Regulation of Insulin Gene Transcription by ERK1 and ERK2 in Pancreatic β Cells*

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We show that the mitogen-activated protein kinases ERK1/2 are components of the mechanism by which glucose stimulates insulin gene expression. ERK1/2 activity is required for glucose-dependent transcription from both the full-length rat insulin I promoter and the glucose-sensitive isolated E2A3/4 promoter element in intact islets and β cell lines. Dominant negative ERK2 and MEK inhibitors suppress glucose stimulation of the rat insulin I promoter and the E2A3/4 element. Overexpression of ERK2 is sufficient to stimulate transcription from the E2A3/4 element. The glucose-induced response is dependent upon ERK1/2 phosphorylation of a subset of transcription factors that include Beta2 (also known as NeuroD1) and PDX-1. Phosphorylation increases their functional activity and results in a cumulative transactivation of the promoter. Thus, ERK1/2 act at multiple points to transduce a glucose signal to insulin gene transcription.

Circulating insulin is produced by the β cells in the pancreatic islets of Langerhans in adult mammals. Insulin regulates glucose metabolism and in turn glucose regulates synthesis and secretion of insulin by the β cells. Insulin synthesis is stimulated by glucose at several steps, including transcription of the insulin gene (1–4). Insulin mRNA is extremely stable with a half-life of more than 24 h (5). Thus, many studies have focused not on regulation of insulin gene transcription but on understanding the translation of its mRNA and its processing to insulin (6, 7). Nevertheless, increased initiation of insulin gene transcription occurs within 10 min of an elevation in blood glucose concentration (8). Thus, insulin gene transcription, which is obviously essential for homeostasis of mRNA stores, is also sensitive to acute stimuli.

The organization of the insulin promoter is complex, allowing both tissue-restricted transcription of the insulin gene and levels of control for contextual regulation by nutrients, hormones, neurotransmitters, and other agents (9–11). The proximal promoter, the sequence within a few hundred base pairs of the transcription start site, has been studied in detail for the rat and human insulin genes (for a review see Ref. 10). The rat insulin I promoter contains multiple sequence elements capable of responding to glucose (12–17), the best studied being the juxtaposed E and A elements, which together can function as a glucose-responsive minienhancer (13, 14). Mutating or deleting these E and A elements from the promoter greatly reduces its glucose responsiveness (12), consistent with the idea that glucose regulates transcription of the insulin gene through its effects on E and A elements. How glucose signals to regulate insulin gene transcription through this glucose-sensitive element is unknown.

E elements are binding sites for heterodimeric complexes formed by the neuroendocrine basic helix-loop-helix (bHLH)1 protein Beta2 (18, 19) and a ubiquitous bHLH protein such as E47 (20–23). Binding of the bHLH heterodimer to the E element increases in response to prolonged glucose stimulation (12). The molecular signals underlying this increase in binding are unknown. Animals lacking the Beta2 gene develop diabetes and die within 3–5 days of birth (24). The development of the β cells is impaired, and the insulin content of the residual cells is low.

A elements bind any of several homeodomain transcription factors found in β cells, the most abundant being PDX-1 (25–28). In contrast to the relatively slow glucose-induced increase in bHLH heterodimer binding to the E element, PDX-1 binding increases acutely in response to a rise in glucose concentration (26, 29). The glucose-stimulated increase in DNA binding by PDX-1 is reportedly due to phosphorylation that is dependent on phosphatidylinositol 3-kinase and the p38 MAP kinase (26, 30). Although neither kinase was found to directly phosphorylate PDX-1. Glucose also causes PDX-1 to shift into the nucleus (31, 32) and increases its transcriptional activation capacity (33). Maturity onset diabetes of the young type 4 (MODY4) has been linked to PDX-1 (34). One PDX-1 mutation identified in a MODY4 patient causes a frameshift in the activation domain. Heterozygotes for this mutation are predisposed to noninsulin-dependent diabetes mellitus.

Despite exhaustive study of the insulin promoter and control of insulin gene transcription by factors that bind to defined promoter elements, the link between glucose sensing and insulin gene transcription has remained enigmatic. We and others have shown that glucose activates the MAP kinases ERK1/2 in...
islet-derived cells (35–38). MAP kinases, also known as ERKs, are components of highly conserved kinase cascades important for transmitting extracellular information to coordinate cellular responses. MAP kinases have been implicated in many physiological events ranging from cellular proliferation and differentiation to cell survival (reviewed in Refs. 39 and 40). ERK1/2 regulate functions throughout the cell, but among the most significant function is the control of gene transcription. ERK1/2 are stimulated by glucose in INS-1, MIN6, and βTC3 pancreatic β cell lines (35–38). ERK1/2 are activated over the same glucose concentrations, from 2 to 20 mM, as those that elicit insulin secretion. Potentiators of insulin secretion potenti ate ERK1/2 activation. Although ERK1/2 are not required for glucose-stimulated insulin secretion, glucose increased the amount and activity of ERK1/2 in the nucleus of β cells, suggesting that ERK1/2 may regulate gene transcription in these cells (36).

In this report, we show that dominant negative ERK2 and inhibitors of the ERK1/2 activators, the MAP/ERK kinases, MEK1/2, suppress glucose-stimulated transcription of the rat insulin I promoter in INS-1 cells, βTC3 cells, and primary islets. ERK2 stimulates transcription from reporter constructs in the absence of glucose via the glucose-responsive element. This process is activated at least in part because ERK2 phosphorylates bHLH transcription factors, E47/E12 and Beta2, and the homeodomain-containing transcription factor PDX-1. Phosphorylation of Beta2 and PDX-1 regulates their transcription activities. In addition, phosphorylation of E47/E12 regulates its heterodimerization with Beta2 and subsequent DNA binding. These findings suggest that ERK1/2 are important components of the mechanism of glucose-responsive insulin gene transcription.

MATERIALS AND METHODS

Isolation of Islets—Islets from male adult Sprague-Dawley rats were isolated as described (41), incubated in glucose-free Krebs-Ringer bicarbonate HEPES buffer for 2 h, and treated as indicated for 30 min at 37 °C. The islets were lysed in lysis buffer (36).

Northern Analysis—INS-1 cells were cultured as described in Ref. 36. They were infected with recombinant adenoviruses expressing either wild type or dominant negative (K52R) ERK2 at a multiplicity of infection of 20 for 48 h. Adenoviruses expressing wild type and K52R ERK2 were constructed as in Ref. 42. The cells were cultured without glucose for 24 h and then with 20 mM glucose for 24 h. Total RNA was isolated using TRI reagent (Molecular Research Center, Inc.). 10–20 μg of total RNA was separated on a denaturing formaldehyde gel of 1.4% agarose. Prehybridization was at 45 °C for 2 h, and hybridization was performed at 45 °C overnight with [α-32P]dCTP-labeled cDNA probe at 106 cpm/ml. The membranes were washed twice in 2× SSC, 0.1% SDS, once in 1× SSC, 0.1% SDS, and twice in 0.2× SSC, 0.1% SDS for 30 min each at 55 °C. The bands were quantitated using a PhosphorImager.

Transfection Studies in INS-1 Cells—The rat insulin I promoter (−410/+1 bp) was subcloned into pGL3-Basic (Promega) (pGL3-rINSp) containing a luciferase reporter gene. The minihenancen containing five copies of E2A3/4 (−247→−188) bp from the rat insulin I promoter was linked to a minimal rat insulin I promoter upstream of a chloram phenicol acetyltransferase (CAT) or luciferase reporter gene. INS-1 linked to a minimal rat insulin I promoter upstream of a chloram phenicol acetyltransferase (CAT) or luciferase reporter gene. INS-1 cells were cultured to a confluency of 70–80% in 1 ml of OPTI-MEM 1 medium (serum-free; Invitrogen). Plasmid DNA (4 μg) in 0.1 ml of medium was mixed with 4 μg of poly(dI–dC) 

Transfection Studies in Primary Islets—Adult mouse islets were picked by hand from collagenase-digested adult female CD-3 mice and cultured overnight in RPMI medium 1640 with 10% fetal bovine serum. The islets were transfected using a modification of the adenovirus-assisted transfection technique previously described (16). Aliquots of 100 islets were placed in 12 × 75-mm polystyrene culture tubes and washed three times with 1 ml of OPTI-MEM 1 medium (serum-free; Invitrogen). Plasmid DNA (4 μg) in 0.1 ml of medium was mixed with 4 μg of poly(dI–dC) 

Kinase Assays and Phosphoamino Acid Analysis—In vitro kinase assays were performed with purified active ERK2, the stress-activated protein-kinases (SAPK) or c-Jun N-terminal-kinases (JNK) MAP kinases (49) in 30 μl of 20 mM HEPES, pH 8.0, 10 mM MgCl2, 100 μM ATP (γ-32P)ATP, 5–15 cpn/ml) with the active kinase and substrates at 30 °C for 30 min. The samples were analyzed on polyacrylamide gels in SDS that were either stained in Coomassie Blue before autoradiography or transferred onto nitrocellulose or polyvinylidene difluoride membranes. Phosphoamino acid analysis was carried out as described (50).

Transactivation Assays—Wild type and the triple phosphorylation mutant of human E12 AD2 were subcloned into pMV (SV40 promoter) to make GAL4-DNA-binding fusion proteins. Wild type and mutated forms of Beta2 (156–355) and PDX-1 (1–149) were subcloned into pG4L (CMV promoter) and cotransfected with GAL4-binding domains linked to a luciferase reporter gene (G5E1bLuc) with the CMV promoter-driven Renilla luciferase gene (CMV-PRL, Promega) into βTC3 cells using the SuperFect transfection system (Qiagen). The cells were harvested 48 h later. The cells were exposed to PD98059 and SB203580 for 24 h as indicated.

Electrophoretic Mobility Shift Assay—Double-stranded probes were correspond to B247 CTTCATCAGGCATTGTGGCCCTTCC (Far wild type) and B247 CTTTATCGGACTATGAGCCCTTCC (Far mutant) in the rat insulin I promoter. Single-stranded oligonucleotide probes were end-labeled using T4 polynucleotide kinase (Invitrogen) and [γ-32P]ATP and annealed with a 5× excess of unlabeled antisense oligonucleotides to create double-stranded, labeled probes. The probe was run on a 6% poly(dI–dC) Micro Bio-Spin columns (Bio-Rad). The binding reaction contained 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 3% Ficoll, 1 mg/ml bovine serum albumin, 100 μg/ml double-stranded poly(dI–dC)poly(dI–dC) (Amersham Biosciences) and 10,000 cpn of labeled probe in 20 μl. Recombinant proteins (10–20 ng) were either expressed in bacteria or protein phosphoconalysis (see E35). For supershift assays, either 1 μl of rabbit polyclonal E47 antibody or 2 μl of rabbit polyclonal anti-Myc antibody (Santa Cruz) was used. The samples were analyzed on 4% acrylamide:bis-acrylamide (39:1) nonde natured gels in 1× TBE.
and filtered through 0.45-μm filters. An infection mixture of 3 ml of recombinant retrovirus supernatant and 2 ml of INS-1 growth medium plus polybrene (Sigma) (final concentration, 4 μg/ml) was used to infect INS-1 cells. After 6 h, 5 ml of INS-1 medium was added for 72 h. 0.5 μg/ml puromycin (Sigma) was added to select cells expressing recombinant retroviruses for 3–5 days.

Miscellaneous Materials and Methods—Site-directed mutagenesis was carried out with the Stratagene QuickChange Kit. Unless otherwise indicated, the protein concentrations were measured using BCA reagent (Pierce) with albumin as standard. Bacterial expression vectors encoding GST-c-Jun (1–221) and GST-ATP (1–254) were kindly provided by Michael Karin (University of California, San Diego). A bacterial expression vector encoding GST-Mnk was generously provided by Tony Hunter (Salk Institute). The glucokinase promoter (~280/+4 bp) was generously provided by Mark Magnuson (Vanderbilt University).

RESULTS

Glucose Activates ERK1/2 in Primary Islets of Langerhans—In previous studies, we showed that glucose activated ERK1/2 in INS-1 insulinoma cells (36). Stimulation was observed in cells preincubated without glucose and in cells preincubated in 3 mM glucose. Before examining effects of ERK1/2 on insulin gene transcription, we first examined the effect of glucose on the activity of ERK1/2 in primary islets and in another glucose-sensitive cell line βTC3. Intact islets were preincubated in the absence of glucose for 2 h. Under these conditions, small amounts of activated ERK1/2 were detected using antibodies that selectively recognize the phosphorylated forms of the kinases. This is consistent with studies in INS-1 cells; in these cells complete inactivation of ERK1/2 by removing glucose required from 1 to 4 h. In isolated islets, increasing the glucose concentration to 15 mM caused a marked activation of ERK1/2, which is potentiated by forskolin (Fig. 1A). Similar findings were observed in βTC3 cells, except that ERK1/2 were activated at the lowest glucose concentration tested, consistent with the left shift in glucose-stimulated insulin secretion in these cells (Fig. 1B).

ERK1/2 Regulate Proinsulin mRNA Levels in Pancreatic β Cells—To determine whether ERK1/2 regulate insulin gene transcription, we first measured the effect of blocking ERK1/2 on insulin mRNA content in INS-1 cells. Wild type or dominant negative (kinase inactive) K52R ERK2 were expressed from adenoviruses in INS-1 cells, and the insulin mRNA content was measured by Northern analysis. K52R ERK2 caused a small but significant reduction in the amount of insulin mRNA after 24 h (Fig. 1C). However, no glucose-stimulated increase in insulin mRNA was observed in these cells, which is most likely due to the high stability and low turnover rate of insulin mRNA in INS-1 cells.

ERK1/2 Regulate Transcription from the Rat Insulin I Promoter in β Cells—Depression of insulin mRNA levels by K52R ERK2 could be through an effect on the stability of insulin mRNA or on the initiation of insulin gene transcription. To determine whether there were effects on transcription, we measured glucose-dependent changes in the expression of a reporter gene coupled to the rat insulin I promoter (~410/+1 bp) with and without blockade of ERK1/2 activity. K52R ERK2 blocked glucose-stimulated transcription from the promoter (Fig. 2A). Neither the CMV nor the glucokinase promoter (52) was substantially affected by K52R ERK2 (data not shown). PD98059 and U0126, two chemically distinct inhibitors of MEK1/2, blocked glucose activation of ERK1/2 in INS-1 cells and suppressed glucose-stimulated rat insulin I promoter activity (Ref. 36 and Fig. 2, B and C). These observations are consistent with the results obtained with K52R ERK2, suggesting that ERK1/2 regulate rat insulin I transcriptional activity in INS-1 cells.

To demonstrate a regulatory role of ERK1/2 in insulin gene transcription in islets, the ability of K52R ERK2 to affect tran-
scription from a rat insulin I promoter construct was tested in freshly isolated adult rat islets. In these experiments the unstimulated condition was 2 mM glucose. The stimulation of transcription from the rat insulin I promoter induced by 16 mM glucose was strongly inhibited by K52R ERK2 (Fig. 2, B and C). Glucose-stimulated transcription from the promoter in rat islets was also suppressed by PD98059 (Fig. 2, B and C). These results support findings in rat insulinoma INS-1 cells suggesting that ERK1/2 are important regulators of insulin gene transcription in pancreatic β cells.

**ERK1/2 Regulate Insulin Gene Transcription via a Glucose-responsive Element**—To determine the portion of the rat insulin promoter that is regulated by ERK1/2, we first examined the effects of blocking ERK1/2 on a well described glucose-responsive element E2A3/4 (−247/−198 bp). K52R ERK2 and the MEK inhibitor PD98059 each suppressed the glucose-dependent transcriptional activity of E2A3/4 in INS-1 cells (Fig. 3A), suggesting that regulation of insulin promoter activity by ERK1/2 occurs at least in part through this glucose-responsive portion of the promoter. Similar results were also found in βTC3 cells (not shown). We also determined whether ERK1/2 regulate insulin gene transcription via the glucose-sensitive element in primary mouse islets. K52R ERK2 had negligible effect on the glucose-sensitive element at basal glucose, whereas it blocked the ability of glucose to stimulate transcription promoted by E2A3/4 at 16 mM glucose (Fig. 3A). These results are consistent with our observations in β cell lines suggesting that ERK1/2 regulate insulin gene transcription at least in part through E2A3/4.

To determine whether ERK1/2 are sufficient for initiating transcription from the insulin gene promoter independently of the glucose stimulus, constitutively active mutants of Raf and MEK1(DE), upstream activators of ERK1/2 were introduced; they stimulated expression of the reporter gene driven by E2A3/4 (not shown). To examine sufficiency of ERK2 itself, independent of its upstream activators, we used ERK2-MEK1 fusion proteins. Fusion of wild type ERK2 to the wild type allele of its upstream activator MEK1 or to MEK1 with a mutated nuclear export signal, ERK2-MEK1A, respectively, yields constitutive ERK2 activity without activating endogenous ERK proteins (53). In the absence of activated ERK2, glucose doubled transcription from this reporter. Both forms of active ERK2 stimulated transcription from this E2A3/4-driven reporter by 3.5–4-fold above the basal glucose control (Fig. 3B). Thus, ERK2 is sufficient to promote gene transcription via a glucose-responsive element as well as or better than glucose alone. These results support the
conclusion that ERK-sensitive motifs are contained within the glucose-responsive element.

**ERK2 Phosphorylates Beta2, E47/E12, and PDX-1 in Vitro**—To begin to elucidate the mechanism of induction of the glucose-responsive element, we tested the ability of ERK2 and other MAP kinase family members to phosphorylate several factors known to bind to the A and E boxes. E box elements bind to factors belonging to the bHLH family. bHLH transcription factors bind to DNA as homodimers or as heterodimers formed between the ubiquitously expressed class A members of the bHLH family, such as E47/E12 (22), and the tissue-restricted class B bHLH members. The currently known class B bHLH protein in \( H9252 \) cells is Beta2 (18, 19). Five copies of the A motif in the rat insulin promoter are recognition sites for homeodomain proteins. Homeodomain-containing transcription factors that bind these A motifs from \( H9252 \) cells include PDX-1 and Lmx1.1 (28, 54, 55). These proteins, E47, Beta2, PDX-1, and Lmx1.1, were used as *in vitro* substrates for activated MAP kinases (49). ERK2 phosphorylates E47, Beta2, and PDX-1 but not Lmx1.1 (*in vitro*) (Fig. 4, B–D, and data not shown). E47 and PDX-1 were also phosphorylated by activated SAPK and p38 (*in vitro*). On the other hand, Beta2 and Lmx1.1 were not phosphorylated by either of these kinases.

Phosphoamino acid analysis of Beta2 showed that ERK2 phosphorylated only serine residues (Fig. 5B). The amino acid sequence of Beta2 revealed four potential serine residues, 162, 259, 266, and 274, that fit a consensus ERK2 phosphorylation motif and that were mutated to alanine (Fig. 5A). Mutation of Ser\(^{274}\), which lies in the most typical ERK2 phosphorylation motif, reduced the phosphorylation of Beta2 to less than one-third of the wild type (Fig. 5C), suggesting that Ser\(^{274}\) is the predominant site for ERK2. Mutation of the other three sites individually reduced Beta2 phosphorylation by one-third or less, suggesting that each is a minor ERK2 phosphorylation site (Fig. 5C). Mutation of all four residues reduced phosphorylation to less than 20% of the wild type protein, indicating that there are one or more additional minor sites.

**ERK2 was phosphorylated on serine and threonine residues by ERK2** (Fig. 6C). Three contiguous potential phosphorylation sites, serines 352, 355, and 359 in human E47, are present in its second activation domain (Fig. 6A). When these residues were mutated to alanine in E47, phosphorylation by ERK2 was partially reduced compared with wild type E47 (Fig. 6B). Phosphorylation of threonine was completely abolished in the triple...
Phosphoamino acid analysis of wild type PDX-1. Assays were performed with activated forms of ERK2 (top panel), SAPKα (second panel), and p38α (third panel). His6-tagged recombinant wild type or mutant PDX-1 were used as substrates. Bottom panel, an immunoblot with an anti-PDX-1 antibody. One of three experiments is shown.

**Phosphorylation of PDX-1 Regulates Its Transactivating Activity**—We tested whether mutation of the serine residues in the activation domain of PDX-1 affected its transactivating activity in β cells. We examined whether phosphorylation of the ERK2 sites is required for the transactivating activity of the transcription factors using GAL4-DNA-binding domain chimeras of AD2 of E12 and the Beta2 activation domain (155–355). AD2 of E47/E12 has been previously shown to be functional in β cells (20). However, the transactivating activity of the E12 triple phosphorylation mutant was unchanged compared with wild type E12 in βTC3 cells or INS-1 cells (data not shown).

In contrast, mutating Ser274 significantly diminished transactivation by Beta2 in the presence of glucose (Fig. 9, A and B). Although S274A Beta2 exhibited a transactivating activity only about 30% of wild type, mutation of the other ERK2 sites individually only reduced transactivation to about 60–70% of wild type. Mutation of all four residues depressed the transactivation activity of Beta2 to 20% of wild type (Fig. 9A), indicating that the other three sites contribute less than Ser274. Similar results were obtained in INS-1 cells (Fig. 9A). To determine whether Beta2 transactivation is dependent on activation of ERK1/2 in β cells, we tested whether blocking ERK1/2 activation will suppress the transactivating activity of Beta2 observed in the presence of glucose. In the presence of the MEK inhibitor PD98059, the activity of wild type Beta2 was reduced to less than 20% of that in its absence. The addition of PD98059 further reduced the transactivation activities of the S274A/S266A/S259A and S274A/S266A/S259A/S162A mutants to less than 10% of the wild type control (Fig. 9B). This suggests that Ser274 has the largest effect on transactivating potential but that the additional sites also contribute to the glucose-enhanced transactivating activity of Beta2.
suggesting that glucose does not regulate the transactivating activity of PDX-1 through p38 in \( \beta \)TC3 cells.

**Heterodimerization and DNA Binding of E47 and \( \beta \)2 Are Regulated by ERK2 Phosphorylation**—We also examined the effects on E47 dimerization and DNA binding by electrophoretic mobility shift assay using recombinant His\(_6\)-tagged proteins. E47 alone bound poorly to the oligonucleotide derived from the E box. Binding was enhanced by phosphorylation of E47 \textit{in vitro} by ERK2 (Fig. 10). Myc-tagged Beta2 by itself, either unphosphorylated or phosphorylated by ERK2, did not bind DNA (data not shown). When unphosphorylated forms of E47 and Myc-Beta2 were mixed together, two bands corresponding to an E47 homodimer-DNA complex and an E47-Beta2 heterodimer-DNA complex were observed (Fig. 10). Under these conditions, the heterodimer-DNA complex was weak. The compositions of the complexes were confirmed by supershifting with anti-E47 and anti-Myc antibodies. An oligonucleotide with mutations in the E box consensus sequence was not shifted by the proteins, indicating that the shifts observed with the wild type oligonucleotide are specific (data not shown).

**DISCUSSION**

ERK1/2 mediate an essential step in the signaling cascade through which glucose regulates insulin gene transcription. The first suggestion that ERK1/2 lie in this pathway was provided by evidence that both kinases are activated by glucose in insulinoma cell lines (35), evidence that we have now extended to islets. The demonstration that preventing ERK1/2 activation with inhibitors or blocking ERK1/2 activity with a dominant negative mutant prevents glucose stimulation of the insulin promoter establishes a necessary role for ERK1/2 in the path-

**Fig. 9.** ERK2 regulates the transactivating activities of Beta2 and PDX-1 in \( \beta \)TC3 cells. A, \( \beta \)TC3 (left panel) and INS-1 (right panel) cells were transfected with the indicated pGAL4-Beta2 (156–355) constructs and G5Eb1Luc. Dual luciferase assays were performed. Relative luciferase units were expressed as percentages of wild type (wt) Beta2 (100%). Averages of five experiments are shown. B, \( \beta \)TC3 cells were transfected with the indicated DNAs as in A. After 24 h, the cells were treated with or without 50 \( \mu \)M PD98059 for another 24 h and then exposed to glucose as indicated. Averages of five experiments are shown. C, \( \beta \)TC3 cells were transfected with pGAL4-PDX-1 (1–149) constructs as indicated. After 24 h, the cells were treated without or with either 50 \( \mu \)M PD98059 or 10 \( \mu \)M SB203580 for another 24 h before exposure to glucose and harvesting the cells. Relative luciferase units were expressed as percentages of wild type PDX-1 (100%). Averages of five experiments are shown.

**Fig. 10.** Phosphorylation of E47 and Beta2 enhances heterodimerization and DNA binding to the E element. Electrophoretic mobility shift assays were carried out with His\(_6\)-tagged recombinant proteins of E47 and Myc-Beta2 and the Far probe. E47 and Myc-Beta2 were subjected to \textit{in vitro} phosphorylation with or without ERK2 prior to the binding reactions for electrophoretic mobility shift assay. Supershift assays were carried out with anti-E47 and anti-Myc antibodies.
way, as has been previously suggested (56). Furthermore, active ERK2 alone is sufficient to induce transcription driven by E2A3/4 in the absence of glucose.

The involvement of the glucose-responsive E2A3/4 minihancer implicates transcription factors that act through E and A elements. Three of these factors, Beta2, PDX-1, and E47/12, are substrates for ERK1/2 and are activated upon phosphorylation by ERK1/2. ERK2 phosphorylates Beta2 at multiple sites within its activation domain that enhance its glucose-sensitive transactivating capability. Phosphorylation of Beta2 by ERK2 also enhances its capacity to heterodimerize with E47/12 and bind to DNA. The fact that blocking ERK1/2 activity using inhibitors or mutants also blocks glucose-stimulated transactivation by Beta2 strongly supports the conclusion that ERK1/2 regulate insulin transcription through effects on Beta2. Petersen et al. (57) have shown that Ser274 influences the nuclear translocation of Beta2; use of PD08959 also implicated ERK1/2 in this event.

Although phosphorylation of the sites identified on E47/12 has no apparent effect on its transactivating capacity, ERK2 phosphorylation of E47/12 increases its propensity to form heterodimers and to bind to DNA. Phosphorylation of E47 also appears to reduce the formation of E47 homodimer-DNA complexes; such complexes may be nonfunctional in promoting insulin transcription in β cells. ERK1/2 phosphorylation also likely regulates dimerization of E47 in other cell types in which different heterodimers arise.

ERK2 also phosphorylates PDX in its activation domain. Phosphorylation of PDX-1 increases its DNA binding and transactivation capacity (33). Mutation of ERK1/2 phosphorylation sites on PDX-1 halves its transactivation capacity and blockade of ERK1/2 activity reduces transactivation to near that in the absence of glucose, indicating that phosphorylation by ERK1/2 enhances its glucose-dependent transactivation. Thus, ERK1/2 act at multiple loci within the machinery that controls insulin gene transcription to transduce changes elicited by glucose. Phosphorylation could stimulate the Beta2 and PDX-1 activation domains in any of several ways. The affinity for coactivators such as p300/CBP (58, 59) could be increased, and interactions with repressors could be reduced. Phosphorylation could also enhance cooperative interactions, such as the interaction between PDX-1 and the bHLH heterodimer (55).

Phosphorylation of PDX-1 is reportedly regulated by a kinase downstream in a p38-dependent pathway (30). The p38 pathway may regulate the localization or DNA binding of PDX-1 but has no apparent effect on glucose-dependent transactivation based on inhibitor studies. In vitro, PDX-1 is phosphorylated by ERK2, c-Jun N-terminal kinase/SAPK, and p38 itself, suggesting that PDX-1 may integrate signals from multiple MAP kinase pathways. p38 phosphorylates the same two sites as ERK2, indicating that it could increase PDX-1 transactivation. However, glucose-stimulated PDX-1 transactivating activity is not blocked by the p38 inhibitor, although it is reduced by preventing ERK1/2 activation. Furthermore, p38 is activated very poorly by glucose (36), consistent with the failure of the p38 inhibitor to interfere with glucose-induced transactivation. Perhaps the p38 cascade impacts PDX-1 in response to agents other than glucose.

Although we have identified functional changes in three factors that will lead to increased insulin gene transcription, other functional changes may also be caused by ERK2 phosphorylation. Regulation of bHLH transcription factors by ERK1/2 occurs at multiple steps. It has been hypothesized that heterodimer complexes that bind to E box elements may synergize with complexes bound to A elements to cause transactivation of the insulin gene. In particular, E47 has been shown to synergistically interact with PDX-1 (47). In addition, ERK1/2 may have other substrates in β cells, either direct or through protein kinase targets such as Rsk.

In conclusion, ERK1/2 are viewed almost monolithically as enzymes activated during and as a necessary part of cell proliferation. In fibroblasts, activation of ERK1/2 has been associated with exit from G0 into G1 of the cell cycle and cell proliferation. Their roles in differentiated cells have often been overlooked. Although most of our understanding of the regulation and functions of these protein kinases comes from fibroblasts, ERK1/2 are highly expressed in most cell types including post-mitotic neurons and neuroendocrine β cells. In cortical neurons, glutamate-induced changes in transcription from the serum response element, thought to be important for long term adaptive changes, are mediated in part by ERK1/2 (60–63). ERK1/2 have been linked to long term potentiation, both directly through the induction of ERK nuclear translocation by glutamate and by inference from the deficiency of an animal lacking the calcium-sensitive Rac exchange factor in acquisition of long term memory (61, 62). The tight relationship between ERK1/2 activation and glucose concentration suggested that these kinases may link glucose sensing to mechanisms that maintain insulin production both short and long term. Transcriptional control appears to be a major target for ERK signaling. We show here that ERK1/2 appear to serve a function in β cells similar to that in neuronal cells by helping to integrate long and short term nutrient sensing information in the nucleus to maintain insulin homeostasis.

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