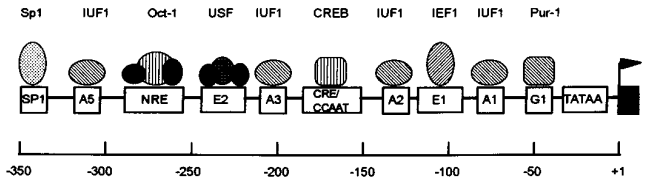

FIG. 1. **The arrangement of regulatory sequences in the human insulin gene promoter.** The *boxes on* the *line* represent discrete regulatory sequences, with their position relative to the transcriptional start site indicated by the negative numbers *below*. Proteins that bind to these sites are shown *above* each *box* (36). IUF1 binds to four sequence elements within the human insulin gene promoter, termed A1, A2, A3, and A5 (according to the nomenclature of German *et al.* (37)) located at positions −76, −130, −215, and −315, respectively, upstream of the transcriptional start (Refs. 10 and 11; W. M. Macfarlane and K. Docherty, unpublished observations). The E2 site not only binds USF but also at least two other factors that have yet to be characterized (12, 38). *NRE*, negative regulatory element.

Antibodies to IUF1—Anti-IUF1 antibodies were raised in sheep against an octameric multiple antigenic peptide of the IUF1 sequence DKKRSSGTTSGGGGGEEPE (amino acids 205–224), synthesized by Alta Bioscience (University of Birmingham, UK).

In Vitro Transcription/Translations—1 μg of linearized plasmid was transcribed *in vitro* for 2 h at 37 °C under reaction conditions of 5 mM dithiothreitol, 10 μg/ml bovine serum albumin, 1 mM ATP, CTP, and UTP, 0.2 mM GTP, 0.01 mM (m7G(5′)ppp(5′)) (Pharmacia Biotech Inc.), 1 × T7 transcription buffer (Pharmacia), 0.2 units/μl RNA Guard (Pharmacia), and 60 units of T7 RNA polymerase (Pharmacia). Transcripts were phenol/chloroform-extracted, ethanol-precipitated, resuspended in diethyl pyrocarbonate-treated water, and quantified spectrophotometrically. *In vitro* translation was performed by incubation of 0.1 μg of RNA at 30 °C for 60 min with the following: 17.5 μl of rabbit reticulocyte lysate (Promega), 0.2 units/μl RNA Guard, and 500 μM amino acid mix (Promega).

Isolation and Treatment of Human Islets of Langerhans—Human islets were isolated from pancreata obtained, with the appropriate consent, from brain-dead heart-beating donors. The organs were perfused *in situ* with hyperosmolar citrate solution at 4 °C and processed by intraductal distension with collagenase (3 mg/ml) and the automated digestion procedure (14). Human islets were separated on the COBE 2991 cell separator using a Ficoll/diatrizoic acid-based continuous density gradient (15). The purified islets were placed in RPMI 1640 medium (Life Technologies, UK) containing 10% (v/v) fetal calf serum and supplemented with 400 IU/ml sodium penicillin G and 200 μg/ml streptomycin sulfate and cultured at 37 °C in a humidified atmosphere of O_2, CO_2 (95:5) for several days prior to use. Selected islets were separated into batches of 120–150 in Hanks' buffered saline (0.12 M NaCl, 5.4 mM KCl, 0.33 mM Na_2HPO4, 0.44 mM KH_2PO_4, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $NaHCO_3$, 5% (w/v) bovine serum albumin) except where specified otherwise.

Preparation of Nuclear Extracts—Nuclear extracts were prepared using a modification of the method of Schreiber *et al.* (16). Islets were centrifuged for 10 s in a microcentrifuge and resuspended in 400 μl of 10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 10 mM NaF, 10 mM sodium molybdate, 10 mM β-glycerophosphate, 10 mM sodium vanadate, and 10 mM *p*-nitrophenyl phosphate. Islets were allowed to swell on ice for 15 min before adding 25 μl of 10% (v/v) Nonidet P-40. The islets were then vortexed for 15 s and centrifuged for 30 s in a microcentrifuge. The pellet, which was enriched in nuclei, was resuspended in 50 μl of 20 mM Hepes, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM *p*-aminobenzoic acid, 10 μg/ml aprotinin, 5% (v/v) glycerol, 10 mM NaF, 10 mM sodium molybdate, 10 mM β-glycerophosphate, 10 mM sodium vanadate, and 10 mM *p*-nitrophenyl phosphate. Nuclear extracts were then centrifuged for 2 min at 4 °C in a microcentrifuge. The supernatant was collected, aliquoted into small volumes, and stored at −70 °C.

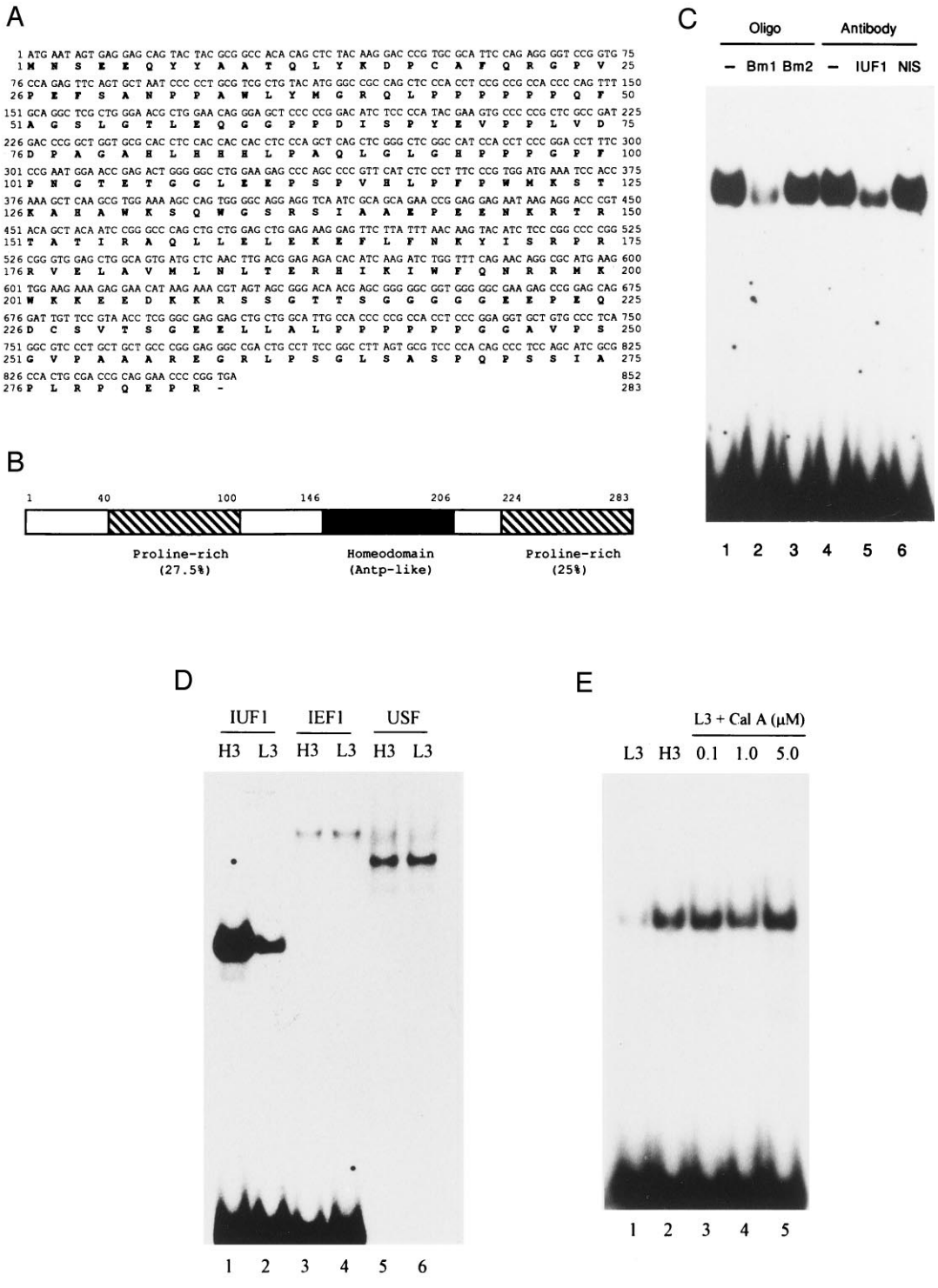

FIG. 2. **Characterization of the human IUF1 cDNA and its glucose-induced DNA binding.** *A*, the cDNA and deduced amino acid sequences of human IUF1. *B*, the predicted protein structure of human IUF1 comprises an antennapedia-type homeodomain flanked by two proline-rich regions. *C*, EMSA competition analysis of IUF1 translated in reticulocyte lysates, using oligonucleotide B as a probe. The indicated unlabeled competitor oligonucleotides Bm1 and Bm2 were used in 50-fold excess; 1 μl of anti-IUF1 antibody or 1 μl of nonimmune sheep serum (*NIS*) was used. *D*, nuclear extracts were prepared from human islets of Langerhans that had been incubated for 3 h in high (20 mM, *H3*) or low (3 mM, *L3*) glucose. Oligonucleotides B, Jr1, and USF (see "Materials and Methods") were then used to measure the DNA binding activities of IUF1, IEF1, and USF, respectively, using an EMSA. *E*, binding of oligonucleotide B to IUF1 was analyzed in nuclear extracts from human islets of Langerhans incubated for 3 h in 20 mM glucose (*H3*), 3 mM glucose (*L3*), or 3 mM glucose supplemented with increasing concentrations of calyculin A (*Cal A*, *lanes 3–5*).

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs were performed as described elsewhere (9). Nuclear extracts (0.5 μg of protein) were incubated with radiolabeled probe for 20 min at room temperature in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM dithiothreitol, 1 mM EDTA, and 5% (v/v) glycerol. For competition experiments, extracts were preincubated for 20 min with 1 μl of a specific anti-IUF1 antibody or with 1 μl of nonimmune serum prior to the addition of probe.

Plasmids—The control construct pGL-LUC is based on the plasmid pGL2 (Promega), with the thymidine kinase promoter from the herpes simplex virus cloned 5′ to the firefly luciferase gene. In the construct pGL-LUC200, a −50 to −250 base pair *Hin*cII-*Pvu*II fragment from the human insulin gene promoter was blunt-ended and cloned into

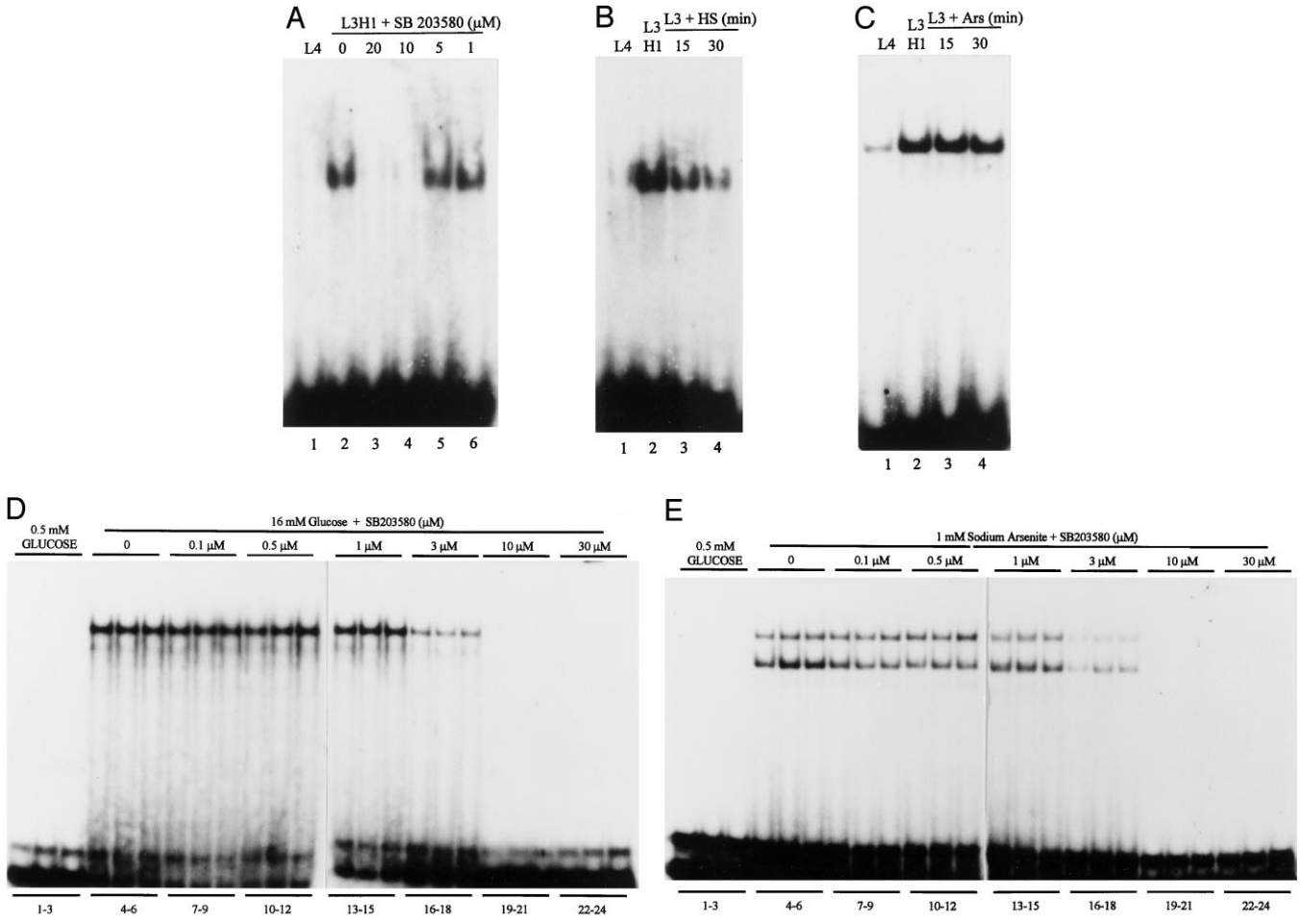

FIG. 3. **Effects of SB 203580, heat shock, and sodium arsenite on IUF1 binding activity in isolated human islets of Langerhans.** *A*, EMSA analysis of IUF1 DNA binding activity in nuclear extracts prepared from human islets of Langerhans incubated for 4 h in 3 mM glucose (*L4*, *lane 1*) or incubated for 3 h in 3 mM glucose followed by 1 h in 20 mM glucose (*L3H1*) in the absence (*lane 2*) or presence of the indicated concentrations of SB 203580 (*lanes 3–6*). Oligonucleotide B was used as a probe. *B*, same as *A*, except that islets were incubated in 3 mM glucose for 4 h (*L4*, *lane 1*), 3 mM glucose for 3 h plus 20 mM glucose for 1 h (*L3H1*, *lane 2*), or 3 mM glucose for 3 h plus 15 or 30 min of heat shock (*HS*) at 45 °C (*lanes 3* and *4*, respectively). *C*, same as *A* except that islets were incubated for 4 h in 3 mM glucose (*L4*, *lane 1*), for 3 h in 3 mM glucose followed by 1 h in 20 mM glucose (*L3H1*, *lane 2*), or for 3 h in 3 mM glucose followed by 15 or 30 min in 1 mM sodium arsenite (*Ars*, *lanes 3* and *4*). *D*, EMSA analysis of IUF1 DNA binding activity in nuclear extracts prepared from MIN6 cells incubated in 0.5 mM glucose for 3 h (*lanes 1–3*), or in 16 mM glucose for 3 h with the indicated concentrations of SB 203580 added 30 min before stimulation of the cells for 30 min with 16 mM glucose. Oligonucleotide B was utilized as probe. *E*, same as *D*, except that the cells were stimulated with 1 mM sodium arsenite in place of 16 mM glucose.

the *Sma*I site of the control construct. DNA was prepared using the Qiagen endotoxin-free Maxiprep method and quantitated spectrophotometrically.

Cell Culture—MIN6 cells (17) were grown in DMEM containing 5 mM glucose, supplemented with 15% heat-inactivated myoclone fetal calf serum (Sigma) and 2 mM L-glutamine, in a humidified atmosphere containing 95% air, 5% CO_2. MIN6 cells were used between passages 26 and 30 for all experiments. Where required, SB 203580, LY294002, wortmannin, or PD 098059 were added 30 min prior to stimulation of the cells with glucose or sodium arsenite, using the concentrations of inhibitors and agonists given in the figure legends.

Immunoprecipitation and Assay of MAPKAP-K2—MIN6 cells were lysed as described for PC12 and A431 cells (7) except that 2 μM microcystin was also present in the lysis buffer. Cell lysate (50 μg of protein) was incubated with 5 μl of protein G-Sepharose beads conjugated to an anti-MAPKAP-K2 antibody (2 μg) raised against residues 356–371 of human MAPKAP-K2 (18). After incubation for 2 h at 4 °C on a shaker, the pellets were washed twice with lysis buffer containing 0.5 M NaCl and twice with lysis buffer and then assayed for MAPKAP-K2 activity using 30 μM of the peptide KKLNRTLSVA (19). One unit of activity was the amount that catalyzed the phosphorylation of 1 nmol of substrate in 1 min.

Transfection—Min6 cells at about 80% confluence in six-well plates were transfected by mixing 4 μg of DNA and 54 μl of a 1 nM lipid suspension containing a 2:1 mixture of dioleoyl-L-α-phosphatidylethanolamine, (Sigma) and dimethyldioctadecylammonium bromide, (Fluka) in 1 ml of serum-free Optimem (Life Technologies). The lipid DNA complexes were allowed to form for 20 min at room temperature before being added to the washed cells. Following 5 h of incubation, 1 ml of complete medium containing 30% heat-inactivated myoclone fetal calf serum was added to the cells. After 12 h, the medium-DNA complexes were replaced by complete medium, and the cells were left for a further 24 h. Treatment of all cells started with a 5-h preincubation in DMEM containing 0.5 mM glucose. Where required, 20 μM SB 203580, 50 μM LY294002, 50 nM wortmannin, or 50 μM PD 098059 were added 30 min prior to stimulation of the cells for 5 h in 16 mM glucose or 1 mM sodium arsenite. Cells were washed twice in phosphate-buffered saline and then removed from the surface of the wells, and a cell pellet was recovered by centrifugation at 7000 rpm for 30 s. The cell pellet was resuspended in 70 μl of 100 mM KH_2PO_4, pH 7.8, 1 mM dithiothreitol solution and lysed by freeze/thawing three times. Cell debris was removed by centrifugation at 13,000 rpm for 1 min.

Luciferase Assay—30 μl of cell extract was added to 350 μl of buffer (15 mM $MgSO_4$, 30 mM glycylglycine, 2 mM Na_2ATP, pH 7.8) containing 0.45 mM coenzyme A and 2.56 mM Triton X-100. To this, 150 μl of 30 mM glycylglycine containing 0.5 mM luciferin (Sigma) was injected, and the luminescence was read at 560 nM using a Berthold Luma LB9501 luminometer. The protein content of the cell extracts was measured according to Bradford (20).

Activation of IUF1 in MIN6 Cell Extracts—Extracts were prepared from MIN6 cells exposed to low (0.5 mM) glucose. His-tagged IUF1 (10 μg) was then incubated in 0.1 ml of 10 mM Tris-HCl, pH 7.0, 50 mM KCl,

10 mM $MgCl_2$, 0.5 mM ATP, 20 μCi of [γ^{32}P]ATP (Amersham), MIN6 extract (0.5 μg), and 10 units of SAPK2 (7), p42 MAPK (21), MAPKAP-K2 (22), or MAPKAP-K3 (8). After incubation at 30 °C for 30 or 60 min, 10 μl of Ni-NTA resin was added, and the suspension was rotated end-over-end for 30 min. Following centrifugation at 13,000 rpm for 30 s, the supernatant was removed and discarded. The pellet was washed twice in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, and 5% (v/v) glycerol) before elution of the proteins by 30-min rotation of the samples in binding buffer containing 250 mM imidazole, pH 7. 10 μl of the eluted material was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography or Coomassie Blue staining of the gel.

Statistical Analysis—Statistical analysis was performed by Student's paired *t* test. A value of $p < 0.05$ was considered significant. All data are expressed as means ± S.D.

RESULTS

Molecular Cloning of cDNA Encoding Human IUF1—We cloned cDNA encoding human IUF1. Its amino acid sequence (Fig. 2*A*) revealed a 283-residue protein with a predicted molecular mass of 31.4 kDa, which contains an antennapedia-like homeodomain flanked by two proline-rich sequences (Fig. 2*B*). Human IUF1 is 95% identical to mouse IPF1 (13), 96% identical to rat STF1/IDX1 (23, 24), and 100% identical to the published partial sequence of X1Hbox8 from *Xenopus laevis* (25).

The IUF1 cDNA expressed in reticulocyte lysates bound to a DNA sequence from the A3 region (oligonucleotide B). Competition for binding was observed with oligonucleotide Bm1, which contains a mutation within the A3 sequence that does not affect IUF1 DNA-binding, but not with oligonucleotide Bm2, which contains a mutation in the A3 sequence that abolishes IUF1 binding (10). In addition, an anti-IUF1 antibody that was able to compete for formation of complexes in nuclear extracts competed for binding of the expressed protein to oligonucleotide B (Fig. 2*C*). These experiments establish that the cDNA does indeed encode IUF1.

Glucose Modulates IUF1 DNA Binding Activity in Human Islets of Langerhans—The ability of IUF1 to bind to DNA in human islets of Langerhans was studied initially after incubation for 3 h in low (3 mM) or high (20 mM) glucose. In 3 mM glucose, the binding activity was approximately 5% of that seen in 20 mM glucose (Fig. 2*D*). This effect was specific for IUF1 because glucose had no effect on the DNA binding activity of the transcription factors IEF1 and USF (Fig. 2*D*), which bind to the E1 and E2 sites in the human insulin gene promoter (Fig. 1). The loss of IUF1 DNA binding after transferring the islets from high (20 mM) glucose to low (3 mM) glucose was reversible, because reexposure to 20 mM glucose resulted in a complete recovery of IUF1 DNA binding activity (data not shown). Moreover, the loss of IUF1 DNA binding at 3 mM glucose was prevented by calyculin A (Fig. 2*E*) or okadaic acid (data not shown), which are potent inhibitors of the Ser/Thr-specific protein phosphatases 1 and 2A. These observations indicate that the glucose-induced activation of IUF1 involves a protein phosphorylation event.

SAPK2 Activity Is Essential for Glucose-induced Activation of IUF1—A high concentration of blood glucose might be considered to represent a stressful stimulus, and we therefore wondered whether one of the SAPK cascades might be involved in mediating the activation of IUF1 by high glucose. Three SAPKs have been identified, which are homologues of the mitogen-activated protein kinases (MAPKs), termed here SAPK1 (also known as SAPK or c-Jun N-terminal kinase), SAPK2 (see Introduction), and SAPK3 (also known as extracellular signal-regulated kinase 6 or p38γ) (reviewed in Refs. 6 and 26). SAPK2 is potently and specifically inhibited by the drug SB 203580, which does not inhibit SAPK1, SAPK3, MAPKs, or many other protein kinases that have been tested (6, 26, 27). We found that the binding of IUF1 to DNA induced by high glucose was prevented by SB 203580 (Fig. 3, *A* and *D*). In contrast, several other protein kinase inhibitors had no effect including 50 μM PD 098059 (which prevents the activation of MAP kinase kinase-1), 2 μM KN62 (which inhibits calcium/calmodulin-dependent protein kinase-2), and 100 nM rapamycin (which prevents the activation of p70 S6 kinase) (data not shown).

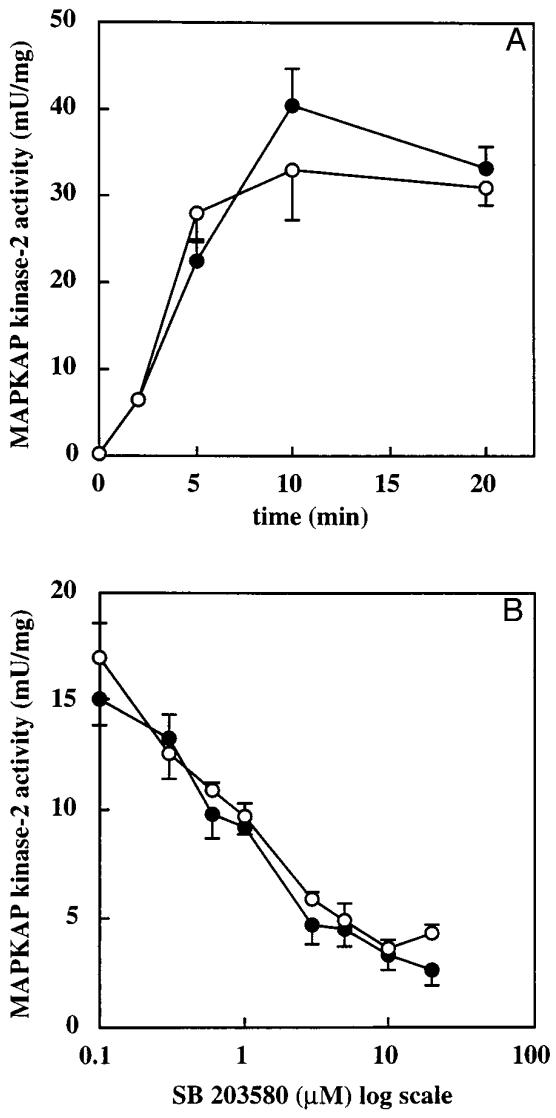

FIG. 4. **Glucose and arsenite stimulate MAPKAP-K2 activity in MIN6 cells: effect of SB 203580.** *A*, MIN6 cells in low (0.5 mM) glucose were transferred to either high (16 mM) glucose (*open circles*) or maintained in low glucose supplemented with 1 mM sodium arsenite (*closed circles*) for the times indicated. MAPKAP-K2 was immunoprecipitated from the cell lysates (50 μg of protein) and assayed as described under "Materials and Methods." The results are shown ±S.E. for experiments using three dishes of cells at each time point. Similar results were obtained in an independent experiment. *B*, MIN6 cells were incubated for 30 min in low (0.5 mM) glucose in the absence or presence of the indicated concentrations of SB 203580 and then exposed for 10 min to 16 mM glucose (*open circles*) or 1 mM sodium arsenite in the presence of 0.5 mM glucose (*closed circles*). MAPKAP-K2 was then assayed as in *A*. The results are shown ±S.E. for experiments using three dishes of cells. Similar results were obtained in an independent experiment.

In other cells, SAPK2 is activated in response to chemical stress (sodium arsenite) and heat shock (7). Both of these stimuli mimicked the effect of high glucose in that they stimulated the binding of IUF1 to DNA (Fig. 3, *B* and *C*); the effects of arsenite (Fig. 3*E*) and heat shock (data not shown) were also prevented by SB 203580.

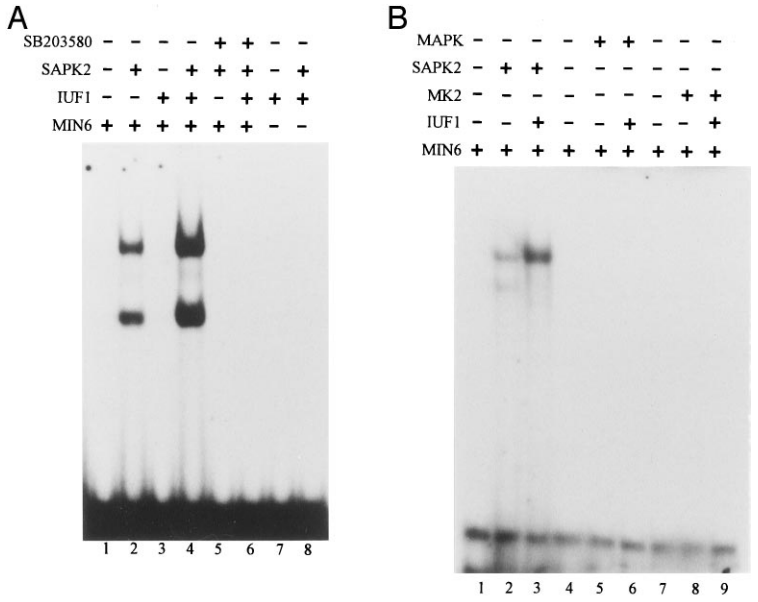

FIG. 5. **SAPK2 stimulates IUF1 DNA binding activity dependent on the presence of MIN6 cell extract.** EMSA analysis of IUF1 DNA binding activity using oligonucleotide B as probe. In all cases, samples were incubated for 30 min with the probe and MgATP. *A*, *lane 1*, 0.5 μg of an extract prepared from MIN6 cells incubated in 0.5 mM glucose; *lane 2*, 0.5 μg of MIN6 extract plus 10 units of activated SAPK2; *lane 3*, 0.5 μg of MIN6 extract plus 1 μg of His-tagged IUF1; *lane 4*, 0.5 μg of MIN6 extract plus 10 units of SAPK2 and 1 μg of His-tagged IUF1; *lane 5*, 0.5 μg of MIN6 extract plus 10 units of SAPK2 and 20 μM SB 203580; *lane 6*, 0.5 μg of MIN6 extract, 1 μg of His-tagged IUF1, 10 units of SAPK2, and 20 μM SB 203580; *lane 7*, 1 μg of His-tagged IUF1; *lane 8*, 1 μg of His-tagged IUF1 plus 10 units of SAPK2. *B*, EMSA analysis of low glucose MIN6 extract (0.5 μg of protein) incubated in the presence of MgATP and 10 units of the indicated kinases and in the presence or absence of 1 μg of His-tagged IUF1 as indicated. *MAPK*, p42 MAP kinase; *MK2*, MAPKAP-K2.

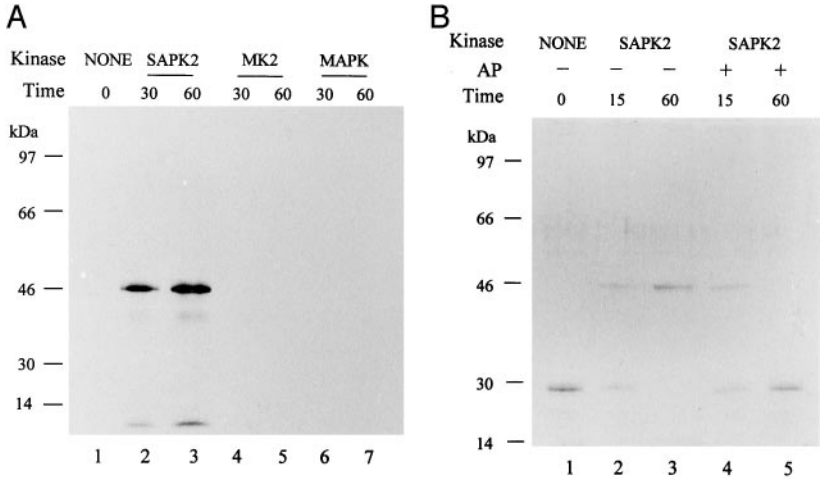

FIG. 6. **Phosphorylation of IUF1 by SAPK2 in the presence of MIN6 cell extract.** *A*, His-tagged IUF1 was incubated for the indicated times (30 or 60 min) in the presence of Mg-γ[^{32}P]ATP, an extract prepared from MIN6 cells incubated in low glucose, and the indicated kinases. *Lane 1* shows His-tagged IUF1 incubated for 60 min with MgATP and MIN6 cell extract in the absence of SAPK2. In *lanes 2–7*, 10 units of SAPK2, MAPKAP-K2 (*MK2*) or p42 MAP kinase (*MAPK*) were included as indicated. Following incubation, His-tagged IUF1 was isolated using Ni-NTA beads (see "Materials and Methods"), and the samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *B*, His-tagged IUF1 was incubated for 15 or 60 min with a MIN6 cell extract and MgATP in the absence or presence of SAPK2 as indicated. The samples were then made 20 mM in EDTA to inactivate SAPK2 and treated for a further 15 or 60 min without or with 10 units of acid phosphatase (*AP*) as indicated. His-tagged IUF1 was isolated using Ni-NTA beads, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

The suppression of IUF1 binding to DNA by SB 203580 implied that high glucose and arsenite should be inducing the activation of SAPK2 in pancreatic β-cells. Fig. 4*A* shows that both agonists activate MAPKAP-K2, an immediate downstream target of SAPK2 (7, 27) within minutes in MIN6 cells (a murine β-cell line). Moreover, the activation of MAPKAP-K2 in MIN6 cells by either glucose or arsenite was prevented by SB 203580, with half-maximal inhibition occurring at a concentration (1–2 μM, Fig. 4*B*) similar to that which prevented the glucose- (Fig. 3*D*) or arsenite- (Fig. 3*E*) induced binding of IUF1 to DNA. IUF1 binding to DNA was partially inhibited at 3 μM SB 203580 and completely inhibited at 10 μM (Fig. 3). The two complexes observed in Fig. 3*E* are only occasionally observed and may represent degradation of IUF1.

Activation of IUF1 by SAPK2 in Vitro Requires the Presence of MIN6 Extracts—IUF1 expressed in *E. coli* as a His-tagged protein was inactive (*i.e.* it failed to bind to its recognition sequence within oligonucleotide B) (Fig. 5*A*, *lane 7*) and could not be activated by incubation with MgATP and SAPK2 (Fig. 5*A*, *lane 8*). However, when an extract from MIN6 cells that had been incubated in low glucose, and therefore contained only inactive IUF1, was incubated with MgATP and SAPK2, the endogenous IUF1 was activated (Fig. 5*A*, *lane 2*). SAPK2 also activated bacterially expressed IUF1 when the latter was added to a MIN6 cell extract (Fig. 5*A*, *lane 4*). Moreover, activation of endogenous or recombinant IUF1 was prevented by SB 203580 (Fig. 5*A*, *lanes 5* and *6*). No activation of IUF1 occurred if SAPK2 was omitted (Fig. 5*A*) or if it was replaced by p42 MAP kinase, MAPKAP-K2 (Fig. 5*B*), or MAPKAP-K3 (data not shown).

The above results suggested that SAPK2 was activating an enzyme in MIN6 cells that then activated IUF1, but it was also possible that SAPK2 was activating a cofactor or accessory protein required for IUF1 activity. To distinguish between these possibilities, the IUF1 was incubated with SAPK2, Mg-[γ-^{32}P]ATP, and a low glucose-treated MIN6 cell extract. The

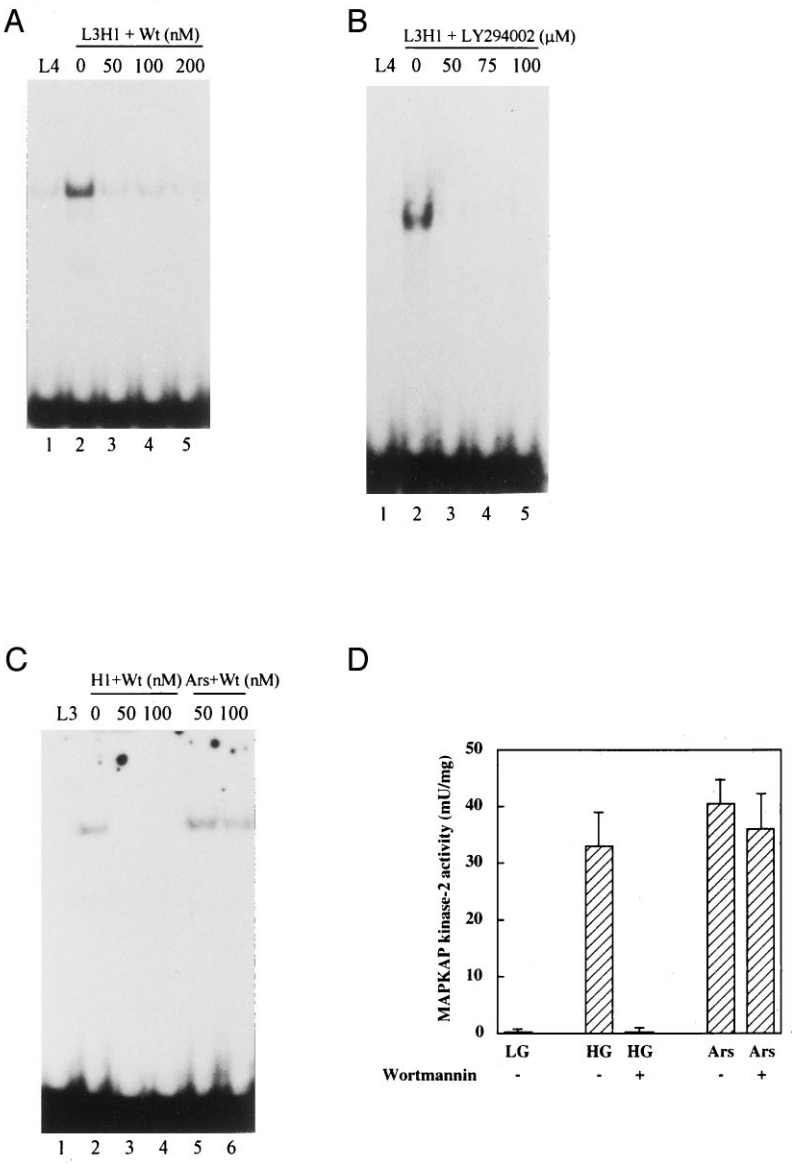

FIG. 7. **Wortmannin and LY 294002 inhibit glucose but not arsenite stimulation of IUF1 DNA-binding and MAPKAP-K2 activity in isolated human islets of Langerhans and MIN6 cells.** *A*, EMSA analysis of nuclear extracts prepared from human islets of Langerhans incubated for 4 h in 3 mM glucose (*L4*, *lane 1*) or 3 mM glucose for 3 h followed by 1 h in 20 mM glucose (*L3H1*) in the absence (*lane 2*) or presence of increasing concentrations of wortmannin (*lanes 3–5*) using oligonucleotide B as probe. *B*, EMSA analysis of IUF1 binding activity in nuclear extracts prepared from human islets of Langerhans incubated for 4 h in 3 mM glucose (*L4*, *lane 1*) or 3 mM glucose for 3 h followed by 1 h in 20 mM glucose (*L3H1*) in the absence (*lane 2*) or presence of increasing concentrations of LY 294002 (*lanes 3–5*), using oligonucleotide B as probe. *C*, EMSA analysis of IUF1 binding activity in nuclear extracts from MIN6 cells incubated for 3 h in 0.5 mM glucose (*L3*, *lane 1*) or 3 h in 0.5 mM glucose followed by 1 h in 16 mM glucose (*H1*, *lanes 2–4*) or 1 mM sodium arsenite (*Ars*, *lanes 5* and *6*) in the absence (*lanes 1* and *2*) or presence (*lanes 3–6*) of 50 and 100 nM wortmannin (*Wt*). *D*, MIN6 cells in low (0.5 mM) glucose (*LG*) were incubated for 30 min in the absence (−) or presence (+) of 100 nM wortmannin and then exposed for 10 min to high (16 mM) glucose (*HG*) or 0.5 mM glucose containing 1 mM sodium arsenite (*Ars*). MAPKAP-K2 was assayed as in Fig. 4*A*. The results are shown ± S.E. for experiments using three dishes of cells at each time point. Similar results were obtained in an independent experiment.

His-tagged IUF1 was then purified using a nickel-NTA affinity resin and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. These experiments demonstrated that the activation of IUF1 was accompanied by the phosphorylation of a 46-kDa protein that bound to the nickel-NTA column (Fig. 6*A*), was recognized by IUF1 antibodies, bound to oligonucleotide B (data not shown), and was of similar size to activated IUF1 in β-cells, as determined by Western blotting (4). No phosphorylation of the 46-kDa protein occurred if SAPK2 was replaced by either p42 MAPK or MAPKAP-K2 (Fig. 6*A*).

The predicted molecular mass of IUF1 is 31.5 kDa, and recombinant His-tagged IUF1 exhibits this size when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 6*B*). The size difference between inactive recombinant IUF1 and activated IUF1 can be attributed in part to phosphorylation because incubation of recombinant IUF1 with MgATP, SAPK2 and MIN6 extract was accompanied by an increase in its apparent molecular mass from 31 to 46 kDa (Fig. 6*B*, *lanes 2* and *3*), which could be reversed by phosphatase treatment (Fig. 6*B*, *lanes 4* and *5*). This 31- to 46-kDa size shift was also observed when recombinant IUF1 was treated with SAPK2 and extracts from PC12 (human phaeochromocytoma) or AtT20 (mouse pituitary corticotrophic) cells, but not with SAPK2 and extracts of HepG2 (human liver) or COS-7 (monkey kidney) cells (data not shown).

Inhibitors of Phosphatidylinositide 3-Kinase Prevent the Glucose-induced Activation of IUF1 to DNA by Inhibiting the Activation of SAPK2—Two structurally unrelated inhibitors of phosphatidylinositide (PI) 3-kinase, wortmannin and LY 294002, prevented the glucose-induced activation of IUF1 DNA binding in MIN6 cells at concentrations similar to those that are known to inhibit growth factor-stimulated PI 3-kinases (Fig. 7, *A* and *B*). This observation was unexpected, since these compounds had not been reported to inhibit the activation of SAPK2 by any other agonist. However, as shown in Fig. 7*D* wortmannin (100 nM) completely prevented the activation of MAPKAP-K2 by glucose. In contrast, wortmannin had no effect on the arsenite-induced DNA binding of IUF1 (Fig. 7*C*) or activation of MAPKAP-K2 (Fig. 7*D*) or on the phosphorylation and activation of IUF1 that occurred when MIN6 cell extracts were incubated with SAPK2 and MgATP (data not shown).

Effect of SB 203580 and Inhibitors of PI 3-Kinase on IUF1-dependent Gene Transcription—To investigate the functional significance of modulations in IUF1 binding activity in terms of the overall transcriptional response to glucose, a construct (pGL-LUC 200) containing the −50 to −250 base pair region of

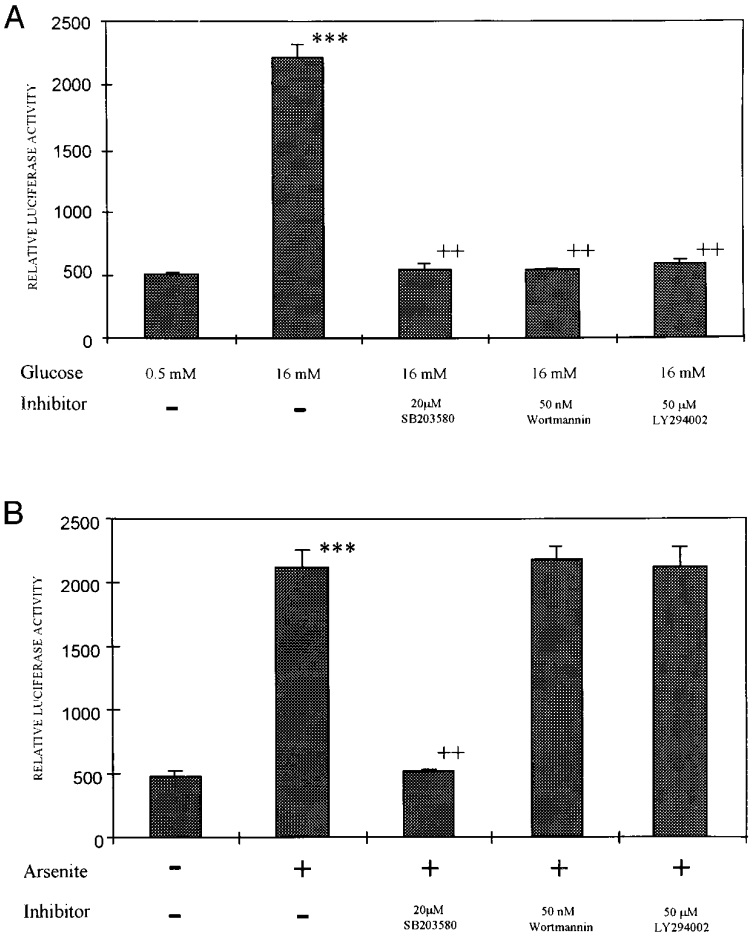

FIG. 8. **Glucose and arsenite stimulate the activity of the human insulin promoter in transfected MIN6 cells: effects of SB 203580, wortmannin, and LY 294002.** *A*, MIN6 cells were transfected with pGL-LUC200 and incubated in 0.5 mM glucose or 16 mM glucose in the absence or presence of the indicated inhibitors. *B*, MIN6 cells were transfected with PGL-LUC200 and incubated in 0.5 mM glucose or 0.5 mM glucose plus 0.5 mM sodium arsenite in the absence or presence of the indicated inhibitors. Values are given as relative luciferase activity and are normalized for protein concentration. *Error bars* represent standard deviation ($n = 4$). ***, $p < 0.00001$ when compared with 0.5 mM glucose; ++, $p < 0.001$ when compared with 16 mM glucose or 1 mM sodium arsenite.

the human insulin gene promoter was transfected into MIN6 cells. A construct lacking the insulin promoter sequence was utilized in control experiments. Stimulation of transfected cells with 15 mM glucose led to a 5-fold increase in expression of the pGL-LUC 200 construct (Fig. 8*A*), an effect that was prevented by SB 203580, wortmannin, or LY 294002 (Fig. 8*A*). Arsenite (1 mM) mimicked high glucose in stimulating IUF1-dependent gene expression (Fig. 8*B*), and the effect of arsenite was prevented by SB 203580, but not by wortmannin or LY 294002, as expected (Fig. 8*B*). The activity of the control construct did not differ significantly from that observed at low (0.5 mM) glucose (data not shown). These results demonstrate that the glucose-induced increase in DNA binding of IUF1 correlates with the glucose-responsive activity of the −50 to −250 base pair region of the insulin gene promoter.

DISCUSSION

The results presented in this paper provide overwhelming evidence that the pancreatic β-cell-specific transcription factor IUF1 only binds to DNA when β-cells are exposed to high concentrations of glucose. Thus, the binding of IUF1 to DNA was 20-fold greater when human islets of Langerhans were exposed to 20 mM glucose than when exposed to 3 mM glucose (Fig. 2, *D* and *E*). Moreover, the binding of IUF1 to DNA triggered by high glucose was prevented by the pyridinyl imidazole SB 203580 (Fig. 3, *A* and *D*), which is now well established as a specific inhibitor of the protein kinase SAPK2 (6, 27). Many other lines of evidence demonstrated that SAPK2 plays a key role in the activation of IUF1 by glucose. First, high glucose triggered the activation of MAPKAP-K2 (Fig. 4*A*), an immediate downstream target of SAPK2, and the activation of MAPKAP-K2 was prevented by SB 203580 (Fig. 4*B*) at a concentration similar to that which prevented the activation of IUF1 DNA binding (Fig. 3, *D* and *E*). Second, other agonists that activate MAPKAP-K2 (sodium arsenite, heat shock) triggered the activation of IUF1 DNA binding, and the activation of IUF1 (Fig. 3*E*) and MAPKAP-K2 (Fig. 4*B*) induced by arsenite was suppressed by similar concentrations of SB 203580. Third, the ability of glucose to stimulate transcription of a reporter gene containing several upstream IUF1 binding sites was inhibited by SB 203580 and mimicked by heat shock and sodium arsenite (Fig. 8). Fourth, SAPK2 triggered the activation of IUF1 when added to extracts prepared from the β-cell line MIN6 (Figs. 5 and 6), and this was prevented by SB 203580. Taken together, these results demonstrate that SAPK2, or a closely related homologue, plays an essential role in mediating the activation of IUF1 by high glucose. The stimulation of insulin gene expression by high glucose can therefore be thought of as a stress response, and high glucose can be added to the growing list of stresses that activate SAPK2, which include osmotic shock, ultraviolet radiation, and bacterial endotoxins as well as sodium arsenite and heat shock.

Although SAPK2 triggered the activation of IUF1 when added to MIN6 cell extracts, it was unable to phosphorylate and activate bacterially expressed IUF1. The requirement for both SAPK2 and MIN6 extract to induce activation of IUF1 indicates that SAPK2 exerts its effect indirectly by first activating an IUF1-modifying enzyme, which then activates IUF1. However, the IUF1-modifying enzyme does not appear to be MAPKAP-K2 or the closely related MAPKAP-K3 (which has a very similar substrate specificity (8)), because neither of these enzymes induced the phosphorylation and activation of either purified, bacterially expressed IUF1 or endogenous IUF1 in

MIN6 extracts. In addition, IUF1 does not contain a consensus sequence for phosphorylation by MAPKAP-K2/MAPKAP-K3 (Hyd-Xaa-Arg-Xaa-Xaa-Ser-, where Hyd is a bulky hydrophobic residue such as Leu or Phe) (8, 19).

An intriguing and important finding made during the present study was that the high glucose-induced activation of IUF1 DNA binding and the transcriptional activity of the −50 to −250 insulin promoter construct were not only prevented by SB 203580 but also by concentrations of wortmannin and LY 294002, which inactivate PI 3-kinase (Figs. 7 and 8). These observations led to the discovery that the glucose-induced activation of MAPKAP-K2 is also prevented by wortmannin and LY 294002 (Fig. 7*D*), indicating that the suppression of IUF1 activation by these compounds results from their ability to prevent the activation of SAPK2 by glucose. However, the arsenite-induced activation of MAPKAP-K2 in MIN6 cells, as well as the arsenite-induced activation of IUF1 DNA binding and IUF1-dependent gene transcription are unaffected by wortmannin or LY 294002 (Figs. 7 and 8). This not only provides further evidence for an important role of SAPK2 in the activation of IUF1 DNA binding but indicates that glucose induces the activation of SAPK2 by a novel pathway. The activation of IUF1 is known to require the metabolism of glucose (4), suggesting that a glucose-derived metabolite activates PI 3-kinase in β-cells. Clearly, the wortmannin/LY 294002-sensitive component must lie above the point at which the effects of arsenite and glucose converge.

The results obtained with okadaic acid and calyculin A imply a role for protein phosphatases 1 and/or 2A in the regulation of IUF1 DNA binding activity. Since protein phosphatase 2A has been shown to inactivate MAPKAP-K2 (22) as well as SAPK2 (7) and its upstream activators (28), the ability of these compounds to prevent the inactivation of IUF1 at low glucose concentrations (Fig. 2*E*) may be explained by their ability to activate the SAPK2 pathway. Alternatively (or in addition), protein phosphatases 1 and 2A might dephosphorylate IUF1 directly.

Although our studies and those of others (29) clearly implicate IUF1 in mediating the response of the insulin gene to glucose, other transcription factors may also be involved. The factor RIPE3B1, which binds to the C1 element of the rat insulin 2 gene may be regulated by glucose (30), and other data have been presented that indicate that several other sequence elements within the insulin promoter may be glucose-responsive (31, 32). In this connection, it is of interest that the insulin gene promoter contains a cyclic AMP response element (CRE), which should bind the transcription factor CREB. CREB is only active when it has been phosphorylated at Ser-133. In SK-N-MC cells, the phosphorylation of CREB induced by fibroblast growth factor or sodium arsenite is prevented by SB 203580, and CREB-dependent gene transcription can be triggered by cotransfection with SAPK2. MAPKAP-K2 phosphorylates CREB at Ser-133 *in vitro* and may be the enzyme that mediates the activation of CREB by fibroblast growth factor and arsenite in SK-N-MC cells (33). It will be interesting to determine whether glucose induces the activation of CREB (and other transcription factors that bind to the insulin gene promoter) via the SAPK2 pathway in β-cells.

Finally, it should be mentioned that the islets of Langerhans are not the only cells in which glucose regulates gene transcription. Glucose is known to control the expression of a large number of genes in liver and adipose tissue (34). These include the L-type pyruvate kinase, spot 14, fatty acid synthase, and acetyl-CoA carboxylase genes (35). The results obtained in this paper may therefore be of general significance, and it will be important to find out whether the SAPK2/MAPKAP-K2 cascade mediates the transcriptional regulation of other glucose-responsive genes.

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