ERK1/2-dependent activation of transcription factors required for acute and chronic effects of glucose on the insulin gene promoter

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Running title: ERK1/2 and regulation of the insulin promoter by glucose

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The insulin promoter is both positively and negatively regulated in response to conditions to which pancreatic β-cells are exposed. Exposure of intact rat islets and INS-1 pancreatic β-cells to 11 mM glucose for minutes to hours results in an enhancement in the rate of insulin gene transcription assessed with a reporter linked to the insulin gene promoter. By 48 hours, glucose inhibits insulin gene promoter activity. Here we show that not only the acute effect of elevated glucose to stimulate the insulin gene promoter but also the chronic effect of elevated glucose to inhibit the insulin gene promoter depend on ERK1/2 MAP kinase activity. In examining the underlying mechanism, we found that acute exposure to 11 mM glucose resulted in the binding of the transcription factors NFAT and Maf to the glucose-responsive A2C1 element of the insulin gene promoter. An NFAT and C/EBP-β complex was observed in cells chronically exposed to 11 mM glucose. Formation of NFAT-Maf and NFAT-C/EBP-β complexes was sensitive to inhibitors of ERK1/2 and calcineurin, consistent with our previous finding that activation of ERK1/2 by glucose required calcineurin activity and the well documented regulation of NFAT by calcineurin. These results indicate that the ERK1/2 pathway modulates partners of NFAT, which may either stimulate or repress insulin gene transcription during stimulatory and chronic exposure to elevated glucose.

Pancreatic β-cells of the islets of Langerhans produce and store insulin in response to physiological demand. Elevated glucose concentrations result in the activation of a complex network of intracellular signaling pathways that trigger β-cells to release insulin and coordinate an increase in the rate of insulin biosynthesis to replenish its supply (1-3). This is achieved not only by increased stability of preproinsulin mRNA, increased translation of the mRNA, and processing of the protein product, but also by increased transcription of the insulin gene (3,4). Increased insulin gene transcription has been detected within 15 min of exposure of β-cells to a glucose stimulus (3,5). Several trans-acting factors have been identified that bind to the insulin gene promoter and enhance transcription in response to glucose (6-15). These include PDX-1 and a heterodimer containing an E2A gene product (E12, E47) and Beta2 (also known as NeuroD1), which bind to A and E boxes of the insulin promoter to provide a synergistic effect on insulin gene transcription. Negative regulators of insulin...
promoter activity have also been identified, including the CAAT enhancer binding protein-β (C/EBP-β), which is expressed in β-cells during prolonged exposure to high glucose concentrations (16,17).

The highly conserved A2-E1A1 region of the insulin promoter is most responsive to glucose and contains binding sites for each of these factors (18). Interactions among factors binding to composite A2-E1 or E1A1 sites of the A2-E1A1 region are sufficient for a potent glucose response in human islets (18). The response is ablated when the E-box is not present; therefore, E47/Beta2 is critical for glucose-induced insulin gene transcription. Although it has been demonstrated that PDX-1 can bind to A1 and A3/4 sites of the insulin promoter, it is still unclear if there are conditions in which PDX-1 will bind to A2 (19). The A2 site does overlap with binding sites of the RIPE3b region of the insulin promoter including an NFAT binding site (A2), a MaF binding site (C1), and an inverted CEB element, which is embedded within the A2C1 composite site (see Fig. 5B). Each of these factors has been shown to bind to these sites when β-cells are exposed to glucose. Synergistic effects of composite sites, compared to individual sites alone, on transcription implies the existence of interactions among factors which bind to the RIPE3b region and E47/Beta2 as well as between E47/Beta2 and PDX-1 to provide maximal glucose-responsiveness to the insulin gene promoter (19). Evidence for the latter has also been reported for E2A3/4 (14,20,21).

The upstream signaling that regulates these factors begins with glucose metabolism in β-cells. Glucose is the central regulator of β-cell function and underlies effects elicited by circulating fuels and hormones to which β-cells respond. One rapid consequence of glucose metabolism in β-cells is cell depolarization and a subsequent rise in intracellular calcium (22,23). Intracellular calcium metabolism is integrated into multiple signaling pathways that orchestrate insulin release and biosynthesis. One such pathway is the ERK1/2 cascade. We and others have previously demonstrated that ERK1/2 are activated in pancreatic β-cells by stimulatory concentrations of glucose, and determined that this activation is calcium dependent (24-26). Moreover, activation of ERK1/2 by glucose is sensitive to inhibitors of calmodulin and the class 2B calcium/calmodulin-dependent phosphatase, calcineurin (27). Hence, calcineurin is an upstream regulator of the ERK1/2 pathway in pancreatic β-cells.

Analysis of mRNA levels showed that blockade of ERK1/2 activity using kinase-defective ERK2 resulted in a substantial reduction in prepro-insulin mRNA content after 24 hours, which along with insulin gene promoter reporter assays, suggested a role for ERK1/2 in insulin gene transcription (28). Possible actions of this signaling pathway on other key steps of the biosynthetic process have not yet been thoroughly explored. ERK1/2 can phosphorylate and modulate the activity of several factors that may regulate the A2-E1A1 region of the insulin promoter. Beta2 and E47 are phosphorylated by ERK1/2 to promote heterodimerization and binding to E-box sites (28). ERK2 has also been reported to phosphorylate and activate the bZIP transcription factors MaFα and C/EBP-β (29-31); however, evidence for physiological roles for ERK1/2 in regulating MaF in β-cells is lacking, and C/EBP-β has been shown to inhibit not activate insulin transcription. In addition to regulating ERK1/2 in response to glucose, calcineurin also activates NFAT to enhance insulin gene transcription (12,32). Here we report that NFAT and MaF both bind to the A2 and C1 elements within the insulin promoter in response to an acutely elevated glucose concentration in an ERK1/2-dependent manner. In β-cells chronically exposed to high glucose,
C/EBP-β binds to NFAT on the promoter, but the NFAT/Maf complex is lost; this suggests that one action of C/EBP-β is to reduce the effect of NFAT on insulin gene transcription. We conclude that prolonged exposure of β-cells to glucose results in an ERK1/2-dependent change in factors associated with the insulin gene promoter.

MATERIALS AND METHODS

Materials--Early passages and subclones selected for increased glucose-stimulated insulin secretion of the rat pancreatic β-cell line INS-1 were kindly provided by Chris Newgard (Duke) (33). NFAT expression vectors were kindly provided by Chi-Wing Chow (Albert Einstein College of Medicine). Antibodies were as described or from the following sources: ERK1/2 (Y691 (34)), phospho-ERK1/2 (Sigma), NFATc3 (Santa Cruz), c-Maf (Santa Cruz), C/EBP-β (C19, Santa Cruz; Cell Signaling).

Cell culture and isolation of islets--INS-1 cells were grown in RPMI 1640 medium (Sigma) containing 5.5 or 11 mM glucose, 10% fetal bovine serum, 10 mM Hepes, pH 7.4, 10.2 mM L-glutamine, 50 mM sodium pyruvate, 2.5 mM β-mercaptoethanol, streptomycin (0.1 mg/ml), and penicillin (100 U/ml) at 37 °C in 10% CO₂. Pancreatic islets were isolated from Zucker nondiabetic and diabetic rats as described (32).

 Constructs--Vector constructs harboring the full-length 410 bp region (pSYNT) and the A2E1 region (pFOXCAT-4XA2E1) of the rat insulin promoter and a plasmid encoding MafA were kindly provided by Michael German (UCSF). The -410 rInsI and 4XA2E1 fragments were amplified by PCR with primers incorporating 5′-XhoI and 3′-HindIII restriction sites and directionally cloned into the pGL3-Basic luciferase promoter-reporter mammalian expression vector (Promega). The MafA coding sequence was subcloned into pCMV5. The plasmid MSV-C/EBP-β was obtained Fred Robinson (UCSD).

Transfections and reporter assays--INS-1 cells were grown in 6-well plates to 60–80% confluency in 5.5 mM glucose and cotransfected with either pGL3-rInsI or pGL3-A2E1 and pRL-SV40 using the FuGene-6 reagent (Roche Molecular Biochemicals, IN). Eighteen h after transfection, the cells were placed in fresh 5.5 mM glucose or stimulated with 11 mM glucose and harvested at the indicated times (2, 6, 12, 24, 48 h). For inhibitor studies, 25 μM U0126, 1 μM FK506 or FK520, 1 μM rapamycin, or 0.1% DMSO control was added to the medium 30 min prior to cell stimulation. Cells were harvested with passive lysis buffer (Promega, WI) that was supplemented with 100 mM β-glycerophosphate, 2 mM Na₃VO₄, and 100 mM NaF. The lysates were vortexed for 30 sec and the supernatants were collected following centrifugation for 30 min at 14,000 rpm at 4 °C in a microcentrifuge. The supernatants were stored at -80 °C. Samples were then assayed for promoter activity by the Dual Luciferase Assay System (Promega, WI) using a TD-20/20 bioluminometer (Turner Designs) or for ERK1/2 activation by immunoblotting.

Immunoblotting--Extracts from INS-1 cells were prepared by boiling for 5 min in sodium dodecyl sulfate (SDS) electrophoresis sample buffer. Lysate proteins (30 μg) were resolved on polyacrylamide gels in SDS. The proteins were electro-transferred to nitrocellulose membranes and blotted with indicated antibodies. Blocking was typically in 1x Tris-buffered saline (TBS) with 1% bovine serum albumin, 1% milk, and 0.1% polyoxyethylene sorbitan monolaurate (Tween-20) Washes were in TBS-0.1% Tween-20. Enhanced chemiluminescence (ECL) was used as the method of detection by secondary antibodies conjugated to horseradish peroxidase.
Immunofluorescence—Cells were plated onto 24-well dishes and were exposed to glucose under conditions described above. Cells were fixed with ice cold methanol, washed with 1 ml of PBS, and permeabilized with cold 0.5 ml PBS, 0.2% Triton X-100 for 15 min. Prior to addition of antibodies, cells were incubated with PBS, 0.1% Triton X-100, 4% BSA overnight. Primary antibody (1:250) in the same solution was incubated with cells overnight. After washing, cells were incubated with secondary antibody (1:5000) in cold PBS, 0.1% Triton X-100, 1% BSA for 1 h.

Electrophoretic Mobility Shift Assays (EMSAs)—Complementary oligonucleotides containing wild type (A2C1) (wt) A2C1 (5’-GTGTTGGAATTACAGCTTGCCCT) and mutated (m) NFATm (5’-GTGTTGTTCCATTACAGCTTGCCCT), MAREm (5’-GTGTTTGGAAATTACAGCTGACTACCCCT), NFATm/MAREm (5’-GTGTTTGGAAATTACAGCTGACTACCCCT), C/EBP-bm (5’-GTGTTTGGAAATTAGCTTGCCCT), NFATm/CEBP-bm/MAREm (5’-GTGTTTGGAAATTACAGCTGACTACCCCT) consensus sites of the ratI insulin gene promoter were synthesized (IDT Integrated DNA Technologies, IA). The oligonucleotides were hybridized, and end-labeled with T4 polynucleotide kinase (NEB) in the presence of [γ-32P]ATP. INS-1 cells were lysed in buffer A (10 mM Tris (pH 8.0), 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenyl methylsulfonyl fluoride, and 10 μg/ml leupeptin) supplemented with 0.6% NP-40. The pellets were washed with buffer A and collected by centrifugation for 2 min at 14,000 rpm. The nuclear pellets were resuspended in buffer B (10 mM Tris (pH 8.0), 205 mM KCl, 1 mM dithiothreitol, 0.5 mM PMSF, and 10 μg/ml leupeptin) to harvest nuclear extracts. Equal amounts of nuclear extract proteins (20 μg) were incubated for 30 min with double-stranded 32P-labeled A2C1 probe (20,000 cpm) in reaction buffer (10 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 6% glycerol). Where indicated, antibodies were added 15 min after labeled probe. The reactions were subjected to electrophoresis on 6% polyacrylamide gels and bands were detected on film by autoradiography.

Co-immunoprecipitation—INS-1 lysate proteins (300 μg) were incubated with indicated antibodies (1:100 dilution) and 50 μl 1:1 slurry of protein A-Sepharose beads (Amersham) at 4 °C for 1 h. Beads were centrifuged and washed in buffer A and resuspended in 50 μl 1x SDS electrophoresis sample buffer. Samples were analyzed by immunoblotting as described.

Chromatin Immunoprecipitation (ChIP) assays—The standard conditions for treatment of INS-1 cells were exposure to 5.5 mM glucose for 30 min (basal), exposure to 11 mM glucose for 30 min (stimulated), or exposure to 11 mM glucose for at least 48 h (chronic). INS-1 cells were then exposed to 1% formaldehyde and rocked for 8 min for protein-chromatin cross-linking. Glycine (final concentration 125 mM) was added, and the plates were rocked for an additional 2 min. The medium was removed and the cells were washed 2x with ice-cold PBS and harvested in passive lysis buffer. Chromatin was sheared by ultrasonication using a microtip probe (Sonics and Materials, Inc.) with an amplitude setting of 30 for 10 1.5-second pulses. The cross-linked lysates were cleared by centrifugation and stored at -80°C. Samples (300 μg protein) were adjusted to a volume of 0.3 ml with lysis buffer. The input control contained 50 μg of protein adjusted to 0.3 ml with lysis buffer. The samples were pre-cleared with 20 ml protein A-Sepharose beads and immunoprecipitations used 1 μl of the indicated antibodies, 1 μl purified IgG (Santa Cruz) corresponding to host animal antibodies, or no antibody. The reactions were incubated
overnight at 4°C with protein A-Sepharose beads which had been pre-incubated with 1 mg/ml bovine serum albumin. The protein A-Sepharose beads were sedimented and washed with: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 8.0 for 5 min; again with the same buffer only containing 0.5 M NaCl for 5 min; 250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.0 for 5 min; 1 M NaCl, 20 mM Tris, pH 7.4 for 5 min; and finally with 10 mM Tris pH 8.0, 1 mM EDTA twice for 5 min each. The immunoprecipitates were eluted from the beads with 0.5 ml of 1% SDS, 0.1 M NaHCO3. To reverse crosslinking, samples were adjusted to 0.2 M NaCl and incubated at 65°C for 5 h.

Chromatin was extracted with phenol/CHCl3 and precipitated with NaOAc/EtOH. DNA precipitates were used as templates for detecting the presence of a rInsI promoter segment (-329 to –90) by PCR using primers: 5’-CTGGGAAATGAGGTGGAAAA and 5’-AGGAGGGGTTAGGAGGCAGA.

**Statistical Analyses**—Results are expressed as means +/- SEM determined from at least three independent experiments, unless otherwise stated. Statistical significance was calculated by one-tailed unpaired Student’s t-test.

**RESULTS**

ERK1/2 stimulate and repress the activity of the insulin gene promoter--We compared the ability of different concentrations of glucose to stimulate insulin transcription and found that there was little stimulation of transcription of a reporter linked to the rat I insulin gene promoter observed from 3.8 to 5.5 mM glucose. As the glucose concentration was increased to 11 mM glucose, increased activity of the reporter was observed; no further increase was noted at 16 mM glucose (Fig. 1 and data not shown). Thus, for these experiments we cultured INS-1 cells at 5.5 mM glucose and used this as the control or basal condition. As reported in the literature (17,35-39), chronic exposure (24 h or longer) to a high glucose concentration inhibits insulin gene transcription. Because ERK1/2 are required for stimulation of insulin gene transcription by glucose, we examined the potential functions of ERK1/2 in this inhibitory process.

To examine effects on insulin gene transcription, cells were exposed to 11 mM glucose for 2 to 48 h and the activity of the reporter was measured (Fig. 2A-D). Glucose-enhanced insulin gene promoter activity was observed for up to 6 h of exposure to 11 mM glucose; as expected, this stimulation was blocked by inhibition of the ERK1/2 pathway with U0126. These results are in agreement with our earlier work showing that K52R ERK2 (a dead mutant that blocks activation of endogenous ERK1/2) and another pharmacological inhibitor with similar specificity decreases prepro-insulin mRNA content and inhibits the insulin gene promoter (28). U0126 inhibits the MAP kinase kinases MEK1/2, the two enzymes that activate ERK1/2, and at higher concentrations also MEK5, the enzyme that activates ERK5 (40). It is one of the most selective protein kinase inhibitors because, unlike the majority of such drugs, U0126 is not an ATP competitor (41).

We have found no effect of glucose on the activity of ERK5 in INS-1 cells (data not shown); thus the events inhibited by U0126 are not due to preventing activation of ERK5.

Within 12 h, the effect of glucose on the insulin gene promoter-reporter was reduced to near basal levels; by 24 h glucose inhibited insulin gene promoter activity; and at 48 h, glucose repressed insulin gene transcription to approximately half that of transcription at the control glucose concentration of 5.5 mM at the same time (Fig. 2A). Surprisingly, the repression of transcription below basal activity by chronic exposure to 11 mM glucose was also reversed by U0126; activity in the presence of
the inhibitor was consistently close to that observed at 5.5 mM glucose. These findings suggest that ERK1/2 are required not only for the stimulatory but also for the inhibitory effect of glucose on insulin gene transcription.

As we showed previously, activation of ERK1/2 by glucose is dependent upon calcineurin (27). Therefore, we examined the effects of the calcineurin inhibitors FK506 and FK520, as well as rapamycin, which inhibits the mammalian target of rapamycin mTOR, on glucose-regulated insulin promoter activity. All three agents inhibited the acute increase in insulin gene reporter activity induced by glucose (Fig. 2B-D). Rapamycin does not inhibit ERK1/2 activation; thus, rapamycin controls events required for promoter activity that are independent of ERK1/2-regulated signal transduction pathways. Both the MEK inhibitor and the calcineurin inhibitors (FK506 and FK520, Fig. 2B) also reversed glucose-induced repression of basal insulin promoter activity at 48 h, indicating that inhibition by glucose requires ERK1/2. In contrast, rapamycin, which does not block ERK1/2 activation, had no effect on the repressed transcription (see model of Fig. 5A).

Lysates from each sample of transfected cells were immunoblotted to examine the activation state of ERK1/2 using antiphospho-ERK1/2 antibodies (Fig. 2E). ERK1/2 was phosphorylated and activated in cells treated with both 5.5 mM and 11 mM glucose; in both cases activation was blocked by the addition of either U0126 or FK520. Consistent with our earlier findings, rapamycin had no effect on ERK1/2 activation by glucose (27).

To determine if these findings using β-cell lines are representative of the behavior of β-cells in intact islets, we examined pancreatic islets isolated from wild type and Zucker fatty diabetic rats. After isolation, islets were transfected with the rat insulin promoter construct and then incubated in medium containing either 5.5 or 11 mM glucose for 6 or 48 h (Fig. 3). The stimulatory effect of glucose observed in islets from normal animals exposed to 11 mM glucose for 6 h was lost after 48 h of incubation in 11 mM glucose. The stimulatory effect observed at 6 h was blocked by U0126 and FK520. The inhibitory effect of 48 h of incubation in 11 mM glucose was alleviated with the MEK and calcineurin inhibitors, supporting the findings in β-cell lines. Promoter activity was substantially lower in islets from Zucker diabetic rats, which also displayed no glucose-stimulated activity (Fig. 3, right). However, U0126 and FK520 enhanced promoter activity under both basal and stimulatory conditions. These data suggest that culturing islets from ZDF diabetic rats for 24 h in 5.5 mM glucose is not sufficient to reverse ERK1/2-dependent inhibitory effects imposed on insulin promoter activity.

**Regulation of the A2-E1 region of the insulin gene promoter.** We have previously shown that ERK1/2 can stimulate the E2-A3/4 region of the insulin gene promoter via E47/Beta-2 and PDX-1 (28). Therefore, we examined effects of ERK1/2 on the more glucose-responsive A2-E1 region of the promoter (Fig. 5B). The glucose-induced changes in reporter activity in INS-1 cells transfected with the reporter driven by tandem copies of A2-E1 were similar to those observed using the full-length promoter-reporter (Fig. 4A-C). The short-term stimulation of A2-E1-driven reporter activity by glucose was sensitive to inhibition by U0126 (Fig. 4A). As was observed for the full length promoter, prolonged exposure to glucose, for 24 h or more, repressed reporter activity driven by A2-E1 below basal activity by up to 70%.

**ERK1/2 regulate binding of transcription factors to the insulin gene promoter.** To continue our identification of mechanisms by which ERK1/2 regulate the insulin gene promoter, we determined if ERK1/2 could affect binding of factors to the A2-E1 region (Fig. 5B). A DNA-binding
complex from INS-1 nuclear extracts was detected by electrophoretic mobility shift assays (EMSAs) which bound to the A2C1 component of the A2-E1 region specifically in response to acute exposure to 11 mM glucose (Fig. 6A). A distinct complex bound to A2C1 in INS-1 nuclear extracts from cells cultured in 11 mM glucose (Fig. 6A). Detection of these complexes was blocked by U0126 and FK506, but not by rapamycin, indicating a dependence on ERK1/2 activity for their formation. Non-specific bands were observed (numbered 1 and 3 in Fig. 6) that were either drug insensitive and/or present with the control probe. The first of these was often more intense in extracts from cells cultured in 11 mM glucose or with probes in which NFAT and MARE sites were mutated. A2C1 has been shown to contain binding sites for NFAT, Maf, and C/EBP-β, all of which have been linked either to ERK1/2 or calcineurin in other cell types (29,31,42,43). To determine if these factors were present in the identified complexes, we preincubated nuclear extracts isolated from INS-1 cells exposed to basal, stimulatory, and chronic glucose conditions as above with antibodies directed against NFAT, c-Maf, and C/EBP-β (Fig. 6B). Formation of the complex found under stimulated conditions was disrupted by NFAT and c-Maf antibodies. Formation of the inhibitory complex from cells cultured in 11 mM glucose was blocked by NFAT and c-Maf antibodies. These findings suggest that NFAT is present in both complexes. If the cells were treated with either U0126 or FK506, the formation of both the stimulatory and inhibitory complexes was prevented, indicating that one or more events controlled by ERK1/2 are required for complex formation.

To determine which of these factors bind directly to DNA, we mutated DNA bases within their respective DNA-binding motifs (see sequences in Fig. 5B). The NFAT-Maf complex did not form if either the NFAT or the Maf binding site was disrupted (Fig. 6C), indicating that both must bind to DNA for detection of complex formation under these conditions. However, mutation of both the NFAT and the Maf binding sites did not prevent the formation of the NFAT-C/EBP-β complex in extracts from cells chronically exposed to 11 mM glucose. Thus, these complexes appear capable of forming as long as the C/EBP-β site is intact. Consistent with this expectation, mutation of the CEB site prevented formation of the NFAT-C/EBP-β complex. Collectively, these data suggest that one mechanism by which ERK1/2 regulate the A2-E1 region of the insulin promoter is by inducing the binding of NFAT-Maf and NFAT-C/EBP-β complexes.

NFAT co-immunoprecipitates with Maf and C/EBP-β. To obtain independent evidence that NFAT interacts with Maf and C/EBP-β, we immunoprecipitated with antibodies directed against C/EBP-β, c-Maf and NFAT itself (Fig. 7A). Two NFAT forms were immunoprecipitated with antibodies to NFAT, one of approximately 160 kDa and a second of 120 kDa (Fig. 7A). Multiple splice forms and phosphorylation states generate NFAT species with a range of molecular weights (44). The larger NFAT form was present in C/EBP-β as well as Maf immunoprecipitates, confirming that NFAT interacts with both proteins.

Because interpretation of some of our results depends on the specificity of the antibodies that were used, we characterized the ability of the antibodies to recognize recombinant proteins expressed in 293 cells. The C/EBP-β antibody detected species of ~35 and 45 kDa in lysates of transfected cells consistent with the sizes of the human and rat C/EBP-β species known as LAP molecules (Fig. 7B); a 45 kDa band, presumably endogenous C/EBP-β was also detected in untransfected lysates. Two species of ~43 and 40 kDa were detected with the c-Maf antibody in lysates from cells transfected with MafA (Fig. 7C); no proteins were detected in untransfected lysates.
We conclude that the c-Maf antibody clearly recognizes MafA, the major Maf form found in β-cells. Thus, our co-immunoprecipitation studies indicate that MafA or other Maf species interact with NFAT.

Changes in NFAT and Maf occupancy of the A2-E1 region in intact cells. Chromatin immunoprecipitation (ChIP) assays were used to measure binding of NFAT, Maf, and C/EBP-β directly to the A2-E1 region of the insulin promoter in intact cells (Fig. 8). Cross-linked and sheared chromatin from cells exposed to basal, stimulatory, and chronic glucose conditions was immunoprecipitated through its association with C/EBP-β, Maf, and NFAT. The A2-E1 region present in the immunoprecipitates was amplified following reversal of the crosslinking to determine which factors had been crosslinked to this region. Antibodies against NFAT and Maf immunoprecipitated DNA from the A2-E1 region primarily from cells acutely exposed to 11 mM glucose. Antibodies against C/EBP-β immunoprecipitated A2-E1 DNA only under inhibitory conditions. These results suggest that NFAT and Maf bind to A2-E1 DNA in a glucose-dependent manner, while C/EBP-β is only associated with this region of the insulin promoter under inhibitory conditions (model Fig. 10), as previously reported (16).

Finally, to examine the expression of C/EBP-β, we immunoblotted lysates of INS-1 cells under basal, stimulated, and chronic conditions (Fig. 9A). The 35 kDa form of C/EBP-β was only detected in cells chronically exposed to 11 mM glucose. Immunofluorescence also showed that the amount of C/EBP-β was substantially increased in cells chronically exposed to 11 mM glucose (Fig. 9B).

Effects of ectopically expressed factors on activity of the A2-E1 reporter--Islet-enriched factor Beta2 and the β-cell specific activator MafA have been previously shown to confer tissue-specific expression of insulin in β-cells (45,46). To confirm that NFAT-MafA could activate the A2-E1 region of the rat I insulin promoter and that C/EBP-β could repress this activity, we transfected a non-β-cell line (293 cells) with either NFAT, MafA, C/EBP-β, Beta2 or a combination of these factors (Fig. 10). The cells were stimulated with epidermal growth factor and ionomycin to activate ERK1/2. NFAT expressed alone did not stimulate the A2-E1 region, whereas NFAT-MafA caused a modest increase compared to MafA alone. This increase could be disrupted by the overexpression of C/EBP-β with these factors. Moreover, NFAT-MafA-Beta2 synergistically activated the A2-E1 promoter-reporter compared to effects of each factor expressed alone. This stimulation was inhibited by U0126 or prevented by overexpressing C/EBP-β. These data indicate that the NFAT-MafA contribution to the A2-E1 insulin promoter region is most pronounced when Beta2 is present and that the synergistic effects of these factors on promoter activity are ERK1/2-dependent. The results also support the conclusion from β-cells that C/EBP-β represses the enhancement of NFAT-MafA promoter activation by Beta2.

DISCUSSION

In the β-cell, as in other cells, many signal transduction events are coordinated to elicit cellular outputs. None are likely to be sufficient to regulate complex processes such as glucose-dependent insulin gene transcription, but many are likely to be required. Thus, it will be important to catalog each of these events and their mechanisms of action. We find that ERK1/2 are required for this process and have begun to define the ERK1/2-mediated events that impinge on the insulin gene promoter.

We showed that freshly isolated rat islets and β-cell lines display an increase in reporter
activity driven by the full length promoter when exposed to 11 mM glucose for from 2 to 6 h. We previously showed that glucose-dependent transcription from the E2A4/3 region of the insulin gene promoter required ERK1/2 and that Beta2, PDX-1, and E12/47 were targets for ERK1/2 regulation (28). To determine the sites and mechanisms of action of ERK1/2, here we have studied ERK1/2-sensitive processes involving the glucose-responsive A2-E1 elements of the promoter. We found that Maf-NFAT complexes associate with A2C1 in an ERK1/2-dependent manner under conditions that stimulate insulin gene transcription. Complexes induced by glucose with similar drug sensitivity were also observed in Min6 and βTC3 cells, two other lines with characteristics of pancreatic β-cells (data not shown). MafA binds to this region (10,11,47,48). However, its binding to NFAT on A2C1 has not previously been demonstrated. We conclude that ERK1/2 influence glucose-dependent stimulation of insulin gene transcription through actions on at least five factors that bind to two of the most glucose-sensitive regions, A2E1 and E2A4/3, of the insulin gene promoter (Fig. 11).

In addition to the stimulatory effects of glucose on promoter activity, during more prolonged stimulation of INS-1 cells with glucose, a reduced response was observed if cells were cultured for 24 h or more in 11 mM glucose. Inhibition of insulin gene transcription by prolonged exposure to elevated glucose has been referred to as glucose desensitization, because it is a reversible phenomenon (35,39,49). Blood glucose that remains at 11 mM (200 mg glucose/dl plasma) for several hours is indicative of a diabetic state according to World Health Organization standards (50). Thus, the impaired response of the insulin gene promoter may eventually lead to depletion of the prepro-insulin mRNA pool. The result from a prolonged impairment in insulin gene transcription might be the loss of the ability to synthesize adequate insulin to control blood glucose. Chronic inhibition of transcription by glucose has been associated with increased expression of C/EBP-β, and decreased expression of PDX-1 and MafA (17,39,51-53), all of which may be involved in this system. In our examination of the mechanism, we have found the unexpected result that the inhibition of insulin gene transcription caused by chronic exposure to 11 mM glucose is dependent on ERK1/2 activity. Thus, activation of ERK1/2 in an inappropriate context may decrease insulin gene transcription through actions on these factors.

Blocking ERK1/2 activation restores transcription driven by the full length promoter in the chronic presence of 11 mM glucose to basal levels. Glucose-stimulated transcription from the full length promoter also requires ERK1/2. Therefore, transcription at the stimulated level could not have been observed in the presence of U0126. In the case of isolated A2-E1 elements, blocking ERK1/2 inhibited not only glucose-stimulated transcription but also the basal activity of A2-E1. These results show that both the basal activity and the glucose-induced stimulation of the A2-E1 region of the insulin promoter are critically dependent upon ERK1/2 and calcineurin. Because basal transcription driven by A2-E1 was suppressed by inhibition of ERK1/2, we were not able to assess to what extent the inhibitory effects of chronic glucose on these isolated elements may involve ERK1/2. However, the fact that the ERK1/2 and calcineurin inhibitors blocked the formation of complexes that were induced under chronic glucose conditions suggests that ERK1/2 also participate in suppressing the activity of A2-E1 caused by chronically elevated glucose. The lack of effect of blocking ERK1/2 activation on basal transcription from the full length promoter suggests that there are other contributing, ERK1/2-independent effects on basal promoter activity.

A question that we have not yet answered is what mechanisms control formation
of the stimulatory and inhibitory complexes that require ERK1/2. Presumably, phosphorylation by ERK1/2 is involved. MafA is phosphorylated on at least two sites by ERK1/2 in vitro; these phosphorylations enhance differentiation of avian neuroretina cells to lens (29). Thus, Maf is likely a direct target of ERK1/2 in pancreatic β-cells; phosphorylation may promote the formation or function of the stimulatory complex found here. A decrease in MafA expression, as reported (39), might decrease the amount of the stimulatory complex. C/EBP-β is also phosphorylated by ERK1/2 in other cell types (31); phosphorylation has been associated with enhanced activity of the protein (30,43). In pancreatic β-cells prolonged elevation of glucose induces C/EBP-β which then interacts with E47, another ERK1/2 target (16). Thus, there are several other mechanisms by which C/EBP-β might be impacted by ERK1/2 in cells chronically exposed to 11 mM glucose including phosphorylation by other kinases or other sorts of modifications. NFAT may also be an ERK1/2 target. In either of these cases, we would predict that prolonged exposure to elevated glucose enhances access of ERK1/2 to these factors in a manner that does not occur during acute glucose stimulation.

A question important to the elucidation of the signaling mechanisms will be to determine if there are calcineurin-regulated events that are independent of ERK1/2. In heart and T cells calcineurin regulates the dephosphorylation of NFAT that allows it to accumulate in the nucleus of stimulated cells in a manner most likely unrelated to ERK1/2 (54,55). This has also been found in β-cells (12). We have shown that activation of ERK1/2 by glucose in β-cells depends on calcineurin. Therefore, because NFAT is present in the complexes, calcineurin likely has at least two distinct inputs into the regulation of insulin gene transcription by glucose.

Finally, from the standpoint of understanding signaling mechanisms, we were surprised to find that the amounts of phosphorylated, active ERK1/2 in cells stimulated with 5.5 mM glucose were not substantially less than in cells stimulated with 11 mM glucose. In earlier studies we found that maximal stimulation of ERK1/2 usually occurred around 8 mM glucose. Culture in 11 mM glucose may have altered the sensitivity of ERK1/2 to glucose (25). We suggest two possible explanations for the fact that ERK1/2-dependent differences were observed at 5.5 and 11 mM glucose. First, an undetectable, but functionally significant, increase in ERK1/2 phosphorylation or localization may occur between 5.5 and 11 mM glucose. In this regard, we have recently observed equivalent ERK1/2 activation in primary human fibroblast cells by two ligands which caused distinct localizations of the active enzymes and distinct cellular changes (56). Second, and perhaps more likely, the effects of ERK1/2 may depend on another event that is only triggered at higher glucose concentrations. We are currently attempting to distinguish between these possibilities. Whatever the reason for the similarity in the amount of activated ERK1/2 at 5.5 and 11 mM glucose, the stimulation of insulin gene transcription observed at 11 mM glucose is sensitive to both U0126 and FK520, indicating that ERK1/2 are required.
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**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig. 1.** Effect of glucose concentration on rat I insulin promoter activity and effects of the MEK inhibitor U0126 on glucose-induced stimulation. INS-1 cells were transfected with rInsI-Luc and stimulated for 4 h with indicated concentrations of glucose. Luciferase activity was measured and values were normalized to SV40 promoter activity as an internal control. Average of three experiments.

**Fig. 2.** Time course of regulation of the rInsI promoter activity by glucose in transfected INS-1 cells. A) Effects of glucose on insulin gene promoter activity in the absence or the presence of U0126 from 2 to 48 h. B) Comparison of the effects of FK506, FK520, and rapamycin on insulin promoter activity after 4 h of stimulation. C) Effects of glucose on insulin gene promoter activity in the absence or the presence of FK520 from 2 to 48 h. D) Effects of glucose on insulin gene promoter activity in the absence or the presence of rapamycin from 2 to 48 h. Results in A-D are the average of four experiments. E) Lysates of transfected cells exposed to 5.5 mM glucose, 11 mM glucose, or 11 mM glucose plus 25 μM U0126 for from 2 to 48 h immunoblotted with antibodies to phosphorylated ERK1/2 (P-ERK1/2) and antibodies that recognize all forms of the proteins.

**Fig. 3.** Islets from Zucker normal and diabetic rats were transfected with rInsI-Luc and exposed to glucose for 6 or 48 h in the presence of the indicated drugs. Assays were as in Figure 1. Average of four experiments.

**Fig. 4.** Time course of regulation of the A2-E1 region of the rat I insulin promoter by glucose in transfected INS-1 cells. A) Effects of glucose on A2-E1 activity in the absence or the presence of U0126 from 2 to 48 h. B) Effects of glucose on A2-E1 activity in the absence or the presence of FK520 from 2 to 48 h. C) Effects of glucose on A2-E1 activity in the absence or the presence of rapamycin from 2 to 48 h. Results in A-C are the average of four experiments.

**Fig. 5.** Summary of regulation of promoter activity and schematic diagram of the glucose-responsive A2-E1-A1 region of the insulin gene promoter. A) Model of glucose regulation of the
insulin promoter. Scheme is based on previous studies of the mechanism of ERK1/2 regulation and analysis here. B) DNA binding sites of transcription factors which bind to indicated cis-acting elements. Arrows indicate reported synergy between complexes. C) DNA sequence alignment of elements comprising the A2C1 component of the rat/I, mouse/I, and human promoters with respect to RIPE3B. A2 and C1 are indicated by dotted lines, and NFAT, C/EBP-β, and Maf binding sites are indicated by boxes. Asterisks indicate bases mutated in A2C1 probes used for EMSA shown in Figure 6.

Fig. 6. Binding of factors to the A2C1 region of the rat I insulin gene promoter. Nuclear extracts from INS-1 cells were isolated to identify complexes that bind to the A2C1 region of rInsI. Cells were cultured at 5.5 mM glucose (basal); treated for 30 min with 11 mM glucose after culture at 5.5 mM glucose (stimulated); and cultured in 11 mM glucose (chronic). A) EMSA with A2C1 oligonucleotide probe or random probe (right) and nuclear extracts from cells treated as indicated. Lane 1 in each panel contains free probe. B) Antibodies to the indicated factors were used to determine if they shifted or blocked complexes bound to the A2C1 probe. The positions of complexes containing NFAT, C/EBP-β, and Maf, from the antibody analysis, are indicated. C) The A2C1 probe was mutated at specified base pair regions (designated with factor name and ‘m’; see Fig. 5C) to determine which binding sites were required for complex formation. Data shown in A-C are representative of a minimum of five experiments. The following bands are indicated from the top of the gel: 1) not always present, sometimes associated with chronic exposure; 2) stimulatory complex; 3) non-specific, drug-insensitive complex; and 4) chronic complex.

Fig. 7. Co-immunoprecipitation of NFAT with factors found in A2C1 complexes. Antibodies against C/EBP-β, c-Maf, and NFAT were used to immunoprecipitate associated proteins from lysates of cells cultured in 11 mM glucose. The immunoprecipitates were blotted with an anti-NFAT antibody. Arrows indicate the positions of two NFAT species. Data are representative of three experiments. A nonspecific immunoprecipitate is shown for comparison on the right. B) Specificity of the C/EBP-β antibody was confirmed by expression of C/EBP-β in 293 cells. Endogenous C/EBP-β was also detected with the antibody. C) The capacity of the c-Maf antibody to recognize MafA was determined by expression of MafA in 293 cells.

Fig. 8. Chromatin immunoprecipitation (ChIP) assay. INS-1 cells were cultured at 5.5 mM glucose (basal); treated for 30 min with 11 mM glucose after culture at 5.5 mM glucose (stimulated); and cultured in 11 mM glucose (chronic). Binding of the A2-E1 region was assessed in immunoprecipitates using antibodies to NFAT, Maf, C/EBP-β, and the two control antibodies, normal mouse and rabbit IgGs, mlG and rIgG. rInsI is the pGL3-rInsI plasmid DNA. Input is cross-linked and sheared chromatin DNA. Data are representative of three experiments.

Fig. 9. C/EBP-β is induced by chronic exposure to 11 mM glucose in INS-1 cells. A) Nuclear extracts of cells cultured at 5.5 mM glucose (basal), treated for 30 min with 11 mM glucose after culture at 5.5 mM glucose (stimulated), and cultured in 11 mM glucose (chronic) were immunoblotted with antibodies to C/EBP-β. The protein was only detected in lysates from cells grown in 11 mM glucose. The data are representative of four experiments. B) Immunofluorescence of INS-1 cells under basal (left) or chronic (center) conditions also revealed a substantial increase in C/EBP-β protein and a significant nuclear localization. (Right) Differential interference contrast images of the cells shown in the center panel.

Fig. 10. Effect of ectopic expression of transcription factors on A2-E1 promoter activity in 293 cells. The cells were transfected with the A2-E1 promoter-reporter and mammalian expression
vectors encoding NFAT, MafA, Beta2, C/EBP-β, alone or in the indicated combinations. After 5 h cells were stimulated with epidermal growth factor and ionomycin. Average of four experiments. Bars show standard deviation.

**Fig. 11.** Model for the regulation of the A2-E1 region of the insulin gene promoter. Schematic depiction of the arrangement of factors binding to the A2-E1 region during basal, stimulatory, and chronic glucose conditions.
**Fig. 2**

**A.**

Time (h) vs. Fold Luciferase Activity for rIns-Luc under different conditions: 5.5mM Glc, 5.5mM Glc+U0126, 11mM Glc, and 11mM Glc+U0126.

**B.**

Fold Luciferase Activity for rIns-Luc in various treatments: 5.5mM Glc, 5.5mM Glc+100 nM FK506, 5.5mM Glc+100 nM RAPA, 11mM Glc, 11mM Glc+100 nM FK506, 11mM Glc+100 nM RAPA.
Fig. 2

C.

D.

Fold Luciferase Activity

rInsI-Luc

0 1 2 3 4 5 6

Time (h)

2 6 12 24 36 48

Fold Luciferase Activity

rInsI-Luc

0 1 2 3 4 5 6

Time (h)

2 6 12 24 36 48

Legend:

- □ 5.5mM Glc
- □ 5.5mM Glc + FK520
- ■ 11mM Glc
- □ 11mM Glc + FK520

Legend:

- □ 5.5mM Glc
- □ 5.5mM Glc + RAPA
- ■ 11mM Glc
- □ 11mM Glc + RAPA
Fig. 2

E.

| Time (h) | 2 | 6 | 12 | 24 | 36 | 48 |
|----------|---|---|----|----|----|----|
| P-ERK1/2 |   |   |    |    |    |    |
| ERK1/2   |   |   |    |    |    |    |

Lanes: 1) 5.5 mM glucose; 2) 11 mM glucose; 3) 11 mM glucose + U0126
Fig. 3

Fold Promoter Activity

rIns-Luc

6 h 48 h ZDF-fa/fa

Fig. 4

A.

Fold Luciferase Activity

A2-E1-Luc

5.5mM Glc
5.5mM Glc+U0126
5.5mM Glc+FK520
5.5mM Glc+RAPA
11mM Glc
11mM Glc+U0126
11mM Glc+FK520
11mM Glc+RAPA

Time (h)
Fig. 4

B.

C.

Fold Luciferase Activity

Time (h)

A2-E1-Luc

5.5mM Glc
5.5mM Glc+FK520
11mM Glc
11mM Glc+FK520

Fold Luciferase Activity

Time (h)

A2-E1-Luc

5.5mM Glc
5.5mM Glc+RAPA
11mM Glc
11mM Glc+RAPA
Fig. 5

A.

Glucose → Ca^{2+} → FK506 → Calcineurin

FK506 → Calcineurin

U0126 → ERK1/2 → NFAT

Insulin Gene Promoter
Fig. 5

B.

C.

-143 GTGTTTGGAAATTACAGCTTCAGC
-131 GTGTTTGGAAACTGCAGCTTCAGC
-137 GTGTTTGGAAACTGCAGCTTCAGC
-137 GTGTTTGGAAACTGCAGCTTCAGC
-143 TGGTTCGGAAATTACAGCTTCAGC

* * * * * * * * *

NFAT MARE

A2 C1
Fig. 6A
Fig. 6B

- NFAT-Maf
- NFAT-C/EBP-β

|       | Free | No Ab | NFAT Ab | C/EBP Ab | Maf Ab |
|-------|------|-------|---------|----------|--------|
| Basal |      |       |         |          |        |
| Stimulated |   |       |         |          |        |
| Chronic |   |       |         |          |        |

1, 2, 3, 4
Fig. 6C

NFAT-Maf

NFAT-C/EBP-β

A2-C1 (wt)  NFATm  MAREm  NFATm/MAREm  CEBm  NFATm/CEBm/MAREm

Free  Basal  Stimulated  Chronic  Free  Basal  Stimulated  Chronic  Free  Basal  Stimulated  Chronic  Free  Basal  Stimulated  Chronic

1  2  3  4
Fig. 7

A. IP: C/EBP-β, c-Maf, NFAT

B. C/EBP-β, vector

C. MafA-myc, MafA

Densities shown in kDa:
- C/EBP-β: 26.6
- c-Maf: 36.5
- NFAT: 48.5
- NFATIP: 180

IB: NFAT, rLAP

IP: NEAT, IgG

hLAP: 48.5
rLAP: 36.5
MafA: 58.0
Fig. 9B

Basal

C/EBP-β

Chronic

C/EBP-β

Chronic

D.I.C.
Fig. 10

![Graph showing Fold Control Reporter Activity for different conditions involving NFAT, MafA, C/EBPβ, and Beta2. The graph illustrates the increase in activity with the addition of various combinations of these factors, with a significant peak when NFAT and MafA are combined with Beta2 and C/EBPβ.]
Fig. 11

Basal

Stimulated

Chronic
Supplementary figures

IP: Lysate
mIgG
rIgG

IB: GAD153 mouse Ab

Nonspecific band(s)
Heavy chain

IP: IgG

IB: NFAT Ab

180 116 84 58
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