

Glucagon-Like Peptide 1 Stimulates Insulin Gene Promoter Activity by Protein Kinase A-Independent Activation of the Rat Insulin I Gene cAMP Response Element

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Glucagon-like peptide 1 (GLP-1), a hormonal activator of adenylyl cyclase, stimulates insulin gene transcription, an effect mediated by the cAMP response element (CRE) of the rat insulin I gene promoter (RIP1). Here we demonstrate that the signaling mechanism underlying stimulatory effects of GLP-1 on insulin gene transcription results from protein kinase A (PKA)-independent activation of the RIP1 CRE. Although GLP-1 stimulates cAMP production in rat INS-1 insulinoma cells, we find accompanying activation of a -410-bp RIP1 luciferase construct (-410RIP1-LUC) to exist independently of this second messenger. GLP-1 produced a dose-dependent stimulation of -410RIP1-LUC (EC_{50} 0.43 nmol/l), an effect reproduced by the GLP-1 receptor agonist exendin-4 and abolished by the antagonist exendin(9-39). Activation of RIP1 by GLP-1 was not affected by cotransfection with dominant-negative $G_s \alpha$, was not blocked by cAMP antagonist Rp-cAMPS, and was insensitive to PKA antagonist H-89. Truncation of -410RIP1-LUC to generate -307-, -206-, and -166-bp constructs revealed 2 segments of RIP1 targeted by GLP-1. The first segment, not regulated by forskolin, was located between -410 and -307 bp of the promoter. The second segment, regulated by both GLP-1 and forskolin, included the CRE and was located between -206 and -166 bp. Consistent with these observations, stimulatory effects of GLP-1 at RIP1 were reduced after introduction of Δ -182 and Δ -183/180 inactivating deletions at the CRE. The action of GLP-1 at -410RIP1-LUC was also reduced by cotransfection with A-CREB, a genetically engineered isoform of the CRE binding protein CREB,

which dimerizes with and prevents binding of basic-region-leucine-zipper (bZIP) transcription factors to the CRE. In contrast, the action of GLP-1 at the CRE was not blocked by cotransfection with M1-CREB, an isoform that lacks a consensus serine residue serving as substrate for PKA-mediated phosphorylation. On the basis of these studies, it is proposed that PKA-independent stimulatory actions of GLP-1 at RIP1 are mediated by bZIP transcription factors related in structure but not identical to CREB. *Diabetes* 49:1156-1164, 2000

Glucagon-like peptide 1 (GLP-1) is an intestinally derived blood glucose-lowering hormone currently under investigation for use as a therapeutic agent in the treatment of type 2 diabetes (1). GLP-1 stimulates insulin gene transcription and proinsulin biosynthesis and potentiates glucose-dependent secretion of insulin from β -cells located in the pancreatic islets of Langerhans (2). GLP-1 also acts as a β -cell glucose competence factor (3). It restores the functionality of β -cells under conditions in which cells are refractory to stimulatory influences of extracellular D-glucose (4). Because glucose is the primary regulator of insulin biosynthesis (5-9), any action of GLP-1 to correct for a dysfunction of glucose-dependent insulin gene expression in the diabetic pancreas would be of particular interest. Here, we focus on identifying cellular signal transduction pathways that mediate stimulatory influences of GLP-1 on insulin gene expression.

GLP-1 increases cellular levels of preproinsulin mRNA by stimulating transcription of the insulin gene (10-13). GLP-1 also increases insulin mRNA stability (13) and posttranslational biosynthesis of proinsulin (11). GLP-1 receptors are members of the secretin family of GTP binding protein-coupled receptors (14) and effects of GLP-1 on β -cell function are mediated in part by cAMP (15-18). The GLP-1 receptor interacts with heterotrimeric G_s proteins (19) to stimulate adenylyl cyclase (20), to increase production of cAMP (10), and to activate protein kinase A (PKA). A-kinase-anchoring proteins target PKA to specific subcellular compartments in which serine/threonine protein phosphorylation is catalyzed (21,22). One substrate of PKA is the cAMP response element (CRE) binding protein CREB, a basic-region-leucine-zipper (bZIP) transcription factor that interacts with CREs found within cAMP-sensitive gene promoters (23,24). Because human and rat insulin I gene promoters contain one or more CREs

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Received for publication 17 December 1999 and accepted in revised form 22 March 2000.

-410RIP1-LUC, -410-bp rat insulin I gene promoter luciferase construct; ATF-1, activating transcription factor 1; bZIP, basic-region-leucine-zipper; CRE, cAMP response element; CREM, CRE modulator; FBS, fetal bovine serum; GLP-1, glucagon-like peptide 1; GST, glutathione S-transferase; HSA, human serum albumin; HBSS, Hank's balanced salt solution; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; RIA, radioimmunoassay; RIP1, rat insulin I gene promoter; STAT, signal transducer and activator of transcription.

(25,26), it has been speculated that stimulatory effects of GLP-1 on promoter activity are mediated via a conventional cAMP signaling mechanism (11,27). However, a rigorous test of this hypothesis has not been reported. What is known is that the rat insulin I gene promoter (RIP1) contains a CRE-like octamer motif (TGACGTCC) similar to the consensus CRE (TGACGTCA) known to mediate stimulatory actions of cAMP on gene expression (23,24). RIP1 is stimulated modestly by activators of cAMP signaling (26–30) and interacts not only with CREB, but with the CCAAT box binding protein NF-Y (31). These unusual properties of RIP1 suggest that it might serve as a useful tool for analyses of novel forms of GLP-1 signal transduction independent of the conventional cAMP and PKA signaling pathways.

Intestinally derived peptides such as GLP-1 are classified not only as hormones, but also as growth factors—peptides capable of regulating diverse cellular processes, including mitosis, growth, and differentiation. GLP-1 receptors interact with multiple subtypes of G proteins, including G_s , G_i , and G_q (32). GLP-1 stimulates phosphatidylinositol 3-kinase (33) and upregulates DNA binding activity of transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) (33,34). GLP-1 also stimulates transcription of immediate early response genes, including *c-fos* and *c-jun* (35). This effect of GLP-1 may be related to its ability to stimulate mitogen-activated protein kinase (MAPK) and to induce phosphorylation of MAPK kinase (MEK) (32,36). GLP-1 also counteracts inhibitory effects of leptin on insulin gene expression (37,38). This result suggests an ability of GLP-1 to influence components of a growth factor-like signaling pathway—the leptin receptor, its associated Janus kinases, and the signal transducers and activators of transcription family of DNA binding proteins (STATs) they control.

Based on this disparate set of observations, an emerging consensus has developed: the signaling properties of GLP-1 at the β -cell are unlikely to be limited to a conventional cAMP signaling pathway. With this in mind, we sought to determine if GLP-1 exerts stimulatory effects on insulin gene transcription independent of cAMP. Here we report that GLP-1 acts in INS-1 cells to stimulate transcriptional activity of a reporter gene construct in which –410 bp of RIP1 is fused to the coding sequence of firefly luciferase (–410-bp RIP1 luciferase construct [–410RIP1-LUC]). GLP-1 is shown to stimulate RIP1 via PKA-independent activation of the CRE. This effect is mediated by bZIP transcription factors related in structure to CREB. A preliminary report of these findings has been published (39).

RESEARCH DESIGN AND METHODS

Cell culture. INS-1 cells (passage numbers 95–110) were cultured in RPMI 1640 containing 10 mmol/l HEPES, 11.1 mmol/l glucose, 10% fetal bovine serum (FBS), 100 μ U/ml penicillin G, 100 μ g/ml streptomycin, 2.0 mmol/l L-glutamine, 1.0 mmol/l sodium pyruvate, and 50 μ mol/l 2-mercaptoethanol (40). Cells were maintained at 37°C in a humidified incubator gassed with 5% CO₂. Cell cultures were passaged by trypsinization and subcultured once a week. **Plasmid DNA constructs.** RIP1 (Fig. 1A) was provided by Dr. M. German (University of California at San Francisco Medical School, San Francisco, CA). It was fused to the coding sequence of luciferase in the pGL3-basic vector (Promega, Madison, WI) to generate –410RIP1-LUC. Truncations of the promoter were obtained by restriction digests to generate –307-, –206-, and –166-bp derivatives (Fig. 1A). Constructs containing deletions (Δ -182 and Δ -183/180) of the RIP1 CRE were prepared by site-directed mutagenesis (Fig. 1B). The dominant-negative G_s α expression plasmid (41) in pcDNA1 (Invitrogen, Carlsbad, CA) was obtained from Dr. H. Bourne (University of California at San Francisco Medical School). Wild-type and M1 rat CREB expression plasmids obtained from Dr. M. Montminy (Joslin Diabetes Center, Boston, MA) were subcloned into pcDNA3 (Invitrogen). A-CREB expression

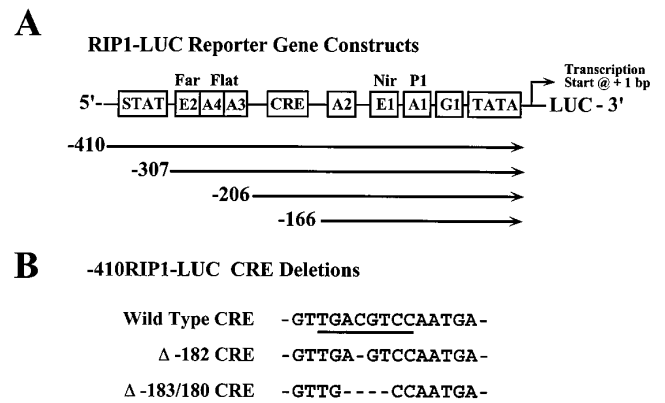


FIG. 1. A: Design of –410, –307, –206, and –166 RIP1-LUC reporter gene constructs. Numbering of base pairs within RIP1 is as described by Seufert et al. (38). Regulatory elements within RIP1 are depicted. B: Design of the –410RIP1-LUC CRE deletion constructs. The CRE-like octamer motif is underlined. Single-base (Δ -182) or 4-base deletions (Δ -183/180) were introduced into the wild-type CRE of RIP1.

plasmid (42) in pRCCMV (Invitrogen) was obtained from Dr. C. Vinson (National Institutes of Health, Bethesda, MD). pCRE-LUC expression plasmid was obtained from Stratagene (La Jolla, CA).

Transfection of INS-1 cells. Adherent INS-1 cells grown to 40–60% confluence in Falcon 35-mm tissue culture dishes (Becton Dickinson, Plymouth, U.K.) were transfected using commercially available reagents consisting of Lipofectamine Plus (transfection efficiency 10–15%) or Lipofectamine 2000 (transfection efficiency 30–40%) (Gibco BRL, Grand Island, NY). Cells were rinsed twice in serum-free culture medium before the addition of 1.0 ml transfection cocktail containing 1.0 μ g plasmid DNA. Cells were incubated in this mixture for 4 h. The transfection cocktail was supplemented with an additional 1.0 ml culture medium containing 20% FBS and incubated an additional 6–8 h in this medium. Thereafter, transfected cells were trypsinized and resuspended in 4.5 ml Hank's balanced salt solution (HBSS) containing 0.1% human serum albumin (HSA) and 5.6 mmol/l glucose. Cells were transferred to 96-well plates (Costar 3610; Corning, Acton, MA) at 100 μ l cell suspension per well and incubated overnight (10–14 h) for expression of the reporter gene before each experiment. Transfection efficiency was determined using a plasmid constructed by our laboratory in which expression of enhanced green fluorescent protein (pEGFP; Clontech, Palo Alto, CA) was placed under control of the rat insulin II gene promoter (G.G.H., unpublished data).

Measurements of luciferase activity. INS-1 cells transfected with –410RIP1-LUC exhibited a high basal luciferase activity when equilibrated 10–14 h in RPMI 1640 culture medium containing FBS and 11.1 mmol/l glucose. This activity decreased in a time-dependent manner to a steady-state level when cells were incubated in HBSS-HSA. Thus, the high background luciferase activity observed in RPMI 1640 was due to factors present in culture medium. Because this background activity masked the stimulatory effect of GLP-1, we incubated transfected cells in HBSS-HSA 10–14 h before each experiment. The viability of cells treated in this manner was confirmed by the trypan blue exclusion method.

Test substances were dissolved in HBSS-HSA and added to 96-well plates at a final volume of 100 μ l/well. Cells were exposed to test substances for 4 h at 37°C in a humidified incubator. Inhibitory test substances such as exendin(9-39), Rp-cAMPS, and H-89 were added 30 min before addition of GLP-1. After 4 h, cells were lysed, and measurements of luciferase activity were performed using a luciferase assay kit (Tropix, Bedford, MA) in conjunction with a dual injection port luminometer (TR-717; Perkin Elmer Applied Biosystems, Foster City, CA) with automated application of ATP and luciferin assay solutions. All experiments were carried out in triplicate. Statistical analyses were performed using analysis of variance combined with the Fisher's Poisson least squares determination test.

Sources of test substances. GLP-1(7-37), exendin(9-39), and exendin-4 were from Peninsula (Belmont, CA). Forskolin, HBSS, RPMI 1640, FBS, and HSA were from Sigma (St. Louis, MO). Rp-cAMPS and Sp-cAMPS were from BioLog Life Sciences (La Jolla, CA). H-89 and KT5720 were from Calbiochem (San Diego, CA).

Electrophoretic mobility shift assay. Synthetic oligonucleotides corresponding to the RIP1 CRE were annealed, end-labeled with ³²P using T4 polynucleotide kinase, and purified on Sephadex G-50 spin columns (Boehringer

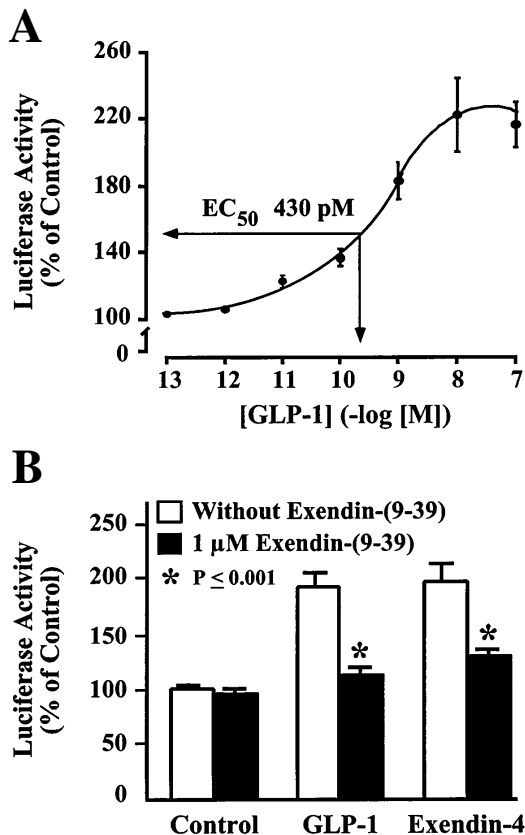


FIG. 2. A: Concentration-response relationship for stimulation of insulin gene promoter activity by GLP-1(7-37). INS-1 cells were transfected with -410RIP1-LUC. At 36 h after transfection, cells were equilibrated for 4 h in HBSS containing 5.6 mmol/l glucose and indicated concentrations of GLP-1(7-37). Luciferase activity measured in cellular lysates was quantitated as relative light units. Values of relative light units for lysates obtained from GLP-1-treated cells were normalized relative to control values obtained from lysates of cells not treated with GLP-1(7-37). **B:** The stimulatory action of 10 nmol/l GLP-1(7-37) was reproduced by 10 nmol/l exendin-4, whereas pretreatment of cells with 1 μmol/l exendin(9-39) blocked the actions of GLP-1(7-37) and exendin-4.

Mannheim, Nutley, NJ). Electrophoretic mobility shift assays were performed by incubating ca. 5×10^4 cpm of labeled DNA probe (0.02–0.03 ng) with glutathione S-transferase (GST) fusion protein in binding buffer containing 10 mmol/l Tris-HCl (pH 8.0), 40 mmol/l KCl, 6% glycerol, 1 mmol/l dithiothreitol, and 0.05% Nonidet P-40 for 20 min at 23°C. The reaction mixture was analyzed by 6% nondenaturing PAGE in combination with autoradiography. GST-CREB was prepared as described (43).

Radioimmunoassay for cAMP. INS-1 cells were maintained in 24-well culture plates at 70–80% confluence. Extracellular solution contained GLP-1 and 100 μmol/l isobutylmethylxanthine dissolved in HBSS. Exposure of cells to GLP-1 was for 30 min at 23°C. Ice-cold absolute ethanol was added to each well, and cells were subjected to 3 rounds of freeze-thawing. Lysed cells and extracellular solution were collected, and the content of cAMP was measured by radioimmunoassay (RIA) using an RIA kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

RESULTS

The action of GLP-1 at RIP1 is mediated by specific GLP-1 receptors. Luciferase activity in INS-1 cells transfected with -410RIP1-LUC was stimulated in a concentration-dependent manner by GLP-1(7-37) (EC_{50} 0.43 ± 0.02 nmol/l; Fig. 2A). At the maximally effective concentration, GLP-1 induced a $207 \pm 8\%$ ($P \leq 0.001$) increase in luciferase activity

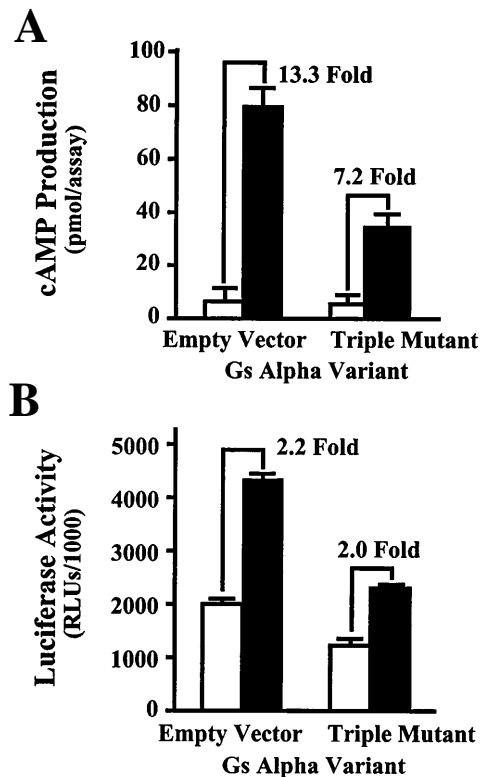


FIG. 3. A: High efficiency transfection of INS-1 cells with a dominant-negative triple mutant $G_s \alpha$ cDNA construct (1 μg/35 mm tissue culture plate) reduced the ability of 10 nmol/l GLP-1(7-37) to increase levels of cAMP in INS-1 cells. Transfection with an equivalent amount of pcDNA1 empty vector was without effect and served as a negative control. **B:** Transfection of INS-1 cells with dominant-negative $G_s \alpha$ plasmid (1 μg/35 mm tissue culture plate) failed to inhibit stimulation of RIP1 by 10 nmol/l GLP-1(7-37) in cells cotransfected with an equivalent amount of -410RIP1-LUC. Transfection efficiency was determined to be 30–40%, as measured by fluorescence microscopy using cultures transfected with plasmid DNA encoding enhanced green fluorescent protein. RLUs, relative light units. □, Basal; ■, GLP-1.

over basal activity. To ensure that the stimulatory action of GLP-1 was directed at the insulin gene promoter, we transfected INS-1 cells with promoterless pGL3 basic vector lacking RIP1. Under these conditions, basal luciferase activity was <0.5% of that observed using -410RIP1-LUC. Furthermore, this residual activity was insensitive to GLP-1 (data not shown). The selectivity of the insulin gene promoter for INS-1 cells was confirmed by noting a very low luciferase activity obtained from HEK293 cells transfected with -410RIP1-LUC (data not shown).

The stimulatory action of GLP-1 at RIP1 was due to its binding to specific GLP-1 receptors present in INS-1 cells. Exendin-4 is a GLP-1 receptor agonist (44), and at a concentration of 10 nmol/l, it induced a $197 \pm 17\%$ ($P \leq 0.001$) increase in luciferase activity—a value comparable to that obtained with 10 nmol/l GLP-1 ($194 \pm 13\%$, $P \leq 0.001$) (Fig. 2B). Exendin(9-39) is a GLP-1 receptor antagonist (45), and at a concentration of 1 μmol/l, it abolished the actions of 10 nmol/l GLP-1 ($P \leq 0.001$) and 10 nmol/l exendin-4 ($P \leq 0.001$) when tested in this assay (Fig. 2B). GLP-1(7-36) amide, which also binds to the GLP-1 receptor, exerted stimulatory effects at RIP1 equivalent to that of GLP-1(7-37) (data not shown).

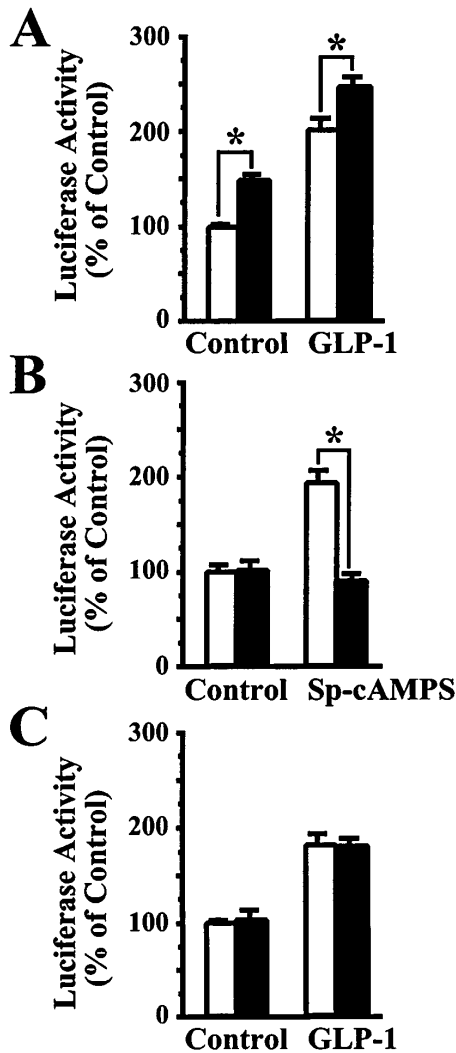


FIG. 4. A: Sp-cAMPS (50 μmol/l) stimulated -410RIP1-LUC activity in INS-1 cellular lysates. The action of Sp-cAMPS was potentiated by 10 nmol/l GLP-1 (7-37). □, No Sp-cAMPS; ■, 50 μmol/l Sp-cAMPS; *P ≤ 0.05. **B:** Rp-cAMPS (50 μmol/l) blocked stimulation of -410RIP1-LUC by 50 μmol/l Sp-cAMPS. □, No Rp-cAMPS; ■, 50 μmol/l Rp-cAMPS; *P ≤ 0.001. **C:** Failure of 50 μmol/l Rp-cAMPS to block stimulation of -410RIP1-LUC by 10 nmol/l GLP-1 (7-37). □, No Rp-cAMPS; ■, 50 μmol/l Rp-cAMPS. Values of luciferase activity are expressed as the percentage of control.

GLP-1 stimulates RIP1 independently of PKA. We next investigated whether GLP-1 stimulates RIP1 by way of a conventional cAMP signaling mechanism. GLP-1 stimulated cAMP production in INS-1 cells, an action reduced by high efficiency transfection with dominant-negative $G_s \alpha$ GTP binding protein (Fig. 3A). This "triple mutant" isoform of $G_s \alpha$ possesses amino acid substitutions ($A^{366}S$, $G^{226}A$, and $E^{268}A$) that disrupt its binding of guanyl nucleotides (41). Consistent with the findings presented here, dominant-negative $G_s \alpha$ was also reported to inhibit luteinizing hormone receptor or β_2 -adrenergic receptor-mediated cAMP production in COS-7 cells (41). In contrast, we found dominant-negative $G_s \alpha$ to be an ineffective antagonist of GLP-1-stimulated luciferase activity in INS-1 cells cotransfected with -410RIP1-LUC (Fig. 3B). We conclude that no positive correlation exists for stimulatory

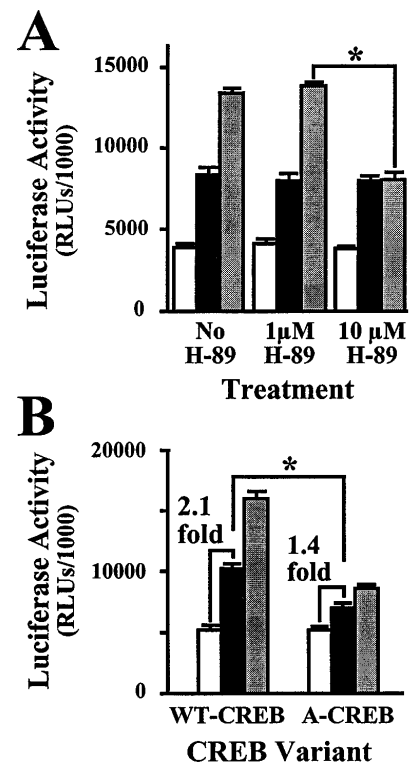


FIG. 5. A: PKA antagonist H-89 produced a concentration-dependent inhibition of 2 μmol/l forskolin-stimulated -410RIP1-LUC activity in transfected INS-1 cells. H-89 did not inhibit luciferase activity stimulated by 10 nmol/l GLP-1 (7-37). *P ≤ 0.001. **B:** Transfection with dominant-negative A-CREB reduced 10 nmol/l GLP-1 (7-37)-stimulated luciferase activity in cells cotransfected with -410RIP1-LUC. A-CREB was also an effective antagonist of 2 μmol/l forskolin-stimulated luciferase activity. *P ≤ 0.01. □, Basal; ■, GLP-1; ▨, forskolin.

effects of GLP-1 on cAMP production and insulin gene promoter activity in the INS-1 cell line.

We next sought to ascertain if the stimulatory action of GLP-1 at RIP1 is mediated by cAMP. Stimulation of -410RIP1-LUC by GLP-1 was a process mimicked by the cAMP agonist Sp-cAMPS (Fig. 4A). This membrane-permeate analog of cAMP activates PKA by binding to the regulatory subunit of the holoenzyme (46). It is important to note that the actions of GLP-1 and Sp-cAMPS were mediated by nonidentical signaling pathways. The stimulatory action of a saturating concentration (10 nmol/l) of GLP-1 was augmented in an additive fashion by simultaneous treatment of INS-1 cells with a saturating concentration (50 μmol/l) of Sp-cAMPS (Fig. 4A). Although the stimulatory action of Sp-cAMPS at RIP1 was blocked by 50 μmol/l of the cAMP antagonist Rp-cAMPS (Fig. 4B), the action of GLP-1 was unaffected by Rp-cAMPS (Fig. 4C). Rp-cAMPS is a stereoisomer of Sp-cAMPS that competes with and blocks the stimulatory action of cAMP at the regulatory subunit of PKA (46). These findings suggest that the primary stimulatory effect of GLP-1 at RIP1 is unlikely to be mediated by cAMP.

The cAMP-mediated regulation of gene expression is assumed generally to involve PKA-dependent phosphorylation of CREB. Consistent with this concept was our determination that the selective PKA antagonist H-89 (47) produced a dose-dependent inhibition of forskolin-stimulated luciferase activ-

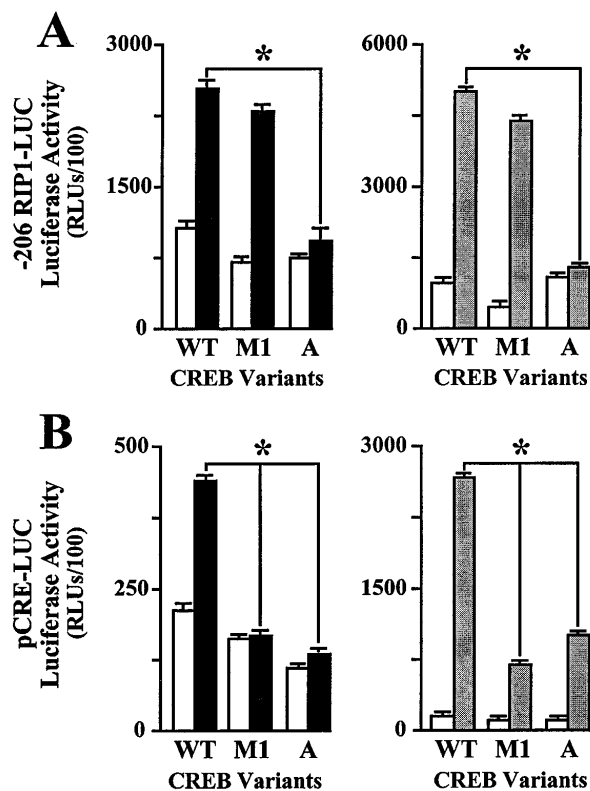


FIG. 6. **A:** Differential effects of CREB expression plasmids on RIP1-dependent luciferase activity stimulated by 10 nmol/l GLP-1 or 2 μ mol/l forskolin in INS-1 cells. Wild-type (WT), M1-type (M1), and A-type (A) CREB expression plasmids were cotransfected with a -206RIP1-LUC construct containing the RIP1 CRE but not more distal regulatory elements. **B:** Actions of WT, M1, and A-type CREB expression plasmids to influence luciferase activity stimulated by 10 nmol/l GLP-1 and 2 μ mol/l forskolin in INS-1 cotransfected with pCRE-LUC. \square , Basal; \blacksquare , GLP-1; \square , forskolin. * $P \leq 0.001$.

ity in INS-1 cells transfected with -410RIP1-LUC (Fig. 5A). In marked contrast, H-89 was ineffective as an antagonist of GLP-1 (Fig. 5A). Similarly, 10 μ mol/l of the selective PKA antagonist KT5720 (48) failed to block the action of GLP-1 in this assay (data not shown). Therefore, GLP-1 exerts stimulatory effects at RIP1 that are PKA independent.

bZIPs of the CREB family mediate stimulatory actions of GLP-1 at RIP1. A-CREB is a dominant-negative isoform of CREB that dimerizes via the leucine zipper with endogenous bZIP transcription factors (CREB, CRE modulator [CREM], and activating transcription factor 1 [ATF-1]). A-CREB blocks binding of bZIPs to promoter elements, thereby preventing CRE-mediated transcriptional activation (42). We observed that the stimulatory action of GLP-1 at -410RIP1-LUC was reduced by cotransfection of INS-1 cells with dominant-negative A-CREB (Fig. 5B). In contrast, transfection with pRCCMV empty vector was without effect (negative control, data not shown). A-CREB was found to also inhibit stimulatory actions of the cAMP-elevating agent forskolin at RIP1 (Fig. 5B, see also Fig. 6A, below). These findings demonstrate that CREB-like bZIPs mediate stimulatory actions of GLP-1 and forskolin at RIP1. However, unlike the action of forskolin, the action of GLP-1 is independent of PKA-mediated phosphorylation of these transcription factors.

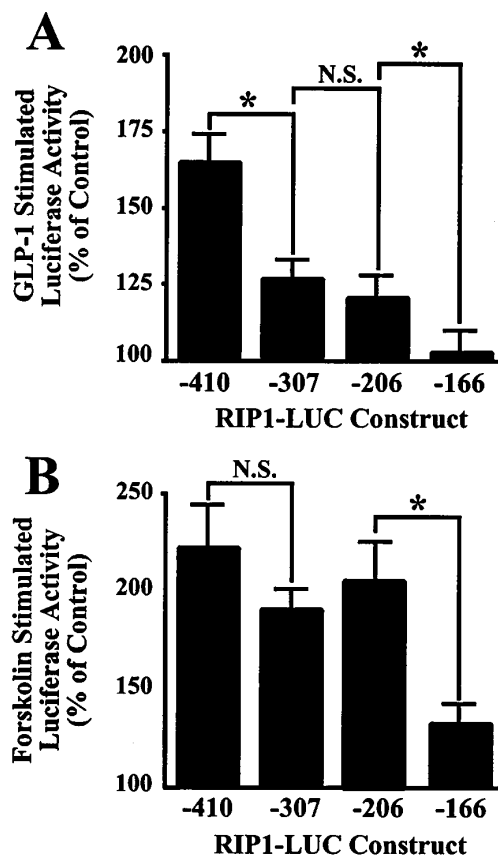


FIG. 7. **A:** A reduction of 10 nmol/l GLP-1 (7-37)-stimulated luciferase activity in INS-1 cellular lysates was observed after truncation of -410RIP1-LUC to generate -307RIP1-LUC or after truncation of -206RIP1-LUC to generate -166RIP1-LUC. **B:** The action of 2 μ mol/l forskolin was also reduced by truncation of -206RIP1-LUC to generate -166RIP1-LUC but not by truncation of -410RIP1-LUC to generate -307RIP1-LUC. * $P \leq 0.005$.

M1 CREB fails to eliminate stimulatory actions of GLP-1 and forskolin at RIP1. M1 CREB is an isoform of CREB derived by site-directed mutagenesis. It binds to the CRE of cAMP-responsive gene promoters, but is unresponsive to PKA because of the conversion of the P-box serine residue to alanine (23,56). To examine in more detail the possible role of bZIPs in regulation of RIP1 at the CRE, INS-1 cells were cotransfected with 2 expression plasmids: one coding for M1 CREB and the other a -206RIP1-LUC construct containing the RIP1 CRE devoid of more distal regulatory elements. M1 CREB failed to block stimulatory actions of GLP-1 and forskolin at -206RIP1-LUC (Fig. 6A). These observations support our contention that the CRE-mediated action of GLP-1 at RIP1 results from stimulatory effects of the hormone independent of the conventional cAMP/PKA/CREB signaling mechanism. It is also important to note the action of M1 CREB was promoter specific. Cotransfection of M1 CREB with pCRE-LUC prevented stimulatory actions of GLP-1 and forskolin at a 4-fold tandem repeat of the consensus CRE found within this genetically engineered promoter (Fig. 6B).

Truncation studies of RIP1 demonstrate a dual mechanism of GLP-1 action. A series of truncated RIP1 constructs were evaluated in an effort to elucidate a role for the CRE in

GLP-1 signal transduction (Fig. 1A). Unexpectedly, truncation of -410RIP1-LUC to generate -307RIP1-LUC resulted in a diminished responsiveness to GLP-1 (Fig. 7A). The -307RIP1-LUC construct lacks a STAT5 regulatory element previously shown to mediate actions of leptin and growth hormone at RIP1 (38,49). In contrast, the action of forskolin at -307RIP1-LUC was not affected significantly by this truncation (Fig. 7B). Additional truncation of RIP1 to generate -206RIP1-LUC did not alter sensitivity to GLP-1 or forskolin as compared with -307RIP1-LUC (Fig. 7A and B). The -206RIP1-LUC construct lacks the E2, A4, and A3 regulatory elements of RIP1. Further truncation to generate -166RIP1-LUC resulted in a drastic reduction in responsiveness to GLP-1 (Fig. 7A) and forskolin (Fig. 7B). The -166RIP1-LUC construct lacks the RIP1 CRE (Fig. 1A). Taken together, these findings emphasize the importance of the RIP1 CRE as a mediator of GLP-1 signal transduction. They also reveal a possible target of GLP-1 action at a more distal region of the promoter located between -410 and -307 bp.

CRE-mediated activation of RIP1 by GLP-1. We also demonstrate that inactivating deletions of the CRE attenuate stimulatory effects of GLP-1 at RIP1 (Fig. 8A). The 2 types of inactivating deletions studied were the $\Delta-182$ single-base pair deletion and the $\Delta-183/180$ 4-base pair deletion (Fig. 1B). Both types of deletions were equally effective when tested versus GLP-1 (Fig. 8A). Similarly, both types of deletions reduced the stimulatory action of forskolin at RIP1 (Fig. 8B). The efficacy of the $\Delta-182$ deletion was confirmed in an electrophoretic mobility shift assay, whereby it was demonstrated that the $\Delta-182$ CRE oligonucleotide failed to bind to recombinant CREB or to compete with wild-type CRE oligonucleotide for binding to recombinant CREB (data not shown).

DISCUSSION

It is now well established that GLP-1 is a potent stimulator of insulin gene expression, although the exact mechanism by which this insulinotropic hormone upregulates transcriptional activity of the insulin gene has remained enigmatic. Previous studies speculated on possible roles of cAMP, PKA, and CREB as mediators of GLP-1 signaling at the insulin gene promoter (10–12,26–31). Here we provide evidence that a bZIP transcription factor of the CREB family (CREB, ATF-1, and CREM) does in fact mediate stimulation of RIP1 by GLP-1. This conclusion is supported by our finding that the action of GLP-1 was diminished by dominant-negative A-CREB, by inactivating deletions of the CRE, or by truncation of RIP1 to remove the CRE. Surprisingly, the action of GLP-1, but not of forskolin, at RIP1 is shown to be entirely independent of PKA. Taken together, these findings emphasize the multifunctional nature of a family of transcription factors (bZIPs) and their cognate DNA binding elements (CREs) that serve to mediate stimulatory actions of GLP-1 at RIP1.

Evidence for compartmentalization of cAMP signaling in the β -cell. Stimulation by GLP-1 of a -410RIP1 reporter gene construct expressed in transfected INS-1 cells was observed to be mediated by receptors with pharmacological properties consistent with that of the GLP-1 receptor cloned from rat islets (14). However, stimulation of RIP1 by GLP-1 was insensitive to dominant-negative $G_s \alpha$, was not blocked by cAMP antagonist Rp-cAMPS, was insensitive to PKA antagonist H-89, and was not blocked by M1 CREB. In con-

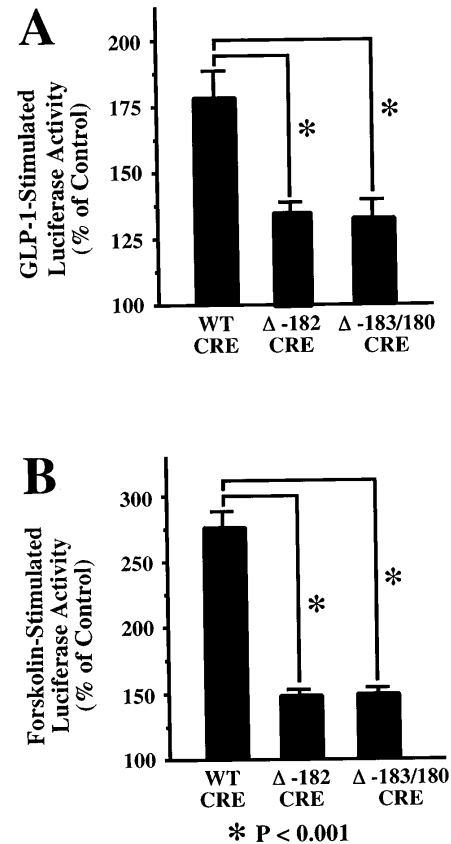


FIG. 8. A: Single-base pair ($\Delta-182$) or 4-base pair ($\Delta-183/180$) deletions of the RIP1 CRE reduced 10 nmol/l GLP-1(7-37)-stimulated luciferase activity in INS-1 cells transfected with -410RIP1-LUC . **B:** The stimulatory action of 2 $\mu\text{mol/l}$ forskolin was also reduced by introduction of $\Delta-182$ or $\Delta-183/180$ deletions of the RIP1 CRE.

trast, dominant-negative $G_s \alpha$ was an effective antagonist of GLP-1-stimulated cAMP production, consistent with the reported expression of this GTP binding protein in β -cells (19). Therefore, the action of GLP-1 at RIP1 does not appear to be mediated by a conventional $G_s \alpha$ signaling system involving adenylyl cyclase, cAMP, PKA, and CREB.

It is interesting to note that stimulation of -410RIP1-LUC by Sp-cAMPS or forskolin was inhibited by Rp-cAMPS and H-89, respectively. When compared with the actions of GLP-1, such findings argue for a compartmentalization of cAMP signaling in β -cells. We propose that subcellular microdomains of cAMP are produced in response to GLP-1. Effects of GLP-1 at the plasma membrane include facilitation of voltage-dependent Ca^{2+} channels and insulin granule exocytosis (17). These actions of GLP-1 are augmented by A-kinase-anchoring proteins that target PKA to the plasma membrane (21,22). Spatially delimited actions of GLP-1 may also extend to discreet subsets of cytosolic or nuclear transcription factors. We provide evidence for an action of GLP-1 to stimulate the tandem consensus CRE found within pCRE-LUC. The action of GLP-1 at pCRE-LUC was mimicked by forskolin and abrogated by coexpression with dominant-negative M1 CREB insensitive to PKA. Such actions of GLP-1 at pCRE-LUC are likely to be mediated by CREB, an established PKA substrate. In contrast, the action of GLP-1 at RIP1 was not affected by PKA inhibitor H-89, was not

altered by M1 CREB, but was blocked by coexpression with A-CREB. These findings suggest an important role for CREB-like transcription factors, possibly ATF-1 or CREM, as mediators of GLP-1 signaling at RIP1.

Our findings also indicate that PKA-mediated stimulation of RIP1 can result from an increase of cAMP concentration after exposure of cells to forskolin. As is the case for GLP-1, the action of forskolin is shown to be relatively insensitive to M1 CREB and is blocked by A-CREB. However, unlike the effect of GLP-1, the action of forskolin is mediated by PKA, thereby suggesting a convergence of PKA-dependent and PKA-independent signaling at the CRE. Although the subcellular localization of PKA accessible to forskolin remains to be determined, it can be speculated that perinuclear PKA activated by a global increase of [cAMP]_i translocates from the cytoplasm to the nucleus, where it interacts with bZIPs at the CRE (50).

cAMP and PKA as regulators of insulin gene expression. A large body of evidence exists demonstrating stimulatory effects of cAMP on insulin gene expression (7,11,25,26, 28–31). Pharmacological activators of cAMP signaling such as forskolin stimulate insulin gene promoter activity, an action mediated by binding of bZIPs to the CRE. Forskolin stimulates phosphorylation of CREB at Ser residue 133 in hamster insulinoma cells (29), an action proposed to upregulate insulin gene expression. Mutation of Ser 133 to Ala blocks activation of a Gal-4-CREB fusion protein by forskolin (51), whereas overexpression of dominant-negative CREB (K-CREB) reduces the stimulatory action of forskolin at RIP1 (31). An important role for the insulin gene CRE as a mediator of cAMP signaling is indicated because introduction of the Δ -183/180 inactivating deletions reduces stimulatory effects of forskolin at RIP1 (31). Therefore, there seems to be little doubt that cAMP can influence insulin gene expression at the CRE, an action mediated by bZIPs of the CREB family.

Despite these observations, it is surprising that GLP-1, a cAMP-elevating hormone, appears to act at the CRE in a cAMP- and PKA-independent manner. This conclusion is based on pharmacological studies using known antagonists of cAMP and PKA. H-89 and KT5720 are broad-spectrum protein kinase antagonists that are highly effective versus PKA when used at a concentration of 10 μ mol/l (47). Despite this fact, H-89 was without effect when tested against GLP-1, although we did find it to be effective when tested against forskolin. Therefore, a role for PKA as a mediator of GLP-1 signal transduction at RIP1 appears to be unlikely. We also found cAMP antagonist Rp-cAMPS at a concentration of 50 μ mol/l to be ineffective when tested against GLP-1. The efficacy of Rp-cAMPS in our assay was confirmed by demonstrating its ability to block stimulatory actions of Sp-cAMPS at RIP1. Based on this finding, we conclude that the action of GLP-1 at RIP1 may not be mediated by cAMP. This conclusion is strengthened by our observation that transfection with dominant-negative G_s α downregulated cAMP production in response to GLP-1, whereas stimulation of RIP1 by GLP-1 was unaffected.

A-CREB and M1 CREB as probes for bZIP-CRE interactions in the regulation of RIP1. CREB is a member of the bZIP family of transcription factors that self-dimerize via leucine zippers and that bind to DNA promoter elements at the consensus CRE octamer motif (23,24). CREB forms homodimers, but also heterodimerizes with closely related bZIP transcription factors, including ATF-1 (52,53) and possibly CREM (24). This multiplicity of possible protein-protein

interactions raises the question as to whether stimulatory actions of GLP-1 at RIP1 are in fact mediated by CREB, or alternatively some other bZIP transcription factor. Indeed, recent studies suggest that CREM is a stimulator of the human insulin gene promoter (54).

Here we report that the action of GLP-1 at RIP1 was inhibited by A-CREB but not by M1 CREB. A-CREB is an isoform of CREB that dimerizes with endogenous CREB and that prevents its binding to the CRE (42). In contrast, M1 CREB is a variant that binds to the CRE but that is insensitive to PKA and does not transactivate gene transcription in response to cAMP (23,55). A-CREB acts as a buffer of endogenous CREB and related bZIPs, whereas M1 CREB suppresses PKA-mediated transactivation by competing with endogenous CREB for the CRE. The inhibitory effect of A-CREB, as reported here, is most simply interpreted in terms of its ability to dimerize with bZIPs of the CREB family—possibly ATF-1 or CREM. This conclusion is supported by our finding that M1 CREB failed to block the action of GLP-1 at RIP1, thereby undermining any putative role for CREB in the regulation of RIP1 at the CRE.

It should be noted that a major role for CREB in the stimulation of RIP1 by forskolin was previously suggested (28,31). The action of forskolin at RIP1 was shown to be inhibited by K-CREB or by inactivating deletions of the CRE (31). However, interpretation of these findings must take into account the possibility that K-CREB is not absolutely specific for endogenous CREB, thereby leaving open a possible role for structurally related bZIPs as mediators of this response. Our findings also lead us to suggest that forskolin acts via PKA to target a bZIP related in structure to CREB and that this same transcription factor might serve as substrate for PKA-independent regulation by GLP-1.

We also extend upon earlier studies to show that Δ -182 and Δ -183/180 CRE deletions reduce stimulatory actions of GLP-1 at RIP1. Because the action of forskolin but not GLP-1 at RIP1 is PKA-sensitive, these findings also indicate that CREB-like bZIPs mediate a convergence of PKA-dependent and -independent signaling pathways at the CRE.

Interpretation of RIP1 truncation studies. We provide evidence based on RIP1 truncation studies for a major stimulatory action of GLP-1 at the CRE. However, the action of GLP-1 may not be exclusively CRE-mediated because we also observed a diminished responsiveness of RIP1 after its truncation from -410 to -307 bp. Previous studies localized a stimulatory growth hormone response element to a STAT5 recognition sequence located between -330 and -322 bp of RIP1 (49). This response element was also implicated in the inhibition of insulin gene expression by leptin (38). Such findings are intriguing because they suggest that the STAT5 recognition element might mediate an interaction between GLP-1, growth hormone, and leptin to control insulin gene expression (37,38,56).

Conclusion. In summary, we demonstrate that GLP-1 stimulates transcriptional activity of the insulin gene promoter in a PKA-independent manner. GLP-1 is shown to target the RIP1 CRE, an action mediated by bZIP transcription factors related in structure to CREB. Such findings are interpretable based on recent studies demonstrating that actions of bZIPs at the CRE are regulated not only by PKA (55), but also by the Ca²⁺/calmodulin-regulated protein kinases (57), protein kinase B (58), and pp90^{RSK} (59). Of particular interest is the fact that protein kinase B and pp90^{RSK} are downstream effectors of phosphatidylinositol 3-kinase and MAPK, enzymes

stimulated by GLP-1 (32,33). It remains to be determined if these potential alternative signaling pathways contribute to the insulinotropic actions of GLP-1 at the pancreatic islets.

ACKNOWLEDGMENTS

G.G.H. acknowledges support of the National Institutes of Health (Grants R01DK45817 and R01DK52166) and the American Diabetes Association (Research Award). M.A.H. holds a Juvenile Diabetes Foundation International Career Development Award.

The authors acknowledge the contribution of Dr. J.F. Habener for support in developing the RIP1 reporter gene constructs. Thanks also go to M.A. Castonguay for assistance in developing the RIP II pEGFP expression plasmid and O. Chepurney for assisting in cell culture, transfections, and plasmid purifications.

REFERENCES

- Drucker D: Glucagon-like peptides. *Diabetes* 47:159–169, 1998
- Fehmann HC, Goke R, Goke B: Cell and molecular biology of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulin releasing peptide. *Endocr Rev* 16:390–410, 1995
- Holz GG, Habener JF: Signal transduction crosstalk in the endocrine system: pancreatic β -cells and the glucose competence concept. *Trends Biochem Sci* 17:388–393, 1992
- Holz GG, Kuhlreiter WM, Habener JF: Pancreatic β -cells are rendered glucose competent by the insulinotropic hormone glucagon-like peptide-1(7-37). *Nature* 361:362–365, 1993
- Permutt MA, Kipnis DM: Insulin biosynthesis: on the mechanism of glucose stimulation. *J Biol Chem* 247:1194–1199, 1972
- Itoh N, Sei T, Nose K, Okamoto H: Glucose stimulation of the proinsulin biosynthesis in isolated pancreatic islets without increasing the amounts of proinsulin mRNA. *FEBS Lett* 93:343–347, 1978
- Nielsen DA, Welsh M, Casadaban MJ, Steiner DF: Control of insulin gene expression in pancreatic β -cells and in an insulin-producing cell line, RIN-5F cells. I. Effects of glucose and cyclic AMP on the transcription of insulin mRNA. *J Biol Chem* 260:13585–13589, 1985
- German MS, Moss LG, Rutter WJ: Regulation of insulin gene expression by glucose and calcium in transfected primary islet cultures. *J Biol Chem* 265:22063–22066, 1990
- Docherty K, Macfarlane WM, Read ML, Smith SB, Wilson ME, Bujalska I, Gilligan M: Regulation of insulin gene transcription by nutrients. *Biochem Soc Trans* 24:368–372, 1996
- Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF: Glucagon-like peptide-1 stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci U S A* 84:3434–3438, 1987
- Fehmann HC, Habener JF: Insulinotropic hormone glucagon-like peptide-1(7-37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma β TC-1 cells. *Endocrinology* 130:159–166, 1992
- Fehmann HC, Habener JF: Galanin inhibits proinsulin gene expression stimulated by the insulinotropic hormone glucagon-like peptide-1(7-37) in mouse insulinoma β TC-1 cells. *Endocrinology* 130:2890–2896, 1992
- Wang Y, Egan JM, Raygada M, Nadiv O, Roth J, Montrose-Rafizadeh C: Glucagon-like peptide-1 affects gene transcription and messenger ribonucleic acid stability of components of the insulin secretory system in RIN 1046-38 cells. *Endocrinology* 136:4910–4917, 1995
- Thorens B: Expression cloning of the pancreatic β -cell receptor for the glucagon-like peptide-1. *Proc Natl Acad Sci U S A* 89:8641–8645, 1992
- Holz GG, Leech CA, Habener JF: Activation of a cAMP-regulated Ca^{2+} signaling pathway in pancreatic β -cells by the insulinotropic hormone glucagon-like peptide-1. *J Biol Chem* 270:17749–17757, 1995
- Holz GG, Leech CA, Heller RS, Castonguay MA, Habener JF: cAMP-dependent mobilization of intracellular Ca^{2+} stores by activation of ryanodine receptors in pancreatic β -cells. *J Biol Chem* 274:14147–14156, 1999
- Gromada J, Holst JJ, Rorsman P: Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide-1. *Diabetes* 47:583–594, 1998
- Marie J-C, Boissard C, Skoglund G, Rosselin G, Breant B: Glucagon acts through its own receptors in the presence of glucagon-like peptide-1 receptors on pancreatic β -cells. *Endocrinology* 137:4108–4114, 1996
- Skoglund G, Basmaciogullari A, Rouot B, Marie J-C, Rosselin G: Cell-specific localization of G protein α -subunits in the islets of Langerhans. *J Endocrinol* 162:31–37, 1999
- Leech CA, Castonguay MA, Habener JF: Expression of adenyl cyclase subtypes in pancreatic β -cells. *Biochem Biophys Res Commun* 254:703–706, 1999
- Lester LB, Langeberg LK, Scott JD: Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. *Proc Natl Acad Sci U S A* 94:14942–14947, 1997
- Fraser ID, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, Marrión NV, Scott JD: A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. *EMBO J* 17:2261–2272, 1998
- Montminy M: Transcriptional regulation by cyclic AMP. *Annu Rev Biochem* 66:807–822, 1997
- Daniel PB, Walker WH, Habener JF: Cyclic AMP signaling and gene regulation. *Annu Rev Nutr* 18:353–383, 1998
- Inagaki N, Maekawa T, Sudo T, Ishii S, Seino Y, Imura H: c-Jun represses the human insulin promoter activity that depends on multiple cAMP response elements. *Proc Natl Acad Sci U S A* 89:1045–1049, 1992
- Philippe J, Missotten M: Functional characterization of a cAMP-responsive element of the rat insulin I gene. *J Biol Chem* 265:1465–1469, 1990
- Dumontel F, Philippe J: Insulin gene: organisation, expression, and regulation. *Diabete Metab (Paris)* 22:164–173, 1995
- Oetjen E, Diedrich T, Eggers A, Knepel W: Distinct properties of the cAMP-responsive element of the rat insulin I gene. *J Biol Chem* 269:27036–27044, 1994
- Schwaninger M, Blume R, Kruger M, Lux G, Oetjen E, Knepel W: Involvement of the Ca^{2+} -dependent phosphatase calcineurin in gene transcription that is stimulated by cAMP through cAMP response elements. *J Biol Chem* 270:8860–8866, 1995
- Eckert B, Schwaninger M, Knepel W: Calcium-mobilizing insulin secretagogues stimulate transcription that is directed by the cyclic adenosine 3'5'-monophosphate/calcium response element in a pancreatic islet β -cell line. *Endocrinology* 137:225–233, 1996
- Eggers A, Siemann G, Blume R, Knepel W: Gene-specific transcriptional activity of the insulin cAMP-responsive element is conferred by NF-Y in combination with cAMP response element-binding protein. *J Biol Chem* 273:18499–18508, 1998
- Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H, Levine MA, Schwindinger W, Bernier M: Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. *Endocrinology* 140:1132–1140, 1999
- Buteau J, Roduit R, Susini S, Prentki M: Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in β (INS-1)-cells. *Diabetologia* 42:856–864, 1999
- Wang X, Cahill CM, Pineyro MA, Zhou J, Doyle ME, Egan JM: Glucagon-like peptide-1 regulates the beta cell transcription factor, PDX-1, in insulinoma cells. *Endocrinology* 140:4904–4907, 1999
- Susini S, Roche E, Prentki M, Schlegel W: Glucose and glucagon-like peptides synergize to induce c-fos, c-jun, junB, zif268, and nur-77 gene expression in pancreatic β (INS-1) cells. *FASEB J* 12:1173–1182, 1998
- Frodin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB, Obberghen EV: Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting β -cell line, INS-1. *J Biol Chem* 270:7882–7889, 1995
- Seufert JR, Kieffer TJ, Leech CA, Holz GG, Moritz W, Ricordi C, Habener JF: Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. *J Clin Endocrinol Metab* 84:670–676, 1999
- Seufert J, Kieffer TJ, Habener JF: Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. *Proc Natl Acad Sci U S A* 96:674–679, 1999
- Holz GG, Hussain MA, Skoglund GS: Rat insulin gene promoter activity stimulated by picomolar concentrations of glucagon-like peptide 1: pharmacological characteristics and signal transduction properties of an incretin hormone used for treatment of type-2 diabetes (Abstract). *Diabetes* 48:A242–A243, 1999
- Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercapto-ethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178, 1992
- Iiri T, Bell SM, Baranski T, Fujita T, Bourne HR: A G_s α mutant designed to inhibit receptor signaling through G_s. *Proc Natl Acad Sci U S A* 96:499–504, 1999
- Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C: A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-

- dependent transcription of *c-fos*. *Mol Cell Biol* 18:967-977, 1998
43. Bodor J, Habener JF: Role of transcriptional repressor ICER in cyclic AMP-mediated attenuation of cytokine gene expression in human thymocytes. *J Biol Chem* 273:9544-9551, 1998
 44. Eng J, Kleinman WA, Singh L, Singh G, Raufman J-P: Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom: further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J Biol Chem* 267:7402-7405, 1992
 45. Thorens B, Porret A, Buhler L, Deng S-P, Morel P, Widmann C: Cloning and functional expression of the human islet GLP-1 receptor: demonstration that exendin-4 is an agonist and exendin-(9-39) is an antagonist of the receptor. *Diabetes* 42:1678-1682, 1993
 46. Botelho LH, Rothermel JD, Coombs RV, Jastorff B: cAMP analog antagonists of cAMP action. *Methods Enzymol* 159:159-172, 1988
 47. Hidaka H, Kobayashi R: Pharmacology of protein kinase inhibitors. *Annu Rev Pharmacol Toxicol* 32:377-397, 1992
 48. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M: K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinase. *Biochem Biophys Res Commun* 142:436-440, 1987
 49. Galsgaard ED, Gouilleux F, Groner B, Serup P, Nielsen JH, Billestrup N: Identification of a growth hormone-responsive STAT5-binding element in the rat insulin 1 gene. *Mol Endocrinol* 10:652-660, 1996
 50. Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, Montminy MR: Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol Cell Biol* 13:4852-4859, 1993
 51. Schwaninger M, Lux G, Blume R, Oetjen E, Hidaka H, Knepel W: Membrane depolarization and calcium influx induce glucagon gene transcription in pancreatic islet cells through the cyclic AMP-responsive element. *J Biol Chem* 268:5168-5177, 1993
 52. Reh fuss RP, Walton KM, Loriaux MM, Goodman RH: The cAMP-regulated enhancer-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. *J Biol Chem* 266:18431-18434, 1991
 53. Liu F, Thompson MA, Wagner S, Greenberg MR: Activating transcription factor-1 can mediate Ca²⁺ and cAMP-inducible transcriptional activation. *J Biol Chem* 268:6714-6720, 1993
 54. Inada A, Someya Y, Yamada Y, Ihara Y, Kubota A, Ban N, Watanabe R, Tsuda K, Seino Y: The cyclic AMP response element modulator family regulates the insulin gene transcription by interacting with transcription factor IID. *J Biol Chem* 274:21095-21103, 1999
 55. Gonzalez A, Montminy MR: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59:675-680, 1989
 56. Kieffer TJ, Heller RS, Leech CA, Holz GG, Habener JF: Leptin suppression of insulin secretion by the activation of ATP-sensitive K⁺ channels in pancreatic β -cells. *Diabetes* 46:1087-1093, 1997
 57. Sheng M, Thompson MA, Greenberg ME: CREB: a Ca²⁺-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* 252:1427-1430, 1991
 58. Du K, Montminy M: CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem* 273:32377-32379, 1998
 59. Xing J, Ginty DD, Greenberg M: Coupling of the RAS-MAPK pathway to gene activation by RSK-2, a growth factor-regulated CREB kinase. *Science* 273:959-963, 1996