Characterization of a Novel Calcium Response Element in the Glucagon Gene*

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To maintain blood glucose levels within narrow limits, the synthesis and secretion of pancreatic islet hormones is controlled by a variety of extracellular signals. Depolarization-induced calcium influx into islet cells has been shown to stimulate glucagon gene transcription through the transcription factor cAMP response element-binding protein that binds to the glucagon cAMP response element. By transient transfection of glucagon-reporter fusion genes into islet cell lines, this study identified a second calcium response element in the glucagon gene (G2 element, from −165 to −200). Membrane depolarization was found to induce the binding of a nuclear complex with NFATp-like immunoreactivity to the G2 element. Consistent with nuclear translocation, a comigrating complex was found in cytosolic extracts of unstimulated cells, and the induction of nuclear protein binding was blocked by inhibition of calcineurin phosphatase activity by FK506. A mutational analysis of G2 function and nuclear protein binding as well as the effect of FK506 indicate that calcium responsiveness is conferred to the G2 element by NFATp functionally interacting with HNF-3β binding to a closely associated site. Transcription factors of the NFAT family are known to cooperate with AP-1 proteins in T cells for calcium-dependent activation of cytokine genes. This study shows a novel pairing of NFATp with the cell lineage-specific transcription factor HNF-3β in islet cells to form a novel calcium response element in the glucagon gene.

Activation of gene transcription allows cells to adapt to changes in environmental conditions through a new pattern of expressed proteins. In cells that are electrically excitable, calcium is an important intracellular second messenger that directs the genomic response of the cell. Transcription factors that have been shown to mediate calcium-induced gene transcription include CREB† (1–4), serum response factor (2, 5), and C/EBPβ (6).

Like neurons, endocrine cells of the pancreatic islets are electrically excitable and express L-type voltage-dependent calcium channels (7, 8). By virtue of stimulation of glycogenolysis and gluconeogenesis in the liver, the islet hormone glucagon is an important regulator of blood glucose levels (9). Glucagon-producing islet cells show spontaneous electrical activity (10). Membrane electrical activity and calcium influx into glucagon-producing pancreatic islet cells is tightly controlled by extracellular messengers. Whereas L-arginine increases spike frequency (10), β-adrenergic cell-surface receptor stimulation by catecholamines through cAMP enhances the L-type calcium current, increasing the influx of calcium associated with each action potential (11). It is well known that membrane depolarization and calcium influx increase the cytosolic free calcium concentration, which stimulates hormone secretion by exocytosis (8, 12). The question is whether, and if so, how, this calcium signal reaches also into the nucleus and regulates gene transcription.

Previous experiments have shown that membrane depolarization and calcium influx stimulate glucagon gene transcription (3). A mechanism involved has been characterized. Through a calcium/calmodulin-dependent protein kinase calcium stimulates the phosphorylation of the transcription factor CREB on the same serine residue that is also phosphorylated by cAMP-dependent protein kinase A. CREB binds to a CRE in the 5′-flanking region of the glucagon gene and stimulates transcription (3–15). The present study addressed the question whether there are additional calcium-responsive elements in the glucagon gene. By the results obtained, the G2 element is identified as a second calcium response element. The further characterization suggests that calcium responsiveness is conferred by the calcium/calcineurin-regulated transcription factor NFATp functionally synergizing with the cell-specifically expressed transcription factor HNF-3β. NFAT family proteins are known to cooperate with newly synthesized AP-1 proteins in T cells (16–19). This study shows a novel pairing of NFATp with the constitutively expressed cell-specific transcription factor HNF-3β forming a novel calcium response element in the glucagon gene.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids −350GluLuc (3), −350−297/−292GluLuc (3), −350A-GluLuc (20), −292GluLuc (20), pT81Luc (21), 4xG3T81Luc (22), 4xCST81Luc (23), 4xGluCRETS81Luc (24), 4xG2T81Luc, 4xG2 mTS81Luc, 4xG2 mT81Luc, and 4xG2 m2T81Luc (20) have been described previously. The plasmid −350ΔGluLuc was prepared by subcloning the SrfI fragment of −350ΔGluLuc into the SrfI site of pT81ΔAN (pT81Luc (21) with the AatII site deleted); four bases in the CRE octamer (from −296 to −293) were then deleted with the restriction enzyme AatII and T4 DNA polymerase; after religation the SrfI fragment was subcloned into the SrfI site of pX22 (21). An expression vector encoding the DNA-binding domain of NFATp (amino acids 398–584) (19, 25) (pBK-CMV-NFATpDBD) was prepared by PCR using pLGPm-NFAT1-B (26) as template and the oligonucleotides 5′-GATTGACGC-GAGCTCAGAGGCTGATC-3′ and 5′-GCCGGATCTTCCACCCTGTCATT-3′ as upstream and downstream primers, respectively; the

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†The abbreviations used are: CREB, CRE-binding protein; CRE, cAMP response element; IL-2, interleukin 2; NFAT, nuclear factor of activated T cells; RT-PCR, reverse transcription and PCR amplification; TPA, 12-O-tetradecanoylphorbol-13-acetate.
PCR product was digested with Sacl and EcoRI and subcloned into the SacI-EcoRI sites of pBK-CMV (Stratagene, Heidelberg, Germany). All constructs were confirmed by sequencing.

**Cell Culture and Transfection of DNA**—The pancreatic islet cell line HIT-T15 (27) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. αTC2 cells (28, 29) were grown in Dulbecco’s modified Eagle’s medium (4.5 g of glucose/liter) supplemented with 2.5% fetal calf serum, 15% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were trypsinized and transfected in suspension by the DEAE-dextran method (3) with 2 μg of indicator plasmid per 6-cm dish. Ross sarcoma virus-acetylcholinesterase acetylcholineplasmid (0.4 μg/μl/cm) was added as a second reporter to check for transfection efficiency. When indicated, 0.7 μg of pBK-CMV-NFATpDBD was co-transfected per 6-cm dish; these co-transfections were done with a constant DNA concentration, which was maintained by adding Bluescript (Stratagene, Heidelberg, Germany). Cells were stimulated with high KCl (45 mM final concentration) or TPA (300 nM) for 6 h before harvest. FK506 (167 μM) was added 1 h before transfection. Cell extracts (3) were prepared 48 h after transfection. A chromatographic chloramphenicol acetyltransferase assay was performed as described previously (30). Thin layer chromatography plates were analyzed with a Fuji PhosphorImager. The luciferase assay was performed as described previously (3).

**Cell Extracts**—Nuclear extracts were prepared from αTC2 cells by the method of Schreiber et al. (31), except for the experiments shown in Fig. 3G. In the experiments shown in Fig. 3G, separate nuclear and cytosolic extracts were prepared as described previously (32–34).

**Electrophoretic Mobility Shift Assay**—Syntetic complementary oligonucleotides with 5'-GATC overhangs were annealed and labeled by a fill-in reaction with [α-32P]dCTP and Klenow enzyme. By using 15 μg of protein from cell extracts, the electrophoretic mobility shift assay was performed as described by Klemas et al. (35). In some binding reactions, a specific anti-NFATp antiserum (kindly provided by A. Rao, Harvard Medical School, Boston) was used. This antiserum (anti-67.1) is directed against the 67.1 peptide of murine NFATp; it does not cross-react with NFATc, NFAT3, or NFATx (36). Cell extracts were incubated with 1 μl of a 1:10 dilution of preimmune serum or anti-NFATp antiserum in the reaction buffer with probe for 15 min at room temperature. Following a 15-min incubation on ice, the samples were loaded onto the gels and electrophoresed as described above.

**Oligonucleotides**—The sequences of the G2 oligonucleotides (wild type and mutants 1, 3, and 5) were as described previously (20) and read as follows (only one strand with the 5'-GATC overhang is shown, and mutated bases within the CRE octamer motif, yielding the constructs G2 m1, 5'-GATCCAGGCGCAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'; G2 m2, 5'-GATCCAGGGCAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'; G2 m3, 5'-GATCCAGGCACAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'; G2 m4, 5'-GATCCAGGCGACAGTGAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'; G2 m5, 5'-GATCCAGGCGACAGTGAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'. The sequence of the oligonucleotide (NFATcons) containing a well-characterized NFAT-binding site was as described (36) except that BamHI (upstream) and EcoRI (downstream) with 5'-GATC overhangs were added.

**RT-PCR**—Poly(A) RNA was extracted from αTC2 cells using a commercial kit (Fast Track 2.0™, Invitrogen). RT-PCR was performed using a commercial kit (GeneAmp® 9700 Thermocycle 9700 Reverse Transcriptase RNA PCR Kit, Roche Molecular Systems) with primers and PCR reaction conditions as follows: upstream primer 5'-AGTCTTCCCAAA-GACGAGCT-3' upstream primer 5'-CCGCGTCGAGAAATCGTCCGGGCTCTGA-3'; downstream primer 5'-GATTCACAGTAAAGAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'; G2 m3, 5'-GATCCAGGCGACAGTGAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'; G2 m4, 5'-GATCCAGGCGACAGTGAAGTTTGAAATAAAAAGTTTCCGGGCCTCTGA-3'. The sequence of the oligonucleotide (NFATcons) containing a well-characterized NFAT-binding site was as described (36) except that BamHI (upstream) and EcoRI (downstream) with 5'-GATC overhangs were added.

**Western Blot**—Nuclear extracts were resolved on a 5.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated for 2 h in 10% nonfat dry milk dissolved in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20), incubated in 1 h in 2% gelatin dissolved in TBST, and then incubated with anti-NFATp antiserum (anti-67.1) (36), diluted 1:3,000 in TBST, overnight at 4 °C. Antibody-antigen complexes were detected with ECL reagents (Amersham). Using the same samples, immunoblots were performed with an anti-CREB antiserum as has been described previously (15).

**Materials**—A stock solution of TPA (1 mM) was prepared in dimethyl sulfoxide and further diluted in cell culture medium. FK506 (provided by Fujisawa) was dissolved in ethanol. Controls received the solvent only.

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**RESULTS**

**Identification of a Second Calcium-responsive Element in the Glucagon Gene**—To study the calcium responsiveness of the glucagon gene in the absence of a functional CRE, the CRE in the 5'-flanking region of the rat glucagon gene was removed by 5'-deletion to −292 (plasmid −292GluLuc) or by internal deletion of four bases within the CRE octamer motif (plasmid −290/−297/−292GluLuc). The plasmids −350GluLuc, −350/−290/−297/−292GluLuc, and −292GluLuc were transiently transfected into the pancreatic islet cell lines HIT (B) or αTC2 (C). The cells were stimulated by high potassium-induced membrane depolarization (KCl, 45 mM). Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the respective controls (5 mM KCl). Values are mean ± S.E. of three independent experiments, each done with two (B) or four (C) dishes.

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![Fig. 1. Stimulation of glucagon reporter gene expression by high potassium-induced membrane depolarization in the absence of a functional CRE. A, relative position of the CRE octamer 5'-TGACGCTCA-3' in the rat glucagon gene 5'-flanking region. In the plasmid −350GluLuc, the rat glucagon gene from −350 to +58 was fused to the coding region of the luciferase reporter gene (LUC). The CRE was removed by either 5'-deletion to −292 (plasmid −292GluLuc) or by internal deletion of four bases within the CRE octamer motif (plasmid −290/−297/−292GluLuc). The plasmids −350GluLuc, −350/−290/−297/−292GluLuc, and −292GluLuc were transiently transfected into the pancreatic islet cell lines HIT (B) or αTC2 (C). The cells were stimulated by high potassium-induced membrane depolarization (KCl, 45 mM). Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the respective controls (5 mM KCl). Values are mean ± S.E. of three independent experiments, each done with two (B) or four (C) dishes.**

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**Identification of a Second Calcium-responsive Element in the Glucagon Gene**—To study the calcium responsiveness of the glucagon gene in the absence of a functional CRE, the CRE in the 5'-flanking region of the rat glucagon gene was removed by 5'-deletion to −292 or by internal deletion of four bases within the CRE octamer motif, yielding the constructs −292GluLuc and −350/−297/−292GluLuc, respectively (Fig. 1). The corresponding glucagon-reporter fusion genes were transiently transfected into two pancreatic islet cell lines, HIT and αTC2. These cell lines have been used previously to demonstrate that membrane depolarization and calcium influx in-
duce glucagon gene transcription through the CRE of the glucagon gene (3, 13–15). HIT cells (27) express glucagon, although only at a low level (37, 38), and have the advantage to respond to second messenger stimulation and to be well characterized with respect to electrical activity, voltage-dependent calcium channels, cytosolic calcium concentration, and secretion (see Ref. 3). Increases in extracellular potassium concentration to 40 mM have been shown to depolarize HIT cells with action potentials continuing at the peak of the depolarizing phase of the spontaneous activity; at the same time, high potassium initiates the influx of calcium through dihydropyridine-sensitive, L-type Ca\(^{2+}\) channels in the cell membrane and elevates the cytosolic calcium concentration (39). Membrane depolarization was induced by elevating the potassium chloride concentration in the incubation medium from 5 to 45 mM. As shown in Fig. 1B, the high potassium-induced increase in glucagon gene transcription was decreased in HIT cells by 77% by the 4-base deletion in the CRE octamer motif, as has been reported previously (3). It was decreased by 46% when the CRE was removed by 5’-deletion to −292 (Fig. 1B). Basal reporter activity was not changed by these deletions (data not shown).

Thus, although the depolarization-induced increase in glucagon gene transcription was reduced using both constructs, glucagon gene transcription did still respond to membrane depolarization, resulting in a 2.0- and 3.3-fold-stimulation of transcription in the absence of the CRE, respectively (Fig. 1B). After transfection of −292GluLuc, the stimulation of transcription by high potassium was abolished when extracellular calcium was bound by 1.5 mM EGTA added to the medium (data not shown), suggesting that gene induction by membrane depolarization depends on calcium influx elevating intracellular calcium levels. The pancreatic α-like cell line αTC2 has been established from a glucagonoma arising in transgenic mice expressing the SV40 large T-antigen oncogene (28, 29). These cells express predominantly glucagon in a rather uniform pattern (28). In αTC2 cells, the depolarization-induced stimulation of glucagon gene transcription was only slightly reduced by 5’-deletion to −292 or by the internal deletion of four bases within the CRE octamer (Fig. 1C). When compared with −350GluLuc, basal reporter activity of −350−297/−292GluLuc was not changed, and basal reporter activity of −292GluLuc was reduced to 38 ± 7% (n = 12). The results obtained in both pancreatic islet cell lines thus indicate that besides the CRE the rat glucagon gene 5’-flanking region contains at least one more calcium-responsive element within 292 bases in front of the transcription start site.

Known elements within the enhancer region of the glucagon gene include the G2 element (20, 40, 41), the G3 element (30, 40, 42–44), as well as a binding site for C/EBP proteins (CS) (23) (Fig. 2). To study their role, four copies of oligonucleotides containing the G2 element (from −200 to −165), the G3 element (from −274 to −234), or the CS element (from −241 to −212) were placed in front of the truncated viral thymidine kinase promoter of herpes simplex virus (from −81 to +52) fused to the luciferase reporter gene (plasmid pT81Luc) (21). As shown in Fig. 2, the promoter alone did not respond to membrane depolarization in αTC2 cells. The G3 element and the binding site for C/EBP proteins were also inactive (Fig. 2). However, the G2 element did confer depolarization responsiveness to the promoter (Fig. 2). The blocker of L-type, voltage-dependent calcium channels, diltiazem, inhibited the high potassium-induced transcriptional activation of the G2 element (data not shown). Depolarization responsiveness conferred by the G2 element to the nonresponsive thymidine kinase promoter was only somewhat less than that conferred by the glucagon CRE. When in separate experiments the plasmids

![Fig. 2. The G2 element of the glucagon gene confers depolarization responsiveness.](image-url)

The binding specificity of the depolarization-induced protein binding was consistently observed in five independent experiments, each done in duplicate.

4xG2T81Luc and 4xGluCRET81Luc were transfected in parallel into αTC2 cells, membrane depolarization stimulated transcription to 182 ± 14% of controls (n = 6) through the G2 element and to 225 ± 18% of controls (n = 6) through the glucagon CRE. These data indicate that the G2 element is a calcium-responsive element of the glucagon gene.
Fig. 3. Membrane depolarization induces protein binding to the G2 element adjacent to the binding of HNF-3β as revealed by the electrophoretic mobility shift assay. A, depolarization-induced nuclear protein binding. αTC2 cells were stimulated for 15 or 120 min with high potassium-induced membrane depolarization (KCl, 45 mM) or were left unstimulated. Nuclear extracts were prepared and incubated with labeled G2 oligonucleotide (wild type). It has been shown previously that under basal nonstimulated conditions band h represents the binding of HNF-3β, whereas several weak bands collectively labeled e represent proteins with a binding specificity related to the Ets family of transcription factors (20). f, free probe; ns, nonspecific bands. The arrow indicates the depolarization-induced protein binding. The G2 wild-type sequence is presented at the top of the figure, and the bases mutated in mutants 1, 3, and 5 are indicated. The boxed sequence motifs show the homology to the HNF-3 consensus binding site (20, 45) as well as the GGAA motif, an essential purine-rich core of binding sites for members of the Ets family of transcription factors. Also boxed is a sequence, 5'-GGAACTT-3', which shares extensive homology with a sequence in the murine IL-2 gene that binds transcription factors.
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In mutant 1 and mutant 5, 5 and 2 bases, respectively, have been mutated that are required for the binding of HNF-3β and the Ets-like proteins, respectively (see Ref. 20; see also top of Fig. 3A). Consequently, band h was not formed when G2 mutant 1 was used as probe, and bands e were not detectable when G2 mutant 5 was used as probe (Fig. 3B). This indicates that the depolarization-induced nuclear complex binds to a site within G2 that is distinct from the HNF-3β-binding site; in contrast, the two guanine bases (noncoding strand) exchanged in mutant 5 are essential for binding (see top of Fig. 3A).

The binding specificity was further characterized in competition experiments using G2 wild type, mutant 1, mutant 5, and also mutant 3. In mutant 3, 4 bases have been mutated (see top of Fig. 3A). As shown in Fig. 3C, the binding of HNF-3β (band h) was competed away by mutants 5 and 3 but not by mutant 1, which carries mutations within the HNF-3β-binding sequence. In contrast, mutant 1 competed for the binding of the depolarization-induced protein as well as did the wild-type sequence (Fig. 3C, compare lane 4 with lane 3 and lane 9 with lane 8), whereas mutant 3 and, even more so, mutant 5 were less effective competitors (Fig. 3C). This suggests that the depolarization-induced protein complex has critical contacts with bases that are mutated in mutants 3 and 5.

The mutations in mutants 3 and 5 alter a sequence within G2, 5′-GGAAACTT-3′, which shares extensive homology with a sequence in the murine IL-2 gene that binds transcription factors of the NFAT family (match of 7 bases out of 8) (18, 19).

In T cells, NFAT transcription factors have been shown to undergo a calcium- and calcineurin-dependent translocation from the cytosol to the nucleus (18, 19, 46). Therefore, a possible relationship between the depolarization-induced G2-binding protein complex and NFAT was studied. First, the oligonucleotide NFATcons, which contains a well characterized NFAT-binding site, was used as probe and cold competitor in electrophoretic mobility shift assays. As shown in Fig. 3D, NFATcons specifically competed for the binding of the depolarization-induced nuclear protein to labeled G2 (lane 4). When NFATcons was used as probe, membrane depolarization induced the binding of a protein complex that comigrated with the depolarization-induced nuclear protein binding to labeled G2 (Fig. 3D, compare lane 7 with lanes 5 and 2; the depolarization-induced protein complexes are indicated by arrows); it also showed a binding specificity that was indistinguishable from that of the depolarization-induced nuclear binding to labeled G2, since the depolarization-induced binding to labeled NFATcons was competed away by G2 and NFATcons but not by G2 mutant 5 (Fig. 3D). In addition to the depolarization-induced nuclear protein, several additional complexes were detected with labeled NFATcons; these were not further characterized. The cross-competition between G2 and NFATcons supports the notion that the depolarization-induced nuclear complex on G2 has a binding specificity that is related to NFAT. Second, the effect of FK506 was studied. FK506 is an inhibitor of the calcium-/calmodulin-dependent protein phosphatase calcineurin (47). Previous studies have shown that FK506 and cyclosporin A effectively inhibit calcineurin activity also in pancreatic islet cells (14, 15).

Whereas membrane depolarization induced the binding of a protein complex to the G2 element in controls (Fig. 3E, lanes 5 and 6), treatment of the cells with FK506 (167 nM) blocked this effect (Fig. 3E, lanes 7 and 8), suggesting that the induction by membrane depolarization of nuclear protein binding to G2 depends on calcineurin phosphatase activity. Third, the effect of an antisera directed specifically against NFATp was studied (donated by A. Rao, Boston, MA) (36). Whereas the addition of a preimmune serum had no effect, the addition of the anti-NFATp antisera abolished specifically the depolarization-induced G2-binding protein (Fig. 3F, compare lane 6 with lanes 4 and 2), indicating that this protein complex possesses NFATp-like immunoreactivity. Finally, using another protocol, separate nuclear and cytosolic extracts were prepared and used in electrophoretic mobility shift assays. A G2-binding protein complex was found in cytosolic extracts from unstimulated cells that comigrated with the nuclear complex induced by membrane depolarization (Fig. 3G, compare lane 7 with lane 2). Concomitant with the induction of protein binding in nuclear extracts, membrane depolarization markedly decreased the binding of this complex in cytosolic extracts (Fig. 3G, compare lane 7 with lane 8), consistent with a depolarization-induced translocation from the cytosol to the nucleus. The binding of both the protein complex induced by membrane depolarization in nuclear extracts as well as the binding of the comigrating protein complex was reduced specifically by the anti-NFATp antisera.
in cytosolic extracts from unstimulated cells was not affected by the addition of a preimmune serum but was abolished by the addition of an anti-NFATp antiserum (Fig. 3G). Taken together, these results indicate that the depolarization-induced nuclear G2-binding protein is NFATp which in pancreatic islet cells undergoes a calcium- and calcineurin-dependent translocation from the cytosol to the nucleus in response to membrane depolarization.

The results obtained in the electrophoretic mobility shift assay indicate that NFATp is expressed in pancreatic islet \( \alpha \)TC2 cells. This was further investigated using different approaches. In an RT-PCR analysis, a primer pair that is specific for NFATp generated from poly(A)\(^+\) RNA of \( \alpha \)TC2 cells a product of the expected size (Fig. 4A). Subcloning and sequencing confirmed that this product is NFATp cDNA, indicating that NFATp is expressed at the RNA level. A more detailed analysis using primer pairs, which target the divergent C-terminal ends of NFATp isoforms (19, 26), detected transcripts encoding the B and C isoforms but not the A isoform (not shown). To confirm further the expression of NFATp at the protein level Western blotting was used. NFAT proteins are known to migrate on SDS gels with an apparent molecular mass of 120–140 kDa (19). As shown in Fig. 4B (upper panel), a protein with NFATp-like immunoreactivity and an apparent molecular mass of about 120 kDa was detected in nuclear extracts from \( \alpha \)TC2 cells that had been stimulated by membrane depolarization. No such band was detected in nuclear extracts from unstimulated cells (Fig. 4B, lane 1). As a control for the quality of the nuclear extracts, an antiserum directed against a constitutively nuclear protein, CREB, was also used and yielded bands of about 43 kDa with similar signal intensities in both extracts (Fig. 4B, lower panel). Therefore, the results obtained in the electrophoretic mobility shift assay, in the RT-PCR analysis, and in Western blotting experiments concur that NFATp is expressed in these cells. When taken together, our data suggest that membrane depolarization induces in pancreatic islet cells through activation of calcineurin the nuclear translocation of NFATp, which binds to the G2 enhancer-like element of the glucagon gene.

Functional Analysis of NFATp and HNF-3\( \beta \) Binding to the G2 Element—To study the role of NFATp binding in calcium-induced activation of G2 transcriptional activity, transfection experiments were performed. As shown in Fig. 5, when nuclear

**FIG. 4. Expression of NFATp in pancreatic islet \( \alpha \)TC2 cells.** A, RT-PCR of NFATp transcripts. The relative position of the primers and the size of the expected fragment are indicated. An RT-PCR product of the expected size was obtained as shown by agarose gel electrophoresis (lane 2). Lane 1, size markers. The RT-PCR product was verified by subcloning and sequencing. B, Western blot. Nuclear extracts from \( \alpha \)TC2 cells that had been stimulated for 60 min with high potassium-induced membrane depolarization (KCl, 45 mM) or had been left unstimulated were subjected to immunoblotting. Upper panel, anti-NFATp antiserum; lower panel, anti-CREB antiserum.

**FIG. 5. Effect of FK506 (167 nM) on depolarization-induced activation of G2 transcriptional activity.** The plasmid 4xG2T81Luc was transiently transfected into \( \alpha \)TC2 cells. The cells were stimulated by high potassium-induced membrane depolarization (KCl, 45 mM). Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the controls (5 mM KCl, no FK506). Values are mean ± S.E. of four independent experiments, each done in duplicate.

**FIG. 6. Mutational analysis of sequences within G2 required for depolarization responsiveness.** Four copies of the G2 oligonucleotide with wild-type sequence (G2) or the G2 mutants 1, 3, or 5 (G2 m1, G2 m3, and G2 m5) were placed in front of the truncated viral thymidine kinase promoter of herpes simplex virus fused to the luciferase reporter gene. The plasmids were transfected into \( \alpha \)TC2 cells. KCI, 45 mM; TPA, 300 nM. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control (no treatment). When compared with 4xG2T81Luc (100 ± 6%), the basal activity was not changed by mutants 1 or 5 and was 67 ± 65% for 4xG2 m3T81Luc, as has been reported previously (20). Values are mean ± S.E. of three independent experiments, each done in duplicate.
translocation of NFATp was blocked by FK506 (167 nM), the activation of G2 transcriptional activity by membrane depolarization and calcium influx was markedly reduced. This suggests that calcium-induced activation of G2 transcriptional activity depends on calcineurin phosphatase activity and NFATp binding. The regulatory sequences within G2 that are required for depolarization responsiveness were characterized. Four copies of G2 wild-type sequence or mutants 1, 3, and 5 were placed in front of the heterologous thymidine kinase promoter of herpes simplex virus (pBluescript). In contrast to the G2 wild-type sequence, mutants 1, 3, and 5 failed to confer depolarization responsiveness (Fig. 7). Thus, the depolarization responsiveness of G2 was abolished by mutant 1 as well as by mutants 3 and 5, which interfere with the binding of HNF-3β (mutant 1) as well as with the depolarization-induced NFATp binding (mutants 3 and 5). Membrane depolarization was unable to stimulate transcription through four copies of a high affinity AP-1 site or a composite IL-2 NFAT:AP-1 site (data not shown). Taken together, the results of the present study suggest that the depolarization responsiveness of G2 may depend on the binding of both HNF-3β and NFATp. It is noteworthy that, in contrast to membrane depolarization, the protein kinase C-activating phorbol ester TPA stimulated transcription through mutant 3 at least as well as through G2 wild-type sequence (Fig. 6), consistent with published data (20). This indicates that distinct proteins mediate protein kinase C and depolarization responsiveness of the G2 element. Whereas HNF-3β and an Ets-like protein have been shown to confer Ras and protein kinase C responsiveness (20), NFATp and HNF-3β are required for the stimulation of G2 activity by membrane depolarization and calcium influx.

**Role of the G2 Element and NFATp in the Regulation of the Intact Glucagon Promoter by Membrane Depolarization**—To study the role of the G2 element in depolarization-induced activation of the intact glucagon promoter, glucagon-reporter fusion genes were used that carry an internal deletion of the G2 element without (~350ΔGluLuc) or with an additional internal deletion inside the CRE (~350Δ2ΔGluLuc). Deletion of the G2 element markedly reduced the depolarization-induced increase in transcriptional activity of the glucagon promoter in αTC2 cells (Fig. 7A, left panel), suggesting that G2 is required for depolarization responsiveness of the glucagon promoter. However, interpretation of the data is complicated by the marked decrease in basal activity by the G2 deletion (Fig. 7A, left panel). Therefore, similar transfection experiments were performed in HIT cells. In this pancreatic islet β-cell line, the internal deletion of G2 caused only a slight decrease in basal activity (Fig. 7A, right panel). The internal deletion of G2 reduced the depolarization-induced activation of the glucagon promoter by 76% (Fig. 7A, right panel), suggesting again that G2 is required for depolarization responsiveness of the glucagon promoter. Similar results were obtained with the double deletion mutant (Fig. 7A, right panel). Depolarization responsiveness of the glucagon promoter was inhibited in HIT cells by the internal deletion of G2 (Fig. 7A, right panel) to the same degree (by about 75%) as by the deletion inside the CRE (Fig. 1B), suggesting that both G2 and the CRE are required for full responsiveness of the glucagon promoter.

**Fig. 7. Role of the G2 element and NFATp in the regulation of the glucagon promoter by membrane depolarization.** **A**. effect of deleting G2 (~G2) or G2 plus CRE (~G2CRE) on glucagon promoter activity. The plasmids ~350GluLuc, ~350ΔGluLuc, or ~350Δ2ΔGluLuc were transfected into αTC2 or HIT cells. The cells were stimulated by high potassium-induced membrane depolarization (45 mM KCl, black bars) or were left unstimulated (white bars). Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the controls (unstimulated ~350GluLuc). Values are mean ± S.E. of three independent experiments, each done in duplicate. **B**. effect of FK506 on depolarization-induced activation of glucagon gene transcription. The plasmid ~292GluLuc was transfected into αTC2 cells. The cells were stimulated by high potassium-induced membrane depolarization (KCI, 45 mM). FK506, 16.7 nM. Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the controls (5 mM KCI, no FK506). Values are mean ± S.E. of three independent experiments, each done in duplicate. C. effect of overexpression of the DNA-binding domain of NFATp (NFATpDBD) on depolarization responsiveness of the glucagon promoter. αTC2 cells were co-transfected with ~350GluLuc and the expression vector pBS-CMV-NFATpDBD as indicated. The cells were stimulated by high potassium-induced membrane depolarization (KCl, 45 mM). Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the controls (unstimulated ~350GluLuc alone). Values are mean ± S.E. of three independent experiments.
activation of glucagon gene transcription by membrane depolarization; furthermore, this suggests that the depolarization responsiveness mediated by one element to the promoter depends in part on the other element.

To explore further the role of the G2-binding transcription factor NFATp for glucagon promoter activity, FK506 and the expression vector pBK-CMV-NFATpDBD were used. When nuclear translocation of NFATp was blocked by FK506, the activation of glucagon promoter activity by membrane depolarization and calcium influx was markedly reduced (Fig. 7B), suggesting that calcium-induced activation of the glucagon promoter depends on NFATp binding. In these experiments 292 base pairs of the glucagon promoter were used which lack the CRE, because the transactivation of serine 119-phosphorylated CREB is inhibited by FK506 (13–15). Although the minimal DNA-binding domain of NFATp is sufficient for cooperation with Fos and Jun proteins, it lacks an intrinsic transactivation domain (25) and, in the absence of interaction with AP-1 on composite NFAT-binding sites, is thus expected to inhibit NFAT transactivation when overexpressed by blocking NFAT-binding sites (25). As shown in Fig. 7C, overexpression of the NFATp DNA-binding domain inhibited the depolarization-induced activation of glucagon gene transcription by 40%. The inhibition of depolarization-induced activation of the glucagon promoter by NFATpDBD was less than that by G2 deletion or FK506, which may be explained by a lower efficiency of NFATpDBD overexpression. Taken together, all these data suggest a role of the G2 element and of NFATp in the regulation of the intact glucagon promoter by membrane depolarization.

**DISCUSSION**

In this report we describe experiments that have led to the identification and characterization of a second calcium response element within the glucagon promoter. Evidence is presented that the transcription factor NFATp is expressed in glucagon-producing islet cells and is directed to the nucleus by calcium/calcineurin in response to membrane depolarization. NFATp is known to cooperate with AP-1 proteins in T cells (16–19). By contrast, this study shows a novel pairing of NFATp with the cell-specific transcription factor HNF-3β, building a novel calcium response element.

Calcium influx through voltage-dependent calcium channels stimulates both glucagon secretion (9) and biosynthesis through gene transcription (3, 13, 15). The present study shows that, in addition to the glucagon CRE described previously (3, 13, 15), the glucagon G2 element is a second calcium response element of the glucagon gene. As is indicated by the effects of internal deletions of the CRE and G2, both elements are required for full depolarization responsiveness of the glucagon gene, similar to what has been shown for the calcium regulation of the c-fos gene by the c-fos CRE and the c-fos serum response element (5, 48). Using primary hippocampal neurons, Bading et al. (49) demonstrated that calcium, depending on its mode of entry into neurons, activates distinct signaling pathways that lead to gene activation via different cis-acting regulatory elements. Furthermore, differential activation of transcription factors induced by calcium response amplitude and duration has been shown in B lymphocytes (50). Thus, it is possible that depending on the type of the calcium signal the G2 element and the glucagon CRE could observe distinct functions in calcium signaling to the glucagon gene. The ability of membrane depolarization to potentiate cAMP-induced glucagon gene transcription in a synergistic fashion (3) may be physiologically important. Through synergistic interaction the calcium response of G2 and the CRE may participate in a cross-talk between intracellular signaling systems that in A cells integrates multiple stimuli to an appropriate transcriptional response of the glucagon gene in order to maintain glucose homeostasis.

NFATp was originally characterized as the preexisting cytoplasmic component of a transcription factor in T cell hybridomas implicated in the induction of several cytokine genes during the immune response (19). NFATp has been molecularly cloned (19, 32) and is now known to be a member of a family of transcription factors that contain at least four members and share a ~300-amino acid DNA-binding domain distantly related to the Rel homology region (18, 19, 51). The NFAT family of transcription factors is a target for the clinically important immunosuppressant drugs cyclosporin A and FK506, which inhibit the calcium-/calmodulin-dependent serine/threonine phosphatase calcineurin (47, 52, 53). In resting T cells, NFATp resides in the cytoplasm and is fully phosphorylated; following stimulation, it rapidly becomes dephosphorylated by calcineurin activated by an increase in cytosolic calcium concentration, and NFATp then translocates to the nucleus (19, 26, 46, 54–56). Although some cells lack the requisite mechanisms for regulating NFATp subcellular localization (26), our results suggest that NFATp is expressed and regulated in glucagon-producing islet cells in a similar way as in T cells and binds to the glucagon gene. G2 element within the glucagon promoter contains a typical NFAT-binding site (5′-GGAACCTTT-3′). Using an electrophoretic mobility shift assay, a protein complex with NFATp-like immunoreactivity was found to bind to this site and was detected in cytosolic extracts from unstimulated cells and in nuclear extracts from cells stimulated by membrane depolarization, consistent with depolarization-induced translocation of NFATp from the cytosol to the nucleus. The mutations in G2 mutant 3 were less detrimental for NFATp binding than those of G2 mutant 5, consistent with the finding that the bases exchanged in G2 mutant 3 are less conserved (72–78%) than those of G2 mutant 5 (100%) in aligned sequences derived by PCR-based site selection of an optimal NFAT-binding site (57). Furthermore, NFAT makes sequence-specific major groove contacts in the 5′-half-site (58), which is altered in G2 mutant 5 but not in G2 mutant 3. The antiserum used is specific for NFATp (36) and completely blocked the depolarization-induced nuclear protein binding, providing no evidence that in addition to NFATp other members of the NFAT family of transcription factors may be involved. NFATp has been shown before to be expressed in a wide variety of tissues including pancreas (36, 51, 59). The expression of NFATp in pancreatic islet βTC2 cells was confirmed by RT-PCR of NFATp mRNA as well as by Western blotting. Inhibition of calcineurin phosphatase activity by FK506 blocked both the depolarization-caused induction of nuclear NFATp binding and G2-mediated transcription. The latter was also prevented by mutating the binding sites for NFATp or HNF-3β within G2. Taken together, these data suggest that the molecular mechanism by which glucagon gene transcription is induced by membrane depolarization and calcium influx in pancreatic islet cells through the G2 element may be consistent with a model in which calcium activates calcineurin phosphatase activity which then, probably by direct dephosphorylation, induces the translocation of NFATp from the cytosol to the nucleus where it cooperates with HNF-3β at the G2 element to stimulate transcription (Fig. 8).

The N-terminal transactivation domain of NFATp has been shown to recruit the coactivators p300/CBP (60), which are also bound by CREB after phosphorylation in response to calcium-induced signaling (61). NFATp-CBP interactions may involve the CREB-binding domain and the cysteine-histidine-rich region 3 of CBP (60). Thus, cooperative recruitment of a common coactivator by CREB, binding to the CRE, and NFATp, binding
The binding of HNF-3β to NFATp and HNF-3β involves cooperative binding; it appears to be based on a functional synergism that may or may not include a direct protein–protein interaction. HNF-3β is a member of the winged helix family of transcription factors (64). It is expressed early in development (64–67). In adults, it is expressed in the liver (68), lungs, small intestine (69, 70), and exocrine pancreas (70), and pancreatic islets (20, 41, 70, 71), being involved in cell-specific gene transcription in these endoderm-derived tissues. Instead of integration into Ras/protein kinase C pathways through AP-1 proteins (19, 47, 52, 53, 62), the interaction between NFATp and HNF-3β may not confer the requirement for an input from signaling pathways others than those regulating NFATp. Thus, the present study shows a new combinatorial usage of NFATp with HNF-3β, which ties the calcium/calcineurin/NFAT pathway directly to cell-specific gene transcription.

The present findings expand the functions of the transcription factor HNF-3β to include a role in calcium-regulated gene transcription. HNF-3β was first defined as a transcription factor involved in developmental and cell-specific gene transcription (see above). Subsequently, it has been suggested that HNF-3β synergizes with the glucocorticoid receptor in the liver to confer glucocorticoid responsiveness to the tyrosine aminotransferase gene (72) and with an Ets-like transcription factor in islet cells to confer Ras and protein kinase C responsiveness to the glucagon gene (20). The results presented here suggest that HNF-3β potentiates transcriptional activation by the calcium-regulated transcription factor NFATp. Thus, HNF-3β is able to cooperate with several regulated transcription factors and in this way seems to function as a mediator of environmental signals in endoderm-derived tissues in which it is expressed.

There are several examples where multiple signaling pathways focus on a single DNA control element, although the mechanisms by which these signals are integrated or selected are different (1, 2, 73). The binding sites within G2 of calcium-regulated NFATp and of the Ets-like proteins, probably regulated by protein kinase C and Ras (20), overlap, suggesting that these proteins compete for binding to G2 and thereby determine which signal is mediated through G2.

Diverse calcium response elements have already been described, including elements binding the transcription factors CREB (1–4), serum response factor (2, 5), and C/EBPβ (6). The calcium-responsive glucagon G2 element differs from these elements by DNA sequence, interacting proteins, and the lack of evidence for an involvement of calcium/calmodulin-dependent protein kinases in signaling to this element. It also differs from composite NFAT-binding sites in the IL-2 and other cytokine genes (18, 19) by a novel pairing of NFATp. In this sense, the depolarization-induced combination of NFATp with the cell lineage-specific transcription factor HNF-3β at the glucagon G2 element represents a novel calcium response element.

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