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Exendin-4 as a Stimulator of Rat Insulin I Gene Promoter Activity via *bZIP*/CRE Interactions Sensitive to Serine/Threonine Protein Kinase Inhibitor Ro 31-8220

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Signal transduction properties of exendin-4 (Ex-4) underlying its ability to stimulate rat insulin I gene promoter (RIP1) activity were assessed in the pancreatic β-cell line INS-1. Ex-4 acted via glucagon-like peptide-1 receptors to stimulate RIP1 in a glucose-dependent manner, as measured in cells transfected with a −410-bp RIP1-luciferase construct (RIP1-Luc). The action of Ex-4 was independent of cAMP and PKA because it was not blocked by cotransfection with dominant-negative $G\alpha_s$, was unaffected by pretreatment with the membrane-permeant cAMP antagonist 8-Br-Rp-cAMPS, and remained apparent after treatment with PKA inhibitors H-89 or KT 5720. Similarly, cotransfection with a dominant-negative isoform of the type-2 cAMP-regulated guanine nucleotide exchange factor (*Epac2*) failed to alter the response to Ex-4. Ro 31-8220, a serine/threonine protein kinase inhibitor that targets PKC as as well as the 90-kDa ribosomal S6 kinase (RSK) and mitogen- and stress-activated protein kinase (MSK) family of cAMP response element-binding protein (CREB) kinases, blocked the stimulatory action of Ex-4 at RIP1-Luc. However, selective inhibition of PKC using K-252c, prolonged exposure to phorbol 1,2-myristate-13-acetate, or cotransfection with dominant-negative atypical PKC-ζ, was without effect. A-CREB, a dominant-negative inhibitor of basic region-leucine zipper transcription factors (*bZIPs*) related in structure to CREB, inhibited the action of Ex-4 at RIP1-Luc, whereas A-ATF-2 was ineffective. Similarly, introduction of deletions at the RIP1 cAMP response element (CRE), or truncation of RIP1 to remove the CRE, nearly abolished the action of Ex-4. Inactivating mutations introduced at the A4/A3 elements, binding sites for the glucose-regulated homeodomain transcription factor PDX-1, did not diminish the response to Ex-4, although a marked reduction of basal promoter activity was observed. The glucose-dependent stimulation of RIP1-Luc by Ex-4 was reproduced using a synthetic reporter (RIP1-CRE-Luc) incorporating multimerized CREs of the RIP1 nonpalindromic sequence 5′-TGACGTCC-3′. It is concluded that the *bZIP* and CRE-mediated stimulation of RIP1 by Ex-4 explains, at least in part, how this insulinotropic hormone facilitates transcriptional activity of the rat insulin I gene. (*Endocrinology* 143: 2303–2313, 2002)

TYPE 2 DIABETES MELLITUS is a disorder of blood glucose homeostasis in which there is insulin resistance accompanied by a diminished capacity of pancreatic β-cells to synthesize and secrete the blood glucose-lowering hormone insulin (1). Whereas for healthy individuals the primary stimulus for increased insulin biosynthesis and secretion is the nutrient glucose, the action of glucose at the β-cell is down-regulated, or largely absent, in type 2 diabetic subjects. Such observations have prompted a search for alternative insulinotropic agents, one of which is the blood glucose-lowering hormone glucagon-like peptide-1-(7–36)-amide (GLP-1) (2). GLP-1 acts as a β-cell glucose competence hormone, restoring the ability of β-cells to respond to glucose under conditions in which they are metabolically compromised (3, 4). This effect is measurable as an augmentation of pulsatile insulin secretion and a lowering of blood glucose concentration (5). GLP-1, or its structurally related analog exendin-4 (Ex-4), also acts as a trophic factor, stimulating β-cell neogenesis and proliferation (6, 7).

Actions of GLP-1 at the β-cell are mediated by the GLP-1 receptor (GLP-1-R) (8) and are manifest as increased insulin gene transcription (9, 10), stabilization of preproinsulin mRNA (11), increased translational biosynthesis of proinsulin (10, 11), and a facilitation of insulin exocytosis (12). The GLP-1-R couples to multiple G proteins (13) and activates signaling pathways for cAMP (8, 9), Ca^{2+} (14), PKA (15, 16), PKC (17), IP3 (18), and Ca^{2+}-calmodulin-regulated protein kinases (CaM-kinases) (19). The GLP-1-R also couples to MAPK (13, 20), PI3K (21), and hormone-sensitive lipase (22). How such signaling pathways interact with β-cell glucose metabolism to facilitate insulin biosynthesis and secretion remains poorly understood.

To elucidate the signal transduction pathway by which GLP-1 increases transcriptional activity of the insulin gene, we have used the INS-1 β-cell line that expresses the GLP-1-R and synthesizes and secretes insulin (23). We (24) reported that GLP-1 stimulates transcriptional activity of the rat insulin I gene promoter (RIP1), as assayed in INS-1 cells transfected with a −410-bp fragment of RIP1 fused to the coding sequence of firefly luciferase (RIP1-Luc). This action of GLP-1 appears to be mediated, at least in part, by the interaction of basic region leucine zipper transcription factors (*bZIPs*) with the RIP1 cAMP response element (CRE).

Abbreviations: ATF, Activating transcription factor; *bZIP*, basic region-leucine zipper transcription factor; CaM-kinase, Ca^{2+}-calmodulin-regulated protein kinase; CRE, cAMP response element; CREB, cAMP response element-binding protein; Ex-4, exendin-4; GLP-1, glucagon-like peptide-1-(7–36)-amide; GLP-1-R, GLP-1 receptor; HSA, human serum albumin; MEK, MAPK/ERK kinase; MSK, mitogen- and stress-activated protein kinase; PDX-1, pancreatic duodenal homeobox-1 transcription factor; RIP1, rat insulin I gene promoter; RIP1-Luc, RIP1-luciferase construct; RSK, 90-kDa ribosomal S6 kinase; SOM, somatostatin.

Unlike conventional CRE-mediated signaling, such as occurs at the somatostatin (SOM) gene promoter (25), no role for PKA as a mediator of GLP-1 action at RIP1 is demonstrable (24). It is important to note that the RIP1 CRE (5′-TGACGTCC-3′) deviates from the SOM CRE (5′-TGACGTCA-3′) by a single C/A nucleotide substitution (26, 27). This nonpalindromic nature of the RIP1 CRE confers to it novel signaling properties and a reduced affinity for the CRE-binding protein (CREB) (27). Indeed, available information indicates that unlike the SOM CRE, the relevant *bZIP* active at RIP1 may be related to but not identical with CREB (27). It is also noteworthy that the CRE of RIP1 overlaps at its 5′ end with a binding site for winged helix-loop-helix transcription factors, and at its 3′ end with a site for the transcription factor NF-Y (28). Therefore, *bZIP*/CRE interactions at RIP1 are likely to be influenced by neighboring transcription factors that modify the responsiveness of RIP1 to GLP-1.

Using the −410-bp RIP1-Luc reporter in conjunction with short-term (4 h) exposure to the GLP-1-R agonist Ex-4, we now demonstrate that signaling properties of the receptor are highly selective for the CRE. Promoter mutagenesis and truncation studies also indicate that the action of Ex-4 is unlikely to be mediated by PDX-1, a homeodomain transcription factor that binds to A elements of the promoter, and was previously reported to be regulated by GLP-1 (7, 21, 29–31). Using a synthetic reporter incorporating multimerized RIP1 CREs (RIP1-CRE-Luc) also demonstrated that the action of Ex-4 is facilitated by concentrations of glucose known to be insulinotropic in their own right. Finally, it is demonstrated that a signaling pathway sensitive to Ro 31-8220 most likely mediates stimulatory actions of Ex-4 at RIP1. Ro 31-8220 is a serine/threonine protein kinase inhibitor, and it exhibits selectivity not only for PKC but also the MAPK-activated kinases (RSKs) and mitogen/stress-activated kinases (MSK) that serve as CREB kinases (32).

Materials and Methods

Cell culture

INS-1 cells (passages 70–89) were cultured in RPMI 1640 containing 10 mM HEPES, 11.1 mM glucose, 10% FBS, 100 μU/ml penicillin G, 100 μg/ml streptomycin, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (23). HEK-293 cells stably transfected with the GLP-1-R (33) were cultured in DMEM containing 10% FBS. Cultures were maintained at 37 C in a humidified incubator gassed with 5% $C0_2$ and were subcultured once a week. Culture medium and additives were from Life Technologies, Inc. (Rockville, MD).

Plasmid DNA constructs

A −410-bp fragment of RIP1 was fused to the coding sequence of firefly luciferase in the pGL3-basic vector (Promega Corp., Madison, WI) to generate RIP1-Luc. The pGL3-control vector lacking RIP1 was completely insensitive to glucose or Ex-4. Truncations of RIP1 were obtained by restriction digests to generate −307-, −206-, and −166-bp derivatives (24). RIP1-CRE-Luc consisted of four RIP1 CREs of the sequence 5′-AGCC[TGACGTCC]GAG-3′ (CRE denoted in *parentheses*). The design of RIP1-CRE-Luc is identical with that of a commercially available luciferase reporter (SOM-CRE-Luc, Stratagene Corp., La Jolla, CA) incorporating multimerized somatostatin CREs of the sequence 5′-AGC-C[TGACGTCA]-GAG-3′. Therefore, RIP1-CRE-Luc emulates SOM-CRE-Luc in all respects with the exception of a single nucleotide substitution (C/A) within the CRE. RIP1-CRE-Luc was generated by synthesis of complementary synthetic oligonucleotides. The positive strand contained a *Hin*d-III overhang at its 5′ terminus and corresponded to 5′-AGCTTAGCC-[TGACGTCC]GAGAGCC[TGACGTCC]GAGAGCC[TGACGTCC]GAGAGCC-[TGACGTCC]GAGA-3. The (−) strand contained a *Bgl*-II overhang at its 5′ terminus and corresponded to 5′-GATCTCTC[GGACGTCA]GGCTCTC[GGACGTCA]GGCTCTC-[GGACGTCA]GGCTCTC[GGACGTCA]GGCTA-3′. The (+) and (−) strand oligos were annealed and ligated into *Hin*d-III and *Bgl*-II digested pLuc-MCS Cis-reporting cloning vector (Stratagene). The mutated A4/A3 RIP1-Luc construct (mt-A4/A3-Luc) contained four substitutions between positions −222 and −209 bp in the A4/3A element so that the wild-type sequence was converted from 5′-TTAATA-ATCTAATT-3′ to 5′-TTCATCATCTACCT-3′. EMSAs using recombinant PDX-1 demonstrated that the A4/A3 element mutated in this manner failed to bind PDX-1 (data not shown). The mt-A4/A3/Δ−182CRE-Luc construct contained an additional single inactivating deletion generated by mutagenesis at position −182 bp of RIP1. It fails to bind recombinant CREB (28). Design of dominant-negative plasmids was as described for A-CREB and A-ATF-2 (34), M1-CREB (35), K-CREB (36), $G\alpha_s$ (37), PKC-ζ (38), and the type 2 isoform of the cAMP-regulated guanine nucleotide exchange factor *Epac2* (39). DNA for transfections was purified using the Wizard DNA purification system (Promega Corp.).

Transfection protocol and luciferase assay for INS-1 cells

INS-1 cells grown to 40–60% confluence in Falcon 60-mm tissue culture dishes (Becton Dickinson and Co., Franklin Lakes, NJ) were transfected using commercially available reagents consisting of Lipofectamine Plus (Life Technologies, Inc.). Transfection efficiency was 10–15% as determined by use of a plasmid in which expression of enhanced green fluorescent protein (CLONTECH Laboratories, Palo Alto, CA) was placed under the control of the rat insulin II gene promoter. Cells to be transfected were rinsed twice in PBS, lifted by trypsinization, and suspended in serum-free culture medium containing DNA and transfection reagents (solution 1, Fig. 1A). The cells were plated onto 96-well cell culture plates (Costar 3610, Corning, Inc., Acton, MA) at a volume of 100 μl of cell suspension per well containing 200 ng RIP1-Luc and approximately 5×10^4 cells. INS-1 cells were exposed to this transfection cocktail for 16 h. The transfection cocktail was then removed and replaced with normal cell culture medium (solution 2, Fig. 1A). After a 7-h equilibration in culture medium, the solution was replaced with solution 3 (Fig. 1A) composed of RPMI 1640 containing 2.8 mM glucose and 0.1% human serum albumin (HSA, fraction V, Sigma, St. Louis, MO). After overnight incubation, cells were then exposed to test solution 4 (Fig. 1A) composed of RPMI 1640 containing 11.1 mM glucose, 0.1% HSA, and indicated concentrations of Ex-4 or exendin-(9–39). After a 4-h exposure to solution no. 4, cells were lysed and assayed for luciferase-catalyzed photoemissions using a luciferase assay kit (Tropix, Bedford, MA) and a dual-injection port luminometer allowing automated application of ATP and luciferin solutions (model TR-717, Perkin-Elmer Corp. PE Applied Biosystems, Foster City, CA). To verify that changes of luciferase activity were conferred specifically by the insulin gene promoter, ratiometric assays (DLR assay, Promega Corp.) were performed such that RIP1-Luc was cotransfected with a plasmid-driving expression of *Renilla* luciferase under the control of a constitutively active promoter. It was demonstrated that levels of *Renilla* luciferase activity were unaffected by the treatments described here. Therefore, values of RIP1-Luc activity are expressed as relative light units for firefly luciferase alone. All experiments were carried out in triplicate. Statistical analysis was performed using the ANOVA test combined with Fisher's protected least significant difference test.

Sources and preparation of reagents

H-89, KT 5720, KN-93, SB 203580, PD 98059, U0126, genistein, lavendustin A, wortmanin, LY 294002, K-252c, Ro 31-8220, PP2, and phorbol 1,2-myristate-13-acetate were from Calbiochem (La Jolla, CA). 8-Br-Rp-cAMPS was from BioLog Life Sciences (La Jolla, CA). Ex-4, exendin-(9–39), and nimodipine were from Sigma. Ex-4 was prepared in RPMI 1640 containing 0.1% HSA to protect against adherence of the peptide to glass or plastic surfaces.

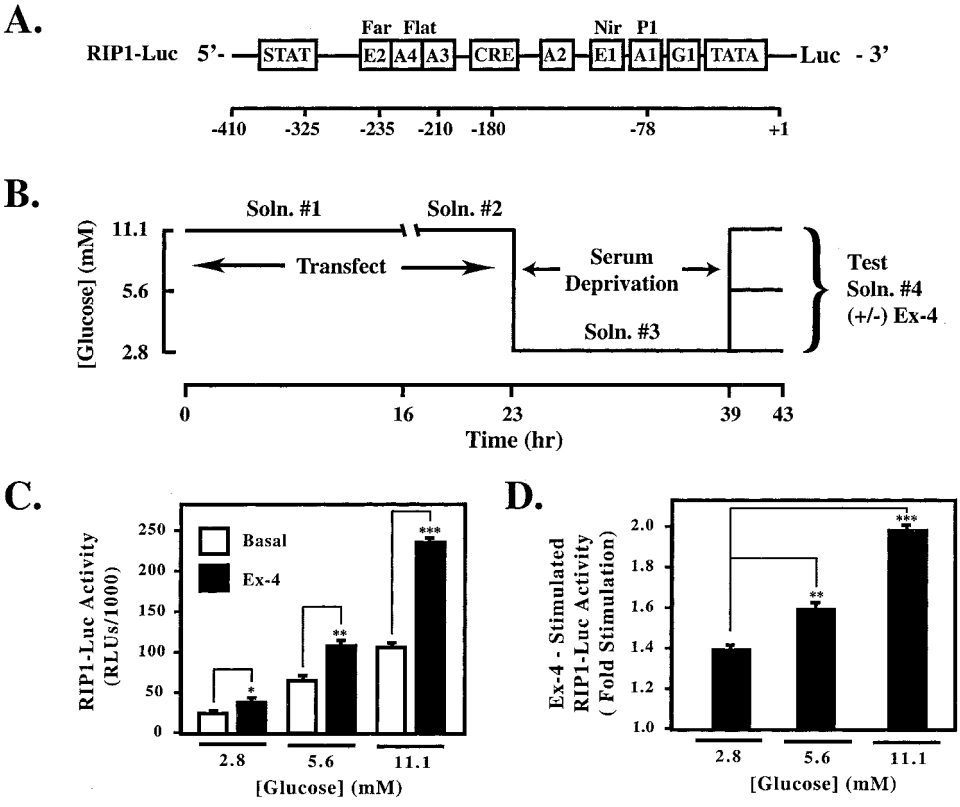

FIG. 1. A, Design of RIP1-Luc incorporating regulatory elements (*boxes*) found within −410 bp of the transcription start. Numbering is not drawn to scale. B, Experimental design for transfection, serum deprivation, and assessment of Ex-4 action. For the composition of solutions, see *Materials and Methods.* C, Stimulation by D-glucose and Ex-4 (10 nM, here and in subsequent figures) of luciferase expression in INS-1 cells transfected with RIP1-Luc. Luciferase activity of INS-1 cell lysates is expressed as relative light units. Statistical significance is expressed relative to values of RIP1-Luc activity obtained in the absence of Ex-4. (*, $P < 0.01$; **, $P < 0.005$; ***$P < 0.001$.) D, Glucose dependence of Ex-4 (10 nM) action expressed as fold-stimulation relative to basal promoter activity. (**, $P < 0.005$; ***, $P < 0.001$.) Each experiment illustrated was repeated a minimum of four times, here and in all subsequent figures.

Results

Analysis of GLP-1-R-mediated signal transduction by use of RIP1-Luc

Signal transduction properties of the GLP-1-R were evaluated in INS-1 cells transfected with RIP1-Luc (Fig. 1A). The experimental protocol involved a three-step solution change in which INS-1 cells were transfected, serum deprived, and exposed to a step-wise increase of glucose concentration in the presence or absence of the GLP-1-R agonist Ex-4 (Fig. 1B). Basal activities of RIP1-Luc were stimulated by glucose over a concentration range of 2.8–11.1 mM of the sugar (Fig. 1C). Under these conditions, a 4-h exposure to 10 nM Ex-4 increased RIP1-Luc activity in a glucose-dependent manner (Fig. 1, C and D).

bZIP/CRE interactions underlie stimulatory effects of Ex-4 at RIP1-Luc

A major role for the RIP1 CRE as a downstream target of GLP-1-R-mediated signal transduction was indicated by the marked reduction of Ex-4 responsiveness after introduction of inactivating Δ-182 or Δ-183/180 deletions at the CRE (Fig. 2A). Furthermore, the stimulatory action of Ex-4 at RIP1-Luc appeared to be mediated by a *bZIP* transcription factor, possibly from the CREB family. This conclusion was supported by the observation that the action of Ex-4 was suppressed by cotransfection of INS-1 cells with dominant-negative A-CREB (Fig. 2B). A-CREB is a genetically engineered isoform of CREB that dimerizes via a leucine zipper and an acidic extension to prevent binding of endogenous *bZIPs* to the CRE (34). In contrast, dominant-negative A-ATF-2 was without effect (data not shown, n = three experiments). ATF-2 is a *bZIP* previously reported to mediate stimulatory effects of Ca^{2+} and CaM-kinase-IV at the human insulin gene promoter (40).

To investigate in greater detail the nature of the *bZIP* active at the CRE of RIP1, two additional dominant-negative CREB isoforms were tested (Fig. 3A). M1-CREB binds to the CRE of cAMP-responsive gene promoters, competes with endogenous *bZIPs* for the CRE and is unresponsive to PKA because of the conversion of the P-box serine residue to alanine (35). K-CREB contains a lysine-to-leucine substitution in the DNA-binding domain of CREB, does not bind the CRE, but dimerizes with endogenous *bZIPs*, thereby blocking their action at the CRE (36). In INS-1 cells transfected with RIP1-Luc, neither M1-CREB nor K-CREB inhibited stimulatory actions of Ex-4 at the insulin gene promoter (Fig. 3A). However, both M1-CREB and K-CREB were effective inhibitors of Ex-4 action when INS-1 cells were transfected with a synthetic reporter (SOM-CRE-Luc) incorporating multimerized CREs of the somatostatin gene promoter (Fig. 3C). It can be concluded that the nonpalindromic nature of the RIP1 CRE (TGACGTCC) confers to it signaling properties not characteristic of the SOM CRE (TGACGTCA). Furthermore, the relevant *bZIP* active at the CRE of RIP1, although being sensitive to inhibition by A-CREB, is not necessarily identical with CREB.

Assessment of a role for the A4/A3 element as a mediator of Ex-4 action

An emerging body of evidence suggests that the stimulatory action of GLP-1 at RIP1 might be mediated not only by the CRE but by A elements of the promoter for which the

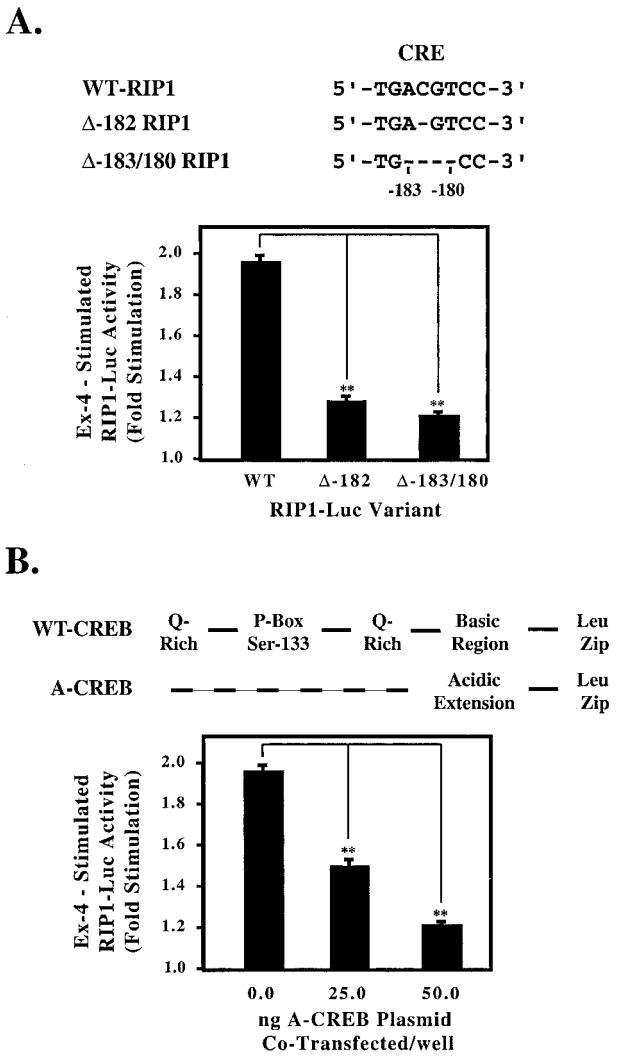

FIG. 2. Analysis of CRE and *bZIP*-mediated signal transduction. A, RIP1-Luc was modified to incorporate Δ-182 and Δ-183/180 inactivating deletions as indicated. The stimulatory action of Ex-4 was then assessed in cells transfected with wild-type (WT) RIP1-Luc, Δ-182 RIP1-Luc, or Δ-183/180 RIP1-Luc. (**, $P < 0.005$). B, Molecular structure of WT-CREB and A-CREB illustrating the glutamine-rich regions (Q-Rich), the phosphorylation box (P-Box), the DNA-binding domain (basic region), and the leucine zipper (Leu Zip). A-CREB contains an acidic extension and Leu ZIP that interacts with the basic region and Leu Zip of endogenous *bZIPs*. Cotransfection with A-CREB suppressed the stimulatory action of Ex-4 at RIP1-Luc. (**, $P < 0.005$.) Control empty vector expression plasmid was added to each transfection cocktail when appropriate to assure that all transfections contained equivalent amounts of DNA.

homeodomain transcription factor PDX-1 exhibits high DNA-binding affinity. We found that inactivating mutations introduced into the A4/A3 (Flat) element (Fig. 4A; plasmid designated as mt-A4/A3-Luc) produced a dramatic reduction of basal RIP1-Luc activity, as detected using INS-1 cells equilibrated in 11.1 mM glucose (Fig. 4B). Furthermore, when transfected with mt-A4/A3-Luc, a step-wise increase of glucose concentration from 2.8 to 11.1 mM produced little or no increase of promoter activity (data not shown). These findings indicate that, as expected, the A4/A3 element plays a major role as a determinant of RIP1-Luc glucose responsiveness (41). A small but statistically significant further decrease of basal promoter activity was also observed when mt-A4/A3-Luc was modified to introduce a Δ-182 inactivating deletion at the RIP1-CRE (Fig. 4A; plasmid designated as mt-A4/A3/-182ΔCRE-Luc). Such observations are consistent with a major role of the A4/A3 element and a small role for the CRE as determinants of glucose-dependent RIP1-Luc activity. Despite these findings, it is notable that the stimulatory action of Ex-4 at RIP1-Luc was increased, not decreased, by mutation of the A4/A3 element (Fig. 4C). In marked contrast, the action of Ex-4 was suppressed by introduction of the Δ-182 CRE deletion into mt-A4/A3-Luc (Fig. 4C). It may be concluded that it is the CRE rather than the A4/A3 element that serves as the primary target for Ex-4 insulinotropic action under the experimental conditions described here.

Truncation to remove the CRE abrogates stimulatory actions of Ex-4

Studies conducted with mt-A4/A3-Luc do not necessarily rule out a possible stimulatory action of Ex-4 at more proximal A elements of RIP1. These effects might be mediated by the E1/A1(Nir-P1) or A2 elements at which binding of PDX-1 does occur (41). To investigate this possibility, truncations of RIP1 were performed to generate luciferase reporters that contain or lack the A4/A3 and CRE elements but retaining the E1/A1 and A2 elements. These reporters are designated −307RIP1-Luc, −206RIP1-Luc, and −166RIP1-Luc, respectively (Fig. 5A). When cells were equilibrated in 11.1 mM glucose, basal activities of −206RIP1-Luc and −166RIP1-Luc were reduced to 7.0% and 4.7% of that measured using −307RIP1-Luc (Fig. 5B). These findings confirm our own observations obtained with mt-A4/A3-Luc and mt-A4/A3/-182ΔCRE-Luc, whereby the A4/A3 and CRE elements were found to play major and minor roles, respectively, as mediators of glucose-dependent promoter activity (Fig. 4B). Of particular interest was the additional observation that the action of Ex-4 at RIP1 was not inhibited when the promoter was truncated to remove the A4/A3 elements (Fig. 5C, compare −307RIP1-Luc and −206RIP1-Luc). Furthermore, truncation of RIP1 to remove the CRE nearly abolished the action of Ex-4 (Fig. 5C, compare −206RIP1-Luc and −166RIP1-Luc), thereby confirming its major importance as a downstream target of GLP-1 receptor-mediated signal transduction.

RIP1-CRE-Luc as a reporter for GLP-1-R-mediated signaling at the CRE

Additional evidence demonstrating a major stimulatory action of Ex-4 at the CRE was provided by our use of a synthetic reporter (RIP1-CRE-Luc) containing multimerized RIP1 CREs fused to the coding sequence of luciferase (Fig. 6A). The nonpalindromic CRE of RIP1 deviates from the palindromic CRE of the somatostatin gene promoter by a single C/A nucleotide substitution (Fig. 6A, see *underlined* nucleotide). This alteration confers novel cAMP- and PKA-independent signaling properties to the CRE of RIP1 (24). When transfected into INS-1 cells, basal activities of

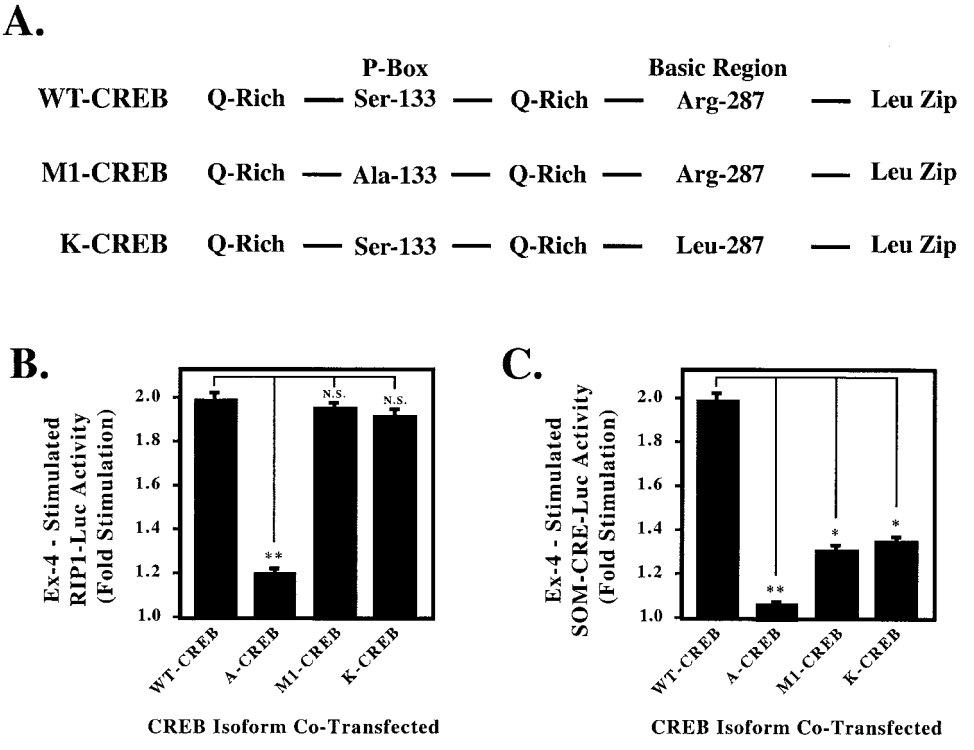

FIG. 3. A, Schematic illustration of the domain structure of CREB isoforms used in this study. B, Effects of CREB isoform overexpression, as evaluated in INS-1 cells transfected with RIP1-Luc. C, Effects of CREB isoform overexpression, as evaluated in INS-1 cells transfected with SOM-CRE-Luc. For B and C, cotransfection was achieved using 200 ng/well of luciferase reporter in combination with 50 ng/well CREB isoform expression plasmid.

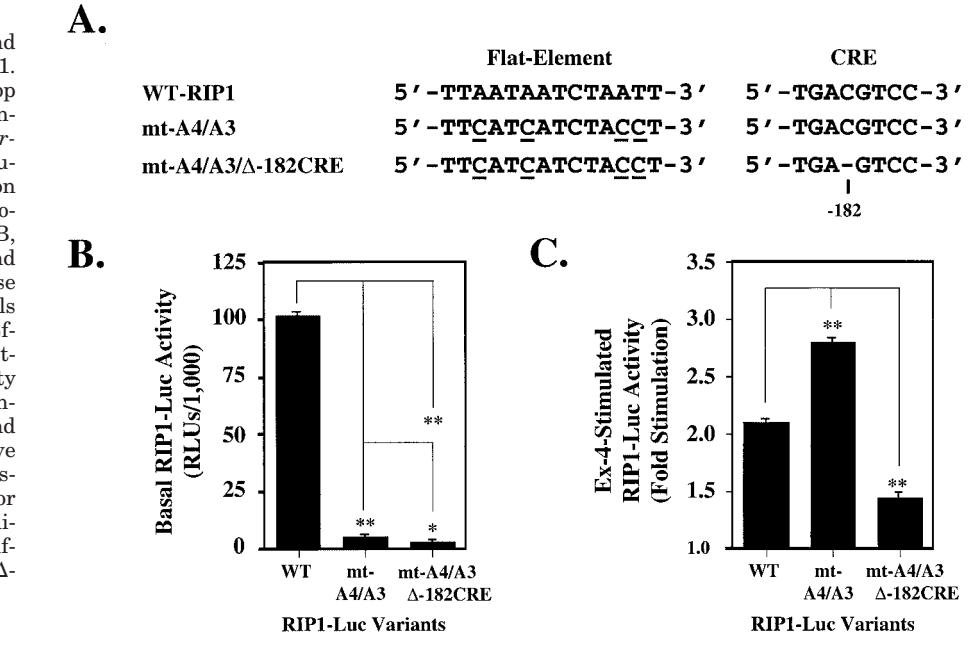

FIG. 4. Analysis of A4/A3 (flat) element and CRE-mediated signal transduction at RIP1. A, Cells were transfected with the −410-bp wild-type RIP1-Luc (WT-RIP1) or variants incorporating inactivating mutations (*underlined*) of the A4/A3 element (mt-A4/A3) or mutations of the A4/A3 element in combination with an inactivating Δ-182 deletion introduced at the CRE (mt-A4/A3/Δ−182CRE). B, Effects of WT-RIP1-Luc, mt-A4/A3-Luc, and mt-A4/A3/Δ−182CRE-Luc on basal luciferase activity measured after a 4-h exposure of cells to medium containing 11.1 mM glucose. C, Effects of WT-RIP1-Luc, mt-A4/A3-Luc, and mt-A4/A3/Δ−182CRE-Luc on luciferase activity measured after 4-h exposure to medium containing 11.1 mM glucose and Ex-4. For B and C, statistical significance is indicated relative to luciferase activity measured in cells transfected with WT-RIP1-Luc (**, $P < 0.001$). For B alone, statistical significance is also indicated when comparing basal values of luciferase activity for mt-A4/A3 and mt-A4/A3/Δ-182CRE (*, $P < 0.05$).

RIP1-CRE-Luc were stimulated by an elevation of glucose concentration within the range of 2.8–11.1 mM (Fig. 6B). RIP1-CRE-Luc was also stimulated by Ex-4 in a glucose-dependent manner (Fig. 6C). Dose-response analysis demonstrated that the maximal stimulatory action of Ex-4 at RIP1-CRE-Luc was 4.8-fold in excess of basal promoter activity, whereas RIP1-Luc was stimulated 2.0-fold when tested in the presence of 11.1 mM glucose (Fig. 6D). Under these conditions, the 50% effective concentration for Ex-4 stimulatory action at RIP1-CRE-Luc approximated 0.8 nM (Fig. 6D).

Ex-4 acts via a PKA-independent signaling pathway sensitive to Ro 31-8220

We previously reported that the stimulatory action of GLP-1 at RIP1-Luc occurs in a cAMP- and PKA-independent manner (24). This was also the case for Ex-4 because its effect at RIP1-Luc was blocked by the GLP-1-R antagonist exendin-(9–39) (42, 43) but was unaffected by the cAMP antagonist 8-Br-Rp-cAMPS (44) or the PKA inhibitors H-89 or KT 5720 (45) (Fig. 7A, Table 1). As a positive control, the efficacy of 8-Br-Rp-cAMPS and H-89 was confirmed using HEK 293 cells stably transfected with

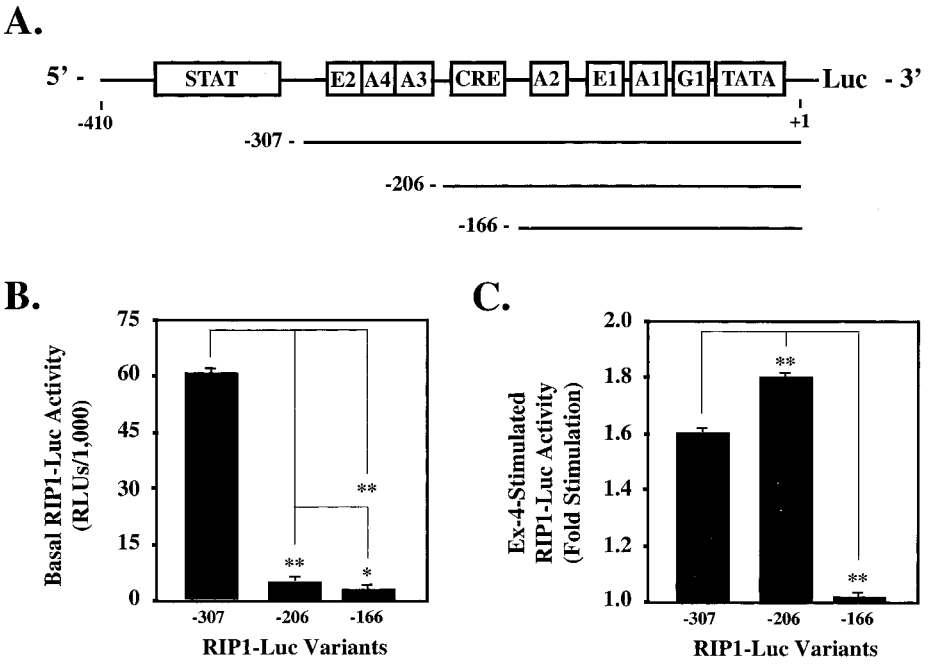

FIG. 5. Effects of RIP1 truncation on basal and Ex-4-stimulated luciferase activity. A, The −410-bp RIP1-Luc construct was truncated to generate −307-, −206-, and −166-bp derivatives that lack the STAT, E2/A4/A3, and CRE regulatory elements, respectively. B, Basal luciferase activity measured in the presence of 11.1 mM glucose was markedly diminished when −307-bp RIP1-Luc was truncated to remove the E2/A4/A3 regulatory elements (compare −307 and −206 bp RIP1-Luc). Truncation of −206 bp RIP1-Luc to remove the CRE resulted in a small, but statistically significant additional decrease of basal luciferase activity (compare −206- and −166-bp RIP1-Luc). C, The stimulatory action of Ex-4 was not inhibited by truncation of −307-bp RIP1-Luc to remove the E2/A4/A3 regulatory elements, whereas very little effect of Ex-4 was observed after truncation of −206-bp RIP1-Luc to remove the CRE. For B and C, statistical significance is indicated relative to luciferase activity measured in cells transfected with the −307-bp RIP1-Luc variant (**, $P < 0.001$). For B alone, statistical significance is also indicated when comparing basal values of luciferase activity for −206- and −166-bp RIP1-Luc (*, $P < 0.05$).

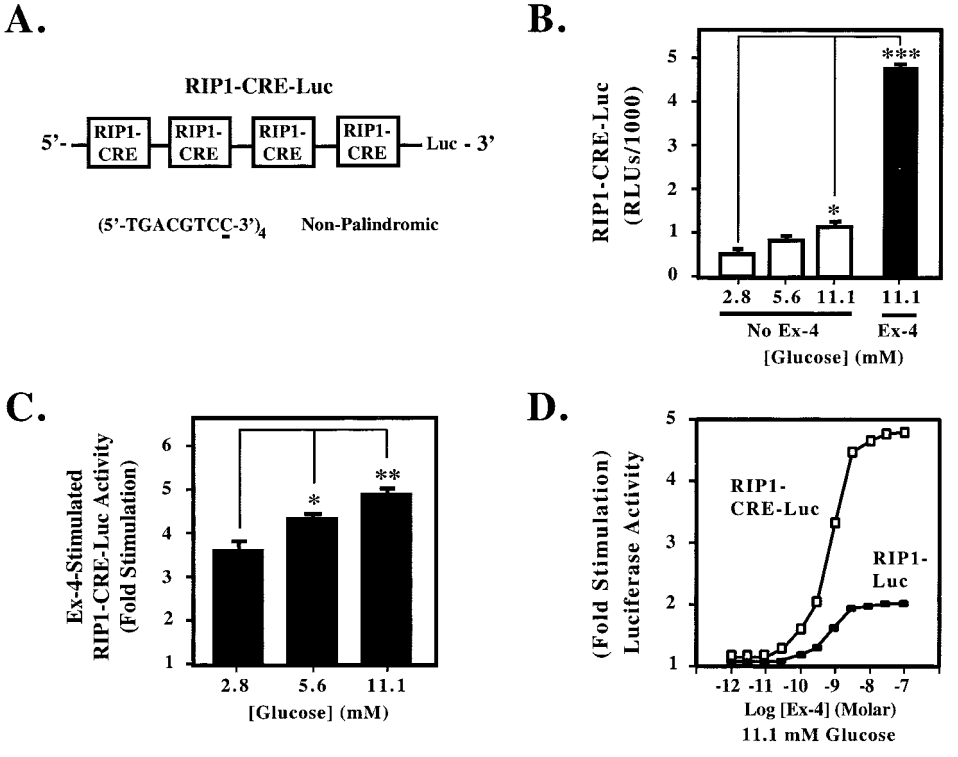

FIG. 6. A, Design of RIP1-CRE-Luc incorporating multimerized CREs of the sequence 5′-TGACGTCC-3′. The RIP1-CRE differs from that found in the somatostatin gene CRE (5′-TGACGTCA-3′) by a single nucleotide substitution (*underlined*). B, RIP1-CRE-Luc activity measured after a 4-h equilibration of cells in medium containing 2.8, 5.6, or 11.1 mM glucose (*open bars*) or after equilibration in medium containing 11.1 mM glucose and Ex-4 (*solid bar*). C, The stimulatory action of Ex-4 at RIP1-CRE-Luc was glucose dependent over a concentration of 2.8–11.1 mM of the sugar. D, Dose-response analysis comparing the stimulatory action of Ex-4 at RIP1-CRE-Luc and RIP1-Luc. For B and C, statistical significance is indicated relative to values of luciferase activity measured in the presence of 2.8 mM glucose alone. (*, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.001$.)

the GLP-1-R, whereby it was demonstrated that these agents block stimulatory actions of Ex-4 at SOM-CRE-Luc (Fig. 7B). More detailed studies using INS-1 cells transfected with RIP1-Luc demonstrated that the stimulatory action of Ex-4 was not reduced by inhibitors of CaM-kinase (KN-93), [MAPK/ERK kinase (MEK) (PD 98059, U10126)], MAPK (SB 203580, PD 98059, U0126), tyrosine kinases (genistein, lavendustin A, PP2), or PI3K (wortmanin, LY 294002) (Tables 1 and 2). Furthermore, the action of Ex-4 was unaffected by treatment with the L-type voltage-dependent Ca^{2+} channel blocker nimodipine (1 μM; n = three experiments, data not shown) or by cotransfection with dominant-negative $G\alpha_s$ or a dominant-negative type-2 cAMP-regulated guanine nucleotide exchange factor (*Epac2*) (n = three experiments each, data not shown). However, the action of Ex-4 at RIP1-Luc was nearly abolished by treatment with Ro 31-8220 (Table 1) (50% inhibitory concentration 0.6 μM).

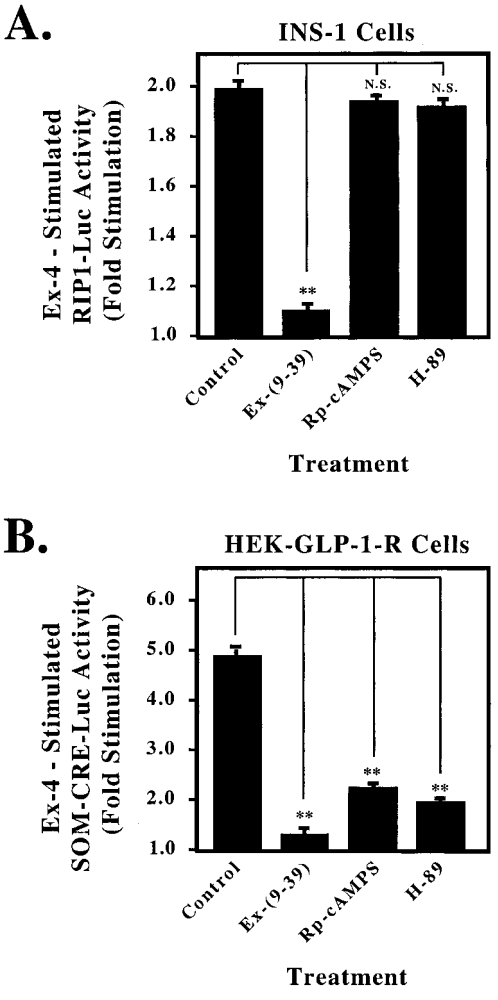

FIG. 7. Pharmacological properties of Ex-4 action. A, INS-1 cells were transfected with RIP1-Luc and subjected to the experimental protocol illustrated in Fig. 1B. Ex-(9–39) (1 μM), 8-Br-Rp-cAMPS (200 μM), H-89 (10 μM), and Ro 31-8220 (10 μM) were administered for 30 min at 37 C before addition of Ex-4 (10 nM) and were also present throughout the subsequent 4-h exposure to Ex-4. These concentrations of 8-Br-Rp-cAMPS and H-89 were chosen because they were previously shown to be fully effective antagonists of cAMP signaling in INS-1 cells (14, 24). B, HEK 293 cells stably transfected with the human GLP-1-R (HEK-GLP-1-R cells) were transfected with a synthetic reporter incorporating multimerized somatostatin gene promoter CREs (SOM-CRE-Luc). The action of Ex-4 was then tested as for INS-1 cells except that the cells were continually exposed to DMEM containing 11.1 mM glucose.

Although Ro 31-8220 has been reported to inhibit PKC as well as the CREB kinases RSK and MSK (32), the action of Ex-4 at RIP1-Luc was entirely unaffected by treatment with PKC inhibitor K-252c (Table 1). Similarly, down-regulation of PKC by prolonged phorbol ester treatment (phorbol 1,2-myristate-13-acetate, 500 nM, 16 h) and the response to Ex-4 altered by cotransfection with dominant-negative atypical PKC-ζ (n = three experiments, data not shown) were without effect.

Discussion

cAMP- and PKA-independent signaling properties of Ex-4 at RIP1

Signal transduction properties of the RIP1 CRE resemble in some ways those of the more conventional somatostatin gene CRE in that both are sensitive to cAMP-elevating agents such as forskolin (26–28). Therefore, it is surprising that GLP-1, a known stimulator of cAMP production, reportedly acts in a cAMP- and PKA-independent manner to stimulate RIP1 (24). This prior conclusion is reinforced by our finding that the stimulatory action of Ex-4 at RIP1 is unaffected by dominant-negative $G\alpha_s$, a G protein that links the GLP-1-R to adenylyl cyclase. Furthermore, treatment with the cAMP antagonist 8-Br-Rp-cAMPS fails to block the action of Ex-4 as is also the case for H-89 and KT 5720, two inhibitors of PKA. It might be argued that Ex-4 targets the CRE in a PKA-independent manner via actions mediated by cAMP-binding proteins known as cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs, *Epac*). However, although cAMPGEFs are expressed in β-cells and INS-1 cells (39, 46, 47), we found the action of Ex-4 at RIP1 to be unaffected by overexpression of dominant-negative cAMPGEF-II under conditions of transfection previously shown by our laboratory to abrogate stimulatory effects of Ex-4 on INS-1 cell Ca^{2+} signaling (14).

CRE mediates glucose-dependent stimulatory actions of Ex-4

The GLP-1-R is functionally coupled to the insulin gene promoter in a manner dependent on prior equilibration of cells in physiologically relevant concentrations of glucose. We found that the action of Ex-4 at RIP1 exhibits glucose dependence over a concentration range of 2.8–11.1 mM of the sugar. These same concentrations of glucose augment the action of Ex-4 when evaluating its action specifically at the CRE, as measured using RIP1-CRE-Luc. Therefore, as is the case for many actions of GLP-1 in β-cells (2–5), the CRE of RIP1 is regulated by GLP-1 in a glucose-dependent manner. This finding may reflect a functional interaction of the GLP-1-R with glucose-regulated protein kinases. Alternatively, the receptor may target transcription factors that bind at, or adjacent to, the CRE in a glucose-dependent manner, as previously suggested for the CRE modulator protein (48).

A bZIP structurally related to CREB is active at the RIP1 CRE

The nature of the transcription factor active at the CRE of RIP1 remains to be elucidated. It was previously reported that the action of forskolin at RIP1 was down-regulated by cotransfection of HIT-T15 cells with dominant-negative K-CREB (28). However, no effect of M1-CREB was observed when assessing stimulatory actions of GLP-1 in INS-1 cells (24). Such findings suggest that the RIP1 CRE might respond to GLP-1 by interacting with a *bZIP* related in structure but not identical with CREB (24). Indeed, previous studies demonstrated that the CRE of RIP1 has a low affinity for CREB (28). Furthermore, in HIT-T15 cells, the RIP1 CRE binds a nuclear factor of 43 kDa (26) immunologically distinct from CREB (27).

In the present study, we found the action of Ex-4 at RIP1 to be blocked by A-CREB, whereas it was unaffected by M1-CREB or K-CREB. The failure of M1-CREB to act is understandable if it exhibits low affinity for the RIP1 CRE as is the case for wild-type CREB (28). In contrast, the failure of

TABLE 1. Effects of PKA, CaM-kinase, and PKC inhibitors

Test compound	Specificity	Concentration tested	Basal activity (% of control)	Fold stimulation by Ex-4
Control	—	—	100	2.0
H-89	PKA	1–10 μM	99	2.0
KT 5720	PKA	1–10 μM	98	1.9
KN-93	CaM-Kinase	1–10 μM	70[a]	3.0[a]
K-252c	PKA + PKC	0.1–1 μM	77[b]	2.1
Ro 31-8220	PKC, RSK, MSK	0.1–10 μM	58[a]	1.1[a]

RIP1-Luc activity in the absence (Basal activity) or presence (Fold stimulation) of Ex-4 (10 nM) was determined over the concentration ranges of kinase inhibitors indicated. Values for basal and fold stimulation are those determined in the presence of the highest concentration of inhibitor indicated. Statistical significance is indicated relative to control (no inhibitor present).

[a], $P \leq 0.001$ or [b], $P \leq 0.01$. Each experiment was performed a minimum of three times.

TABLE 2. Effects of MAPK, Tyr-K, and PI3K inhibitors

Test compound	Specificity	Concentration tested	Basal activity (% of control)	Fold stimulation by Ex-4
Control	—	—	100	2.0
SB 203580	p38 MAPK	0.1–40 μM	79[a]	3.2[b]
PD 98059	MEK	1–50 μM	54[b]	1.8
U0126	MEK	1–10 μM	52[b]	3.1[b]
Genistein	Tyr-kinase	1–10 μM	149[a]	2.1
Lavendustin A	Tyr-kinase	0.1–1 μM	103	2.0
PP2	src tyr kinase	0.5–120 nM	204[b]	1.8
Wortmanin	PI3K	1 nM–1 μM	34[b]	2.5[a]
LY 294002	PI3K	0.5–100 μM	73[a]	1.9

RIP1-Luc activity in the absence (Basal activity) or presence (Fold stimulation) of Ex-4 (10 nM) was determined over the concentration ranges of kinase inhibitors indicated. Values for basal and fold stimulation are those determined in the presence of the highest concentration of inhibitor indicated. Statistical significance is indicated relative to control (no inhibitor present).

[a], $P \leq 0.01$ or [b], $P \leq 0.001$. Each experiment was performed a minimum of three times.

K-CREB to act is understandable if it fails to dimerize with an endogenous *bZIP* active at the CRE. Although K-CREB and A-CREB posses structurally related leucine zipper dimerization domains, K-CREB possesses a modified basic region, whereas A-CREB contains an acidic extension at its N terminus. This region of A-CREB interacts with the basic region of CREB, thereby preventing its binding to the CRE (34). Given that A-CREB is effective in our assays, it appears likely that it exerts its action, at least in part, by interacting with the basic region of an endogenous transcription factor not recognized by the leucine zipper of K-CREB.

Differential signaling properties of the rat and human insulin gene promoters

Studies of the human insulin gene promoter demonstrated the presence of four nonidentical CREs (48, 49), the activity of which is repressed by CREB but stimulated in a Ca^{2+} and CaM-kinase IV-dependent manner by activating transcription factor-2 (ATF-2; a *bZIP* more distantly related to CREB) (40). In the present study, we found that cotransfection with dominant-negative A-ATF-2 failed to block stimulatory actions of Ex-4 at RIP1. Similarly, treatment of INS-1 cells with nimodipine, a blocker of GLP-1-R-mediated Ca^{2+} signaling (14), or KN-93, an inhibitor of CaM-kinases (45), failed to inhibit the responsiveness of RIP1 to Ex-4. Cotransfection with dominant-negative A-CREB, however, blocked the stimulatory action of Ex-4, measured not only at RIP1-Luc but also at the multimerized CRE contained within RIP1-CRE-Luc (Holz, G. G., unpublished observations). It may be concluded that the actions of Ca^{2+} and ATF-2 observed at the human insulin gene promoter are unlikely to explain stimulatory actions of Ex-4 described here for RIP1. Instead, the weight of current evidence indicates that the action of Ex-4 at RIP1 is mediated by a *bZIP* more closely related in structure to CREB (CREM, ATF-1). In this regard, it is noteworthy that multiple isoforms of CREM have been reported to upregulate human insulin gene promoter activity (48).

Ro 31-8220 as an inhibitor of GLP-1-R-mediated signal transduction

We found that the bisindolylmaleimide Ro 31-8220 fully suppresses stimulatory actions of Ex-4 at RIP1 in a dose-dependent manner. This is significant because it is the first identified protein kinase inhibitor that interferes with GLP-1-R-mediated signal transduction at the rat insulin I gene promoter. Ro 31-8220 was originally thought to be a selective inhibitor of PKC but is now recognized to inhibit CREB kinases MSK and RSK as well as glycogen synthase kinase and p70 ribosomal protein S6 kinase (32). Previous *in vitro* assays of kinase activity demonstrated that the IC_{50} for Ro 31-8220 inhibitory action is in the range of 3–38 nM (32). This value is not incompatible with our estimate of an IC_{50} of 600 nM obtained in living cells in which access of Ro 31-8220 to the cytosol is limited by the plasma membrane. However, more detailed studies examining the specificity with which Ro 31-8220 acts in the INS-1 cell system awaits identification of the protein kinase(s) targeted by the GLP-1-R. Despite this caveat, the selectivity with which Ro 31-8220 acts is highlighted by our finding that the action of Ex-4 is not inhibited by micromolar concentrations of kinase inhibitors known to target PKA, CaM-kinases, MEK, MAPK, tyrosine kinases, and PI3K.

Interpretation of studies using protein kinase inhibitors

Studies presented here support a prior report (50) that treatment with the p38 MAPK inhibitor SB 203580 augments, rather than inhibits, effects of GLP-1 at RIP1. Therefore, although the p38 MAPK signaling pathway is reported to mediate stimulatory effects of glucose on insulin gene transcription (51, 52), it may instead serve to counteract stimulatory actions of GLP-1 at RIP1 (50). We also found that the MEK inhibitors PD 98059 and U0126 produced a substantial decrease of basal RIP1 activity, consistent with the suggested role of MEK as a coupling factor linking glucose metabolism to insulin gene transcription (20, 53, 54). However, although GLP-1 is reported to activate ERK (13, 20), the effector of MEK, the action of Ex-4 at RIP1 is resistant to PD 98059 and augmented by U0126. Therefore, a role for the MEK/ERK signaling pathway as a mediator of Ex-4 action remains unsubstantiated. Taken as a whole, these findings using MAPK inhibitors suggest that a beneficial action of Ex-4 to stimulate insulin gene transcription might be preserved under conditions in which β-cell glucose signaling is disrupted, as does occur in type 2 diabetes mellitus.

Additional studies using the PI3K inhibitors wortmanin and LY 294002 demonstrate them to be inhibitors of basal RIP1 activity, as expected given that PI3K is reported to mediate stimulatory effects of glucose on insulin gene transcription (55). However, once again, we found a distinction between the actions of glucose and Ex-4 in that the GLP-1-R apparently couples to RIP1 in a PI3K-independent manner. Moreover, inhibitors of receptor tyrosine kinases (genistein, lavendustin A), or src tyrosine kinase (PP2) fail to abrogate stimulatory actions of Ex-4 at RIP1. These findings demonstrate that the increase of RIP1 activity measured on GLP-1-R stimulation is not secondary to insulin secretion, with concomitant activation of an autocrine signaling mechanism in which INS-1 cell insulin autoreceptors couple to RIP1 in a stimulatory manner (56).

It is also noteworthy that no role for PKC as a mediator of Ex-4 action is demonstrable. Although the GLP-1-R is reported to couple to atypical PKC-ζ (17), we found the action of Ex-4 to be unaffected by a PKC inhibitor (K-252c), by down-regulation of PKC using phorbol ester treatment or by overexpression of dominant-negative PKC-ζ. However, Ro 31-8220 does block the action of Ex-4, thereby indicating that its effect is independent of PKC and more probably mediated by an alternative signaling system, such as is mediated by MSK or RSK. Given that RSK is an H-89-insensitive CREB kinase inhibited by Ro 31-8220 (32), and given the fact that Ex-4 exerts its stimulatory action via a *bZIP* related in structure to CREB, the weight of current evidence suggests a possible role for RSK-like kinases as mediators of GLP-1 action. Such a role for RSK would also be compatible with recent reports that it serves as a modulator of CREB-binding protein function at the CRE (57).

A-element-independent actions of Ex-4 at RIP1

Based on studies of RIN 1046–38 and INS-1 cells, there is reason to believe that the transcription factor PDX-1 serves as a downstream effector of the GLP-1-R. PDX-1 translocates to the nucleus in response to GLP-1, an effect mediated by PKA (31). Levels of PDX-1 mRNA are increased by GLP-1 (29), and binding of PDX-1 to A1 elements of the rat insulin I and II gene promoters is facilitated (21, 29). The transactivation function of PDX-1 is stimulated by GLP-1 (30), and GLP-1 also stimulates a Luc reporter incorporating multimerized E2/A4/A3 elements (30). Given that PDX-1 mediates stimulatory effects of glucose at RIP1 (41), it is reasonable to speculate that it might also mediate stimulatory actions of GLP-1 at the promoter. However, this hypothesis was not tested in a rigorous manner by examining the functional consequences of RIP1 mutagenesis or truncation. We found that inactivating substitutions introduced at the A4/A3 elements of RIP1 produced a decrease of basal promoter activity but failed to abrogate stimulatory actions of Ex-4. This is despite the fact that such substitutions prevent binding of PDX-1 to the promoter (41). Furthermore, truncation of RIP1 to remove not only the A4/A3 elements but also the CRE and simultaneously retaining the A2 and A1 elements results in a reporter that is nearly insensitive to Ex-4. In contrast, inactivating Δ-182 and Δ-183/180 deletions introduced at the CRE produce a suppression of Ex-4 action under conditions in which cells are equilibrated in 11.1 mM glucose. Therefore, the interaction of glucose and GLP-1 to stimulate RIP1 is more likely to be mediated by the CRE, and, indeed, we show that the CRE is a glucose- as well as GLP-1-regulated response element. The nature of the small residual response observed after inactivation of the CRE remains to be determined but might reflect actions of Ex-4 at distal sites of RIP1 independent of the A elements (24).

Conclusion

The complexity of GLP-1-R-mediated signal transduction is consistent with a multifunctional role for this blood glucose-lowering hormone as a stimulator of insulin gene expression; insulin secretion; and β-cell neogenesis, growth, and differentiation. Clearly, the relative contributions of the CRE and A elements as regulators of RIP1 remain not fully understood. Our failure to observe a role for the A elements as targets of GLP-1 insulinotropic action must be interpreted with caution in light of the possibility that such an effect might be insulinoma cell type specific, not reflective of authentic β-cells or dictated by the experimental design employed. Although luciferase-based reporters incorporating RIP1 do not necessarily behave in a manner identical with the endogenous insulin gene promoter, previous studies using Northern blot analysis or semiquantitative RT-PCR demonstrated that the activity of endogenous RIP1 is stimulated by glucose and GLP-1 in a manner identical with that of RIP1-Luc (9–11, 26, 28, 58, 59). Therefore, available evidence indicates the existence of a unique glucose-dependent signaling mechanism by which the GLP-1-R functionally couples to increased transcriptional activity of the insulin gene. This is a cAMP- and PKA-independent process for which a critical role of *bZIP*/CRE interactions at RIP1 is readily evident.

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References

1. **Bell GI, Polonsky KS** 2001 Diabetes mellitus and genetically programmed defects in β-cell function. Nature 414:788–791
2. **Drucker DJ** 1998 Glucagon-like peptides. Diabetes 47:159–169
3. **Holz GG, Habener JF** 1992 Signal transduction crosstalk in the endocrine system: pancreatic β-cells and the glucose competence concept. Trends Biochem Sci 17:388–393
4. **Holz GG, Kuhtreiber WM, Habener JF** 1993 Pancreatic β-cells are rendered glucose competent by the insulinotropic hormone glucagon-like peptide-1(7–37). Nature 361:362–365
5. **Ritzel R, Schulte M, Porksen N, Nauck MS, Holst JJ, Juhl C, Marz W, Schmitz O, Schmiegel WH, Nauck MA** 2001 Glucagon-like peptide 1 increases secretory burst mass of pulsatile insulin secretion in patients with type 2 diabetes and impaired glucose tolerance. Diabetes 50:776–784
6. **Xu G, Stoffers DA, Habener JF, Bonner-Weir S** 1999 Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276
7. **Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, Egan JM** 2000 Insulinotropic GLP-1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes 49:741–748
8. **Thorens B** 1992 Expression cloning of the pancreatic β-cell receptor for the gluco-incretin hormone glucagon-like peptide-1. Proc Natl Acad Sci USA 89:8641–8645
9. **Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF** 1987 Glucagon-like peptide-1 stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci USA 84:3434–3438
10. **Fehmann HC, Habener JF** 1992 Insulinotropic hormone glucagon-like peptide-1-(7–37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma βTC-1 cells. Endocrinology 130:159–166
11. **Wang Y, Egan JM, Raygada M, Nadiv O, Roth J, Montrose-Rafizadeh C** 1995 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
12. **Gromada J, Holst JJ, Rorsman P** 1998 Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide-1. Pflugers Arch 435: 583–594
13. **Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H, Levine MA, Schwindinger W, Bernier M** 1999 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
14. **Kang G, Chepurny OG, Holz GG** 2001 cAMP-regulated guanine nucleotide exchange factor II (Epac2) mediates Ca^{2+}-induced Ca^{2+} release in INS-1 pancreatic β-cells. J Physiol 536:375–385
15. **Lester LB, Langeberg LK, Scott JD** 1997 Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. Proc Natl Acad Sci USA 94: 14942–14947
16. **Fraser ID, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, Marrion NV, Scott JD** 1998 A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. EMBO J 17:2261–2272
17. **Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ, Prentki M** 2001 Protein kinase C-ζ activation mediates glucagon-like peptide-1-induced pancreatic β-cell proliferation. Diabetes 50:2237–2243
18. **Wheeler MB, Lu M, Dillon JS, Leung XH, Chen C, Boyd III AE** 1993 Functional expression of the rat glucagon-like peptide-1 receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C. Endocrinology 133: 57–62
19. **Ding WG, Kitasato H, Matsuura H** 2001 Involvement of calmodulin in glucagon-like peptide 1(7–36) amide-induced inhibition of the ATP-sensitive K+ channel in mouse pancreatic β-cells. Exp Physiol 86:331–339
20. **Frodin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB, Obberghen EV** 1995 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
21. **Buteau J, Roduit R, Susini S, Prentki M** 1999 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
22. **Yaney GC, Civelek VN, Richard AM, Dillon JS, Deeney JT, Hamilton JA, Korchak HM, Tornheim K, Corkey BE, Boyd III AE** 2001 Glucagon-like peptide-1 stimulates lipolysis in clonal pancreatic β-cells (HIT). Diabetes 50: 56–62
23. **Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB** 1992 Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology 130:167–178
24. **Skoglund G, Hussain MA, Holz GG** 2000 Glucagon-like peptide-1 stimulates insulin gene promoter activity by protein kinase A-independent activation of the rat insulin I gene cAMP response element. Diabetes 49:1156–1164
25. **Montminy M** 1997 Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807–822
26. **Philippe J, Missotten M** 1990 Functional characterization of a cAMP-responsive element of the rat insulin 1 gene. J Biol Chem 265:1465–1469
27. **Oetjen E, Diedrich T, Eggers A, Knepel W** 1994 Distinct properties of the cAMP-responsive element of the rat insulin 1 gene. J Biol Chem 269:27036–27044
28. **Eggers A, Siemann G, Blume R, Knepel W** 1998 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
29. **Wang X, Cahill CM, Pineyro MA, Zhou J, Doyle ME, Egan JM** 1999 Glucagon-like peptide-1 regulates the β-cell transcription factor, PDX-1, in insulinoma cells. Endocrinology 140:4904–4907
30. **Hussain MA, Habener JF** 2000 Glucagon-like peptide-1 increases glucose-dependent activity of the homeoprotein IDX-1 transactivating domain in pancreatic β-cells. Biochem Biophys Res Commun 274:616–619
31. **Wang X, Zhou J, Doyle ME, Egan JM** 2001 Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic β-cells by a cyclic adenosine monophosphate/protein kinase-A-dependent mechanism. Endocrinology 142:1820–1827
32. **Davies SP, Reddy H, Caivano M, Cohen P** 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95–105
33. **Gromada J, Rorsman P, Dissing S, Wulff BS** 1995 Stimulation of cloned human glucagon-like peptide 1 receptor expressed in HEK 293 cells induces cAMP-dependent activation of calcium-induced calcium release. FEBS Lett 373:182–186
34. **Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C** 1998 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
35. **Gonzalez GA, Montminy MR** 1989 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680
36. **Walton KM, Rehfuss RP, Chrivia JC, Lochner JE, Goodman RH** 1992 A dominant repressor of cAMP-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter *in vivo*. Mol Endocrinol 6:647–655
37. **Iiri T, Bell SM, Baranski T, Fujita T, Bourne HR** 1999 A G_s α mutant designed to inhibit receptor signaling through G_s. Proc Natl Acad Sci USA 96:499–504
38. **Standaert ML, Bandyopadhyay G, Perez L, Price D, Galoway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J, Toker A, Farese RV** 1999 Insulin activates protein kinases C-ζ and C-λ by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. J Biol Chem 274:25308–25316
39. **Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S** 2000 cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol 2:805–811
40. **Ban N, Yamada Y, Someya Y, Ihara Y, Adachi T, Kubota A, Watanabe R, Kuroe A, Inada A, Kazumasa M, Sunaga Y, Shen ZP, Iwakura T, Tsukiyama K, Toyokuni S, Tsuda K, Seino Y** 2000 Activating transcription factor-2 is a positive regulator in CaM kinase-IV-induced human insulin gene expression. Diabetes 49:1142–1148
41. **McKinnon CM, Docherty K** 2001 Pancreatic duodenal homeobox-1, PDX-1, a major regulator of β-cell identity and function. Diabetologia 44:1203–1214
42. **Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B** 1993 Exendin-4 is a high potency agonist and truncated exendin-(9–39)-amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting β-cells. J Biol Chem 268:19650–19655
43. **Thorens B, Porret A, Buhler L, Deng S-P, Morel P, Widmann C** 1993 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
44. **Botelho LH, Rothermel JD, Coombs RV, Jastorff B** 1988 cAMP analog antagonists of cAMP action. Methods Enzymol 159:159–172
45. **Hidaka H, Kobayashi R** 1992 Pharmacology of protein kinase inhibitors. Annu Rev Pharmacol Toxicol 32:377–397
46. **Leech CA, Holz GG, Chepurny OG, Habener JF** 2000 Expression of cAMP-regulated guanine nucleotide exchange factors in pancreatic β-cells. Biochem Biophys Res Commun 278:44–47
47. **Kashima Y, Miki T, Shibasaki T, Ozaki N, Miyazaki M, Yano H, Seino S** 2001 Critical role of cAMP-GEFII-Rim2 complex in incretin-potentiated insulin secretion. J Biol Chem 276:46046–46053
48. **Inada A, Someya Y, Yamada Y, Ihara Y, Kubota A, Ban N, Watanabe R, Tsuda**

K, Seino Y 1999 The cyclic AMP response element modulator family regulates the insulin gene transcription by interacting with transcription factor IID. J Biol Chem 274:21095–21103

49. **Inagaki N, Maekawa T, Sudo T, Ishii S, Seino Y, Imura H** 1992 c-*Jun* represses the human insulin promoter activity that depends on multiple cAMP response elements. Proc Natl Acad Sci USA 89:1045–1049
50. **Kemp DM, Habener JF** 2001 Insulinotropic hormone glucagon-like peptide-1 (GLP-1) activation of insulin gene promoter inhibited by p38 mitogen-activated protein kinase. Endocrinology 142:1179–1187
51. **Macfarlane WM, Smith SB, James RFL, Clifton AD, Doza YN, Cohen P, Docherty K** 1997 The p38/reactivating kinase mitogen-activated protein kinase cascade mediates the activation of the transcription factor insulin upstream factor 1 and insulin gene transcription by high glucose in pancreatic β-cells. J Biol Chem 272:20936–20944
52. **Macfarlane WM, McKinnon CM, Felton-Edkins ZA, Cragg H, James RFL, Docherty K** 1999 Glucose stimulates translocation of the homeodomain transcription factor PDX-1 from the cytoplasm to the nucleus in pancreatic β-cells. J Biol Chem 274:1011–1016
53. **Benes C, Roisin P, Tan HV, Creuzet C, Miyazaki JI, Fagard R** 1998 Rapid activation and nuclear translocation of mitogen-activated protein kinases in response to physiological concentrations of glucose in the MIN6 pancreatic β-cell line. J Biol Chem 273:15507–1551
54. **Benes C, Poitout V, Marie JC, Martin-Perez J, Roisin M-P, Fagard R** 1999 Mode of regulation of the extracellular signal-regulated kinases in the pancreatic β-cell line MIN6 and their implication in the regulation of insulin gene transcription. Biochem J 340:219–225
55. **Rafiq I, da Silva XG, Hooper S, Rutter GA** 2000 Glucose-stimulated preproinsulin gene expression and nuclear translocation of pancreatic duodenum homeobox-1 require activation of phosphatidylinositol 3-kinase but not p38 MAPK/SAPK2. J Biol Chem 275:15977–15984
56. **Leibiger IB, Leibiger B, Moede T, Berggren PO** 1998 Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 s6 kinase and CaM kinase pathways. Mol Cell 1:933–938
57. **Merienne K, Pannetier S, Harel-Bellan A, Sassone-Corsi P** 2001 Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities. Mol Cell Biol 21:7089–7096
58. **Seufert J, Kieffer TJ, Habener JF** 1999 Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. Proc Natl Acad Sci USA 96:6794–6799
59. **Seufert J, Kieffer TJ, Leech CA, Holz GG, Moritz W, Ricordi C, Habener JF** 1999 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