Proximal cyclic AMP response element is essential for exendin-4 induction of rat EGR-1 gene

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Recently, we (13) reported that exendin-4 induced binding of EGR-1 protein to a cis-regulatory element between −153 and −134 on rat Ccnd1 (cyclin D1) promoter leading to activate cyclin D1 transcription. Other studies using small RNA interference demonstrated that reduced EGR-1 gene expression contributed to the decreased β-cell proliferation and the consequent β-cell failure, supporting the essential role of EGR-1 in normal pancreatic β-cell function (7). EGR-1, also known as Zif268, NF1-A, Tis8, and Krox24, is a 3-zinc-finger protein of a member of transcription factors, and its expression is rapidly and transiently induced by various extracellular stimuli (1–3, 23, 24). The induction of EGR-1 has been primarily known to be regulated at the transcriptional level through cis-regulatory elements of the 5′-regulatory region of the gene, which contains the binding sites for serum response factor, cAMP response element-binding protein (CREB), activating protein-1 (AP1), E26 (ETS), and EGR-1 itself (2, 3, 18–20).

Although the roles of GLP-1 in the proliferation of pancreatic β-cells have been widely studied, the molecular mechanisms by which the signaling cascades lead to the regulation of their target gene such as EGR-1 have been less well known. Therefore, we investigated the cis-acting elements and the trans-acting factor involved in regulating EGR-1 gene expression in response to exendin-4 by use of the INS-1 pancreatic β-cell line.

MATERIALS AND METHODS

Materials. All culture media, exendin-4, and poly(dI-dC) were purchased from Sigma-Aldrich (St. Louis, MO). Wild-type CREB, mutant-type CREB (Ser133Ala), and dominant negative Killer CREB were kindly provided by Dr. R. H. Goodman (Volum Institute, Oregon Health and Science University, Portland, OR). [32P]dCTP and [32P]ATP were obtained from PerkinElmer Life Sciences (Boston, MA). LipofectAMINE-PLUS reagent was from Life Technologies (Grand Island, NY). Antibodies to CREB and phosphorylated CREB were from Cell Signaling Technology (Beverly, MA). Anti-EGR-1, c-JUN, and -Sp1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). QuikChange Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA). Luciferase assay kit, rabbit anti-(mouse IgG)-peroxidase conjugate, and goat anti-

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GLUCAGON-LIKE PEPTIDE-1-(7–36) AMIDE (GLP-1), along with its analog exendin-4, is a potent insulinotropic hormone that enhances insulin secretion and whole body insulin sensitivity (8, 25). They also act as a growth factor that stimulates β-cell proliferation and islet neogenesis in diabetic animal models (4, 15, 21). Activation of the GLP-1 receptor, which is G protein coupled, increases intracellular cAMP level and activates PKA. The cAMP/PKA signaling pathway has been shown to play a pivotal role in maintaining and promoting growth of the β-cells (12). GLP-1 also activates other signaling pathways, such as phosphatidylinositol 3-kinase and mitogen-activated protein kinase, in these cells (15, 21). In addition, GLP-1 induces immediate early response genes, such as c-FOS, c-JUN, and early growth response factor-1 (EGR-1), that are implicated in β-cell growth, differentiation, and apoptosis (21, 22).

Although the roles of GLP-1 in the proliferation of pancreatic β-cells have been widely studied, the molecular mechanisms by which the signaling cascades lead to the regulation of their target gene such as EGR-1 have been less well known. Therefore, we investigated the cis-acting elements and the trans-acting factor involved in regulating EGR-1 gene expression in response to exendin-4 by use of the INS-1 pancreatic β-cell line.
rabbit IgG-peroxidase conjugate were from Promega (Madison, WI). PD-98059 and H-89 were from Calbiochem (San Diego, CA).

Cell culture and treatment. INS-1 cells (between passages 15 and 27) were cultured in RPMI 1640 medium (11.5 mM glucose) supplemented with 10% FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 1 mM pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C under an atmosphere of 95% air-5% CO2. For exendin-4 and inhibitor treatment, cells at an initial density of 1 × 10^5 cells/ml were cultured for 48 h and serum starved with RPMI 1640 (5 mM glucose) for 24 h. After starvation, the cells were treated with exendin-4 or pretreated with inhibitors, as described in the figure legends.

Preparation of nuclear extracts. Cells were washed twice with ice-cold PBS and were pelleted by centrifugation. The pellet was resuspended and incubated for 15 min on ice in a hypotonic solution consisting of 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.4% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 2.5 mM dithiothreitol, 0.5 mM PMSF, and 1 μg/ml leupeptin. Nuclei were collected with centrifugation and incubated for 30 min on ice in a hypertonic buffer consisting of 20 mM HEPES, pH 7.9, 25% glycerol, 0.5 M NaCl, 1.5 mM MgCl2, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 5 mM dithiothreitol, 0.1 mM PMSF, and 1 μg/ml leupeptin. The nuclear extract was prepared by centrifugation and stored at −80°C prior to use.

Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Western blotting. Twenty micrograms from each sample were separated on an SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to PVDF membrane (Amersham Biosciences, Piscataway, NJ). Blocking was performed in 5% nonfat dried milk in PBS containing 0.2% Tween-20. Antibodies used to probe the blots were EGR-1, CREB, phosphorylated CREB, and β-tubulin. The detection was performed by chemiluminescence system (Amersham Biosciences).

Northern blotting. Total cellular RNA was extracted using TRizol reagent (Life Technologies) based on the manufacturer’s instructions. Ten micrograms of each sample were fractionated on a 1% formaldehyde agarose gel and transferred onto a Hybond-N nylon membrane (Amersham Biosciences). The membrane was hybridized in a rapid hybridization buffer (Amersham Biosciences) with a 0.7-kilobase rat EGR-1 promoter fragment spanning the promoter-luciferase reporter gene were transiently transfected with p-462 to form DNA/protein complexes was performed in 20 μl of 10 mM HEPES, pH 8, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 10% glycerol, 1 μg of poly(dI-dC), 32P-labeled oligonucleotide probe (30,000 cpm), and 5 μg of nuclear extract. The reaction was allowed to continue for 15 min at 4°C. In supershift studies, 2 μg of the appropriate antibody were preincubated with the nuclear extract for 15 min at 4°C before addition of the labeled probe. In competition experiments, the nuclear extract was incubated with a 100-fold molar excess of the appropriate unlabeled competitor oligonucleotides. Electrophoresis was carried out on 6% nondenatured polyacrylamide gels with 0.5× TBE (15 V/cm for ~2 h). The gel was dried under vacuum and subjected to autoradiography. Oligonucleotides used were as follows: CRE, 5'-CATGATCACCGTACCGGGGAGG-3'; CRE mutant, 5'-CATGATCACCGGCGGAGG-3'; CRE consensus, 5'-GATGCCGAGGGAGG-3'; CRE consensus, 5'-AGGATGGCCTGACGTCAGAGAGAAGTACGAGATCGAGAAGCAG-3'; and Sp1 consensus, 5'-ATTGGCCTGACGTCAGAGATCGAGAAGCAG-3'.

Chromatin immunoprecipitation assays. Histone deacetylase activity. Histone deacetylase activity was performed as means ± SD. Statistical analysis was performed using one-way ANOVA and Student’s t-test. P < 0.05 was accepted as statistically significant.

RESULTS

Activation of rat EGR-1 gene in response to exendin-4 in INS-1 cells. INS-1 cells transiently transfected with p-462 construct containing full-length EGR-1 promoter (from −462 to +27) were serum-starved and treated with varying concentrations of exendin-4 (10−10, 10−9, 10−8, and 10−7 M). As shown in Fig. 1A, exendin-4 induced the activity of the reporter gene in a dose-dependent manner, starting to induce at 10−9 M and peaking at 10−8 M without a further increase at 10−7 M. In examination of the kinetics of EGR-1 expression, exendin-4 (10−8 M) rapidly and transiently induced the expression of EGR-1 mRNA. The mRNA was detectable within 15 min after exendin-4 treatment, reaching maximum level at 30 min and sharply declining thereafter (Fig. 1B). The time course of the mRNA expression was well correlated with the kinetics of EGR-1 protein induction. The protein level started to increase within 30 min, peaked at 1 h, and returned to basal level by 2 h of exendin-4 treatment (Fig. 1C). In another pancreatic β-cell line, RINm5F, exendin-4 treatment showed a similar response pattern of EGR-1 induction observed in INS-1 cells (data not shown).
ESSENTIAL ROLE OF PROXIMAL CRE IN EXENDIN-4-ACTIVATED EGR-1

Fig. 1. Transcriptional activation of early growth response factor-1 gene (EGR-1) by exendin-4 in INS-1 cells. A: INS-1 cells transfected with 0.5 μg of full-length EGR-1 promoter construct (p-462) were serum starved for 24 h and treated with exendin-4 at the indicated concentrations for 6 h. After cell harvesting, luciferase activities were measured and normalized to β-galactosidase activities. Data are expressed as fold induction of luciferase activity relative to the mean of the untreated control. Values are mean fold induction ± SD of 3 experiments with 2 replicate wells/experiments. *P < 0.05 vs. untreated control. B: Northern blot analysis of EGR-1 mRNA. C: Western blot of EGR-1 protein. Each lane contains 10 μg of total cellular RNA for Northern blot and 20 μg of nuclear extract for Western blot from cells treated with exendin-4 during the indicated time periods. Blots are representative of 3 independent experiments.

Determination of exendin-4 response elements of the rat EGR-1 promoter. The 5′-regulatory region of rat EGR-1 promoter contains several putative binding sites for transcription factors, which are known to mediate transcriptional activation of human EGR-1 gene (18–20). These regulatory sites include five sterol response elements (SREs), two ETSs, and two CREs. To determine the regulatory elements involved in inducing the rat EGR-1 gene in response to exendin-4, a series of 5′ deletion constructs of the rat EGR-1 promoter were transiently transfected, and then the activities of the reporter gene were analyzed. As shown in Fig. 2, two regions were responsive to exendin-4, i.e., between -462 and -324 (designated as 5′-responsive region) and between -73 and -46 (designated as 3′-responsive region). The p-462, a full-length construct, showed an ∼2.1-fold increase in promoter activity by exendin-4. Further serial deletion to −73, which eliminated five SREs and a distal CRE, maintained relatively constant responses of 1.6–1.7-fold induction. However, the deletion to −46, which eliminated the remaining proximal CRE site, resulted in a complete loss of the exendin-4 response. Thus, the 3′ responsive region between −73 and −46 proved to be a principal site for induction of the EGR-1 gene activity in response to exendin-4. On the other hand, the 5′-responsive region between −462 and −324 may be required for the full activation of the EGR-1 promoter.

Significant inhibition of exendin-4-induced EGR-1 transcription by mutation of the proximal CRE. To evaluate the functional significance of the proximal CRE site for the activation of the EGR-1 promoter by exendin-4, the proximal CRE sequences (5′-GTAGCAGCTA-3′) of the p-462 or p-73 construct were mutated to 5′-GTtgGTCA-3′. The distal CRE site located at −139 (5′-TCACGCTA-3′) was also mutated to 5′-TTCtcGTCA-3′, as this distal CRE site was also shown to be involved in the regulation of the human EGR-1 gene by growth factors (5, 6, 18). As shown in Fig. 3, the introduction of the mutated proximal CRE into the p-462 construct (mpCRE-462) reduced the fold induction by ∼28% compared with wild p-462 construct (1.5 ± 0.1 vs. 2.1 ± 0.2, P < 0.05), whereas the introduction of the same mutant CRE into the p-73 construct (mpCRE1–73) abolished the exendin-4 response compared with wild p-73 construct (1.0 ± 0.1 vs. 1.7 ± 0.1, P < 0.05). On the other hand, mutation of the distal CRE (mdCRE-462) decreased the fold induction by only 12% compared with the wild p-462 construct (1.8 ± 0.1 vs. 2.1 ± 0.2, P < 0.05). These results confirmed the critical role of the 3′-responsive region and the importance of the proximal CRE in activating the EGR-1 gene in response to exendin-4.

Induction of CREB phosphorylated on Ser133 associated with the proximal CRE site by exendin-4. To determine the functional significance of constructs used in transient transfection assays is shown on the left. Putative regulatory elements [sterol response element (SRE), ETS, and cAMP response element (CRE)] are indicated. INS-1 cells transfected with the constructs (0.5 μg) were serum starved for 24 h and treated with exendin-4 for 6 h. After cell harvesting, luciferase activities were measured and normalized to β-galactosidase activities. Numbers on the right are mean fold induction of luciferase activity induced by exendin-4 divided by the corresponding untreated control. Filled bars represent means ± SD of 5 experiments with 2 replicate wells/experiments. *P < 0.05 vs. p-462 and p-46.
moiety shift assays (EMSAs) were performed using nuclear extracts from exendin-4-treated INS-1 cells and a radiolabeled probe containing the proximal CRE sequence. As shown in Fig. 4A, the relative intensity of each band was irrelevant to the duration of exendin-4 treatment. The binding specificities of these complexes were determined using unlabeled oligonucleotides. The complexes were completely eliminated by 100-fold excess of unlabeled consensus or wild CRE oligonucleotides but not affected by mutant CRE oligonucleotide (Fig. 4B). Because the proximal CRE sequence of the radiolabeled probe used in this experiment also holds potential binding sites for c-JUN, one of the AP1 subunits, and Sp1 as shown in Fig. 4B, bottom, consensus AP1 and Sp1 oligonucleotides were also tested and proved to be without effect. Next, nuclear factors binding to the proximal CRE site were identified using relevant antibodies. As shown in Fig. 4C, incubation with an antibody against CREB resulted in a supershifted complex formation with similar intensity regardless of exendin-4 treatment. However, from the exendin-4-treated nuclear extract, an antibody (anti-pCREB) that specifically recognizes the CREB phosphorylated on Ser133, picked up a clear, supershifted band that was not detectable from the untreated nuclear extracts. The presence of phosphorylated CREB observed in the supershift assays was confirmed by Western blot analysis using the same nuclear extracts and the anti-phospho-CREB antibody. Exendin-4 treatment substantially induced the phosphorylation of CREB without affecting the levels of CREB proteins (Fig. 4D). Next, the in vivo effect of exendin-4 on the induction of the phosphorylation at Ser133 of CREB bound to rat EGR-1 promoter was examined by ChIP assay. The immunoprecipitated DNAs were amplified by standard PCR using a pair of primers flanking the proximal CREB-binding region (−124 to +27) of the EGR-1 promoter. As shown in Fig. 4E, in vivo binding of the phosphorylated CREB at Ser133 to the region of EGR-1 promoter was clearly detected after exendin-4 treatment, whereas the binding of CREB was not significantly changed. Thus the phosphorylation of CREB on Ser133 appears to be involved in the exendin-4-stimulated EGR-1 transcription.

Requirement of CREB phosphorylation on Ser133 for transcriptional activation of EGR-1 gene. In activating EGR-1 transcription by exendin-4, whether phosphorylation on Ser133 of CREB is required needs to be confirmed. Hence, cotransfection with p-462 construct was performed using a wild-type and two mutant-type CREB-expressing vectors. Two mutant CREB vectors were Ser133Ala and KCREB. Ser133Ala expresses mutant CREB on which Ser133 was substituted with Ala to prevent phosphorylation of endogenous CREB. KCREB expresses an inhibitory protein, which binds to endogenous CREB to prevent the binding of CREB to the CRE site of gene promoters. Although wild-type CREB vector per se increased the luciferase activity, exendin-4 augmented the activity significantly. In contrast, mutant CREB vectors (Ser133Ala and KCREB) showed significantly less increased exendin-4 responses compared with the vector (Table 1). These results suggest that exendin-4 induction of CREB phosphorylation on Ser133 was directly involved in the transcriptional activation of EGR-1 promoter in response to exendin-4.

Exendin-4 induction of EGR-1 expression via PKA and ERK signaling pathways. To investigate the signaling pathway for the exendin-4 induction of EGR-1 expression, specific kinase inhibitors (H-89 for PKA and PD-90059 for MEK) were used. As shown in Fig. 5A, at concentrations commonly used for the inhibition of PKA and MEK activities, pretreatment with H-89 (10 μM) completely inhibited the exendin-4 response, whereas PD-90059 (50 μM) decreased the response by 36%. In parallel with the transfection results, Western blot analysis showed that the exendin-4 induction of phosphorylation of CREB on Ser133 and of EGR-1 protein were completely blocked by H-89 but partly by PD-90059 (Fig. 5B). These results indicate that the PKA signaling pathway is more important for the exendin-4 activation of EGR-1 gene and for the phosphorylation of CREB than the MEK/ERK pathway.

Fig. 4. Activation of the proximal CRE site through binding of phosphorylated CREB on Ser133 by exendin-4. Nuclear extracts from INS-1 cells treated with exendin-4 for the indicated times were used in EMSA with a probe containing the putative CRE sequence. A: proximal CRE binding to nuclear factors at various time points. B: competition assays with unlabeled oligonucleotides. Unlabeled consensus CRE, wild, or mutant CRE and consensus activating protein-1 (AP1) or Sp1 were added at 100-fold molar excess. C: supershift assays with antibodies to CREB (anti-CREB), phospho-CREB on Ser133 (anti-pCREB), c-JUN (anti-c-JUN), or Sp1 (anti-Sp1). Nuclear extracts with exendin-4 treatment for 15 min were preincubated with antibodies for 15 min before addition of radiolabeled probe. D: Western blot analysis of phospho-CREB (p-CREB) and CREB using the same nuclear extracts used for supershift assays. E: ChIP assay was performed to amplify the region from −124 to +27. Input samples represent 0.001, 0.005, and 0.01% of total DNA, whereas PCRs of the immunoprecipitations include 5% of the resuspended DNA. IgG antibody was used as a negative control. Similar results were obtained from 3 independent experiments.
ESSENTIAL ROLE OF PROXIMAL CRE IN EXENDIN-4-ACTIVATED EGR-1

A

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<tr>
<td>mutant API</td>
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B

CRE/API

wild CRE: 5'-CCAATTCAGCACGGGCGAG-3'
mutant CRE: 5'-CCATGTCGTCACGGGCGAG-3'
consensus CRE: 5'-AGAGATGTCATACGGGAGCTAG-3'
mutant API: 5'-CGGTGAGTCAGGGCGGAA-3'
consensus API: 5'-ATTGGATCGGCGGGGCAG-3'

C

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D

untreated | exendin-4
---|---
pCREB | | |
CREB | | |

E

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human and mouse (3, 5, 18), and the region hence appears to
EGR-1
tively. Although the 5
In an effort to clarify the molecular mechanism of GLP-1 in
/H9252
potent analog exendin-4 possess properties of enhancing pan-
/exendin-4 in INS-1 cells.
EGR-1
the transcriptional activation of
/Ser133Ala 0.92
CREB 3.10
DISCUSSION
Table 1. Requirement of CREB phosphorylation on Ser133 for exendin-4 induction of EGR-1 promoter

<table>
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<td>1.6±0.1*</td>
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Values are means ± SD. CREB, cAMP response element-binding protein; EGR-1, early growth response factor-1 gene. Wild (CREB) and 2 mutant CREBs (Ser133Ala and KCREB)-expressing plasmids were cotransfected with p-462 construct into INS-1 cells. After transfection for 18 h, cells were serum starved for 24 h and treated with exendin-4 for 6 h. Luciferase activities were measured and normalized to β-galactosidase activities. Statistical analysis was applied to the fold induction data only. Three experiments were independently performed with 2 replicate wells/experiments. *P < 0.05 vs. vector; †P < 0.05 vs. CREB.

In humans, the rapid and transient activation of EGR-1 gene
EGR-1
expression by many extracellular stimuli is mediated exclu-
sively through the cis-regulatory elements within the 5′-regu-
ulatory region of the gene without involving posttranscrip-
tional regulation such as mRNA stability. The rapid induction of
EGR-1
gene is highly dependent on the binding sites for serum
response factor, ternary complex factors, and CREB on the
EGR-1
promoter. However, the molecular mechanism for the
transient induction of EGR-1 gene is not well known. A
negative feedback loop is suggested to downregulate the
transcription, in which the EGR-1 protein can bind to the EGR-1
binding site of its own gene and allows only a transient
activation of EGR-1 gene. In this study, our determination of
the kinetics for induction of EGR-1 mRNA following exend-
in-4 treatment was well consistent with most other studies
in that EGR-1 mRNA was induced within 15 min and signifi-
cantly reduced after 30 min (2, 5, 22). The rapid and transient
induction of EGR-1 mRNA, together with the activation of
EGR-1 promoter, indicates the transcriptional regulation as a
fundamental mechanism for the exendin-4 induction of EGR-1
gene.

Through serial-deletion analysis of rat EGR-1 promoter, we
mapped the 5′- and 3′-responsive regions, which were located
between −462 and −324 and between −73 and −46, respec-
tively. Although the 5′-responsive region contains the SRE
cluster and ETS sites important for EGR-1 transcription in
human and mouse (3, 5, 18), and the region hence appears to
be required for the full activation of the EGR-1 promoter in
response to exendin-4, we focused our interest on the 3′-
responsive region between −73 and −46. The reasons are as
follows. First, deletion of this particular region eliminated
almost completely the exendin-4 response, providing this re-
region as a minimally essential promoter. Second, this region
contains a proximal putative CRE site that could be the final
target of the classic cAMP/PKA signaling pathway for GLP or
exendin-4 (15).

Our site-directed mutation of the proximal CRE within the
3′-responsive region resulted in a complete loss of the responsi-
siveness to exendin-4, confirming that the CRE is crucial in
inducing the activation of the 3′-responsive region. The
importance of the equivalent CRE in activating the gene was also
reported with the human EGR-1 promoter (14, 19). The 3′-
responsive regions of rat and human hold 100% identically
conserved proximal CRE site (5′-GTACGTCA-3′) extended from
−69 to −62. However, the introduction of the mutated proximal CRE into p-462 construct resulted in only a one-third
loss of the exendin-4 response instead of abolition of the
response. This result might be attributed to the action of intact
SRE cluster and ETS sites located within the 5′-responsive
region between −462 and −324.

On the other hand, the distal CRE of human EGR-1 pro-
moter has been shown to be involved in inducing EGR-1 genes
(2, 5, 6, 18). In support of these studies, we also observed that

Fig. 5. Involvement of PKA and ERK signaling pathways in exendin-4 induction of EGR-1 expression. A: INS-1 cells transfected with p-462 construct were serum starved for 24 h and then incubated for 1 h in the presence of H-89 (10 μM) or PD-98059 (50 μM). Cells were then treated with exendin-4 for 1 h. After cell harvesting, luciferase activities were measured and normalized to β-galactosidase activities. Data are expressed as fold induction of luciferase activity relative to the untreated wild p-462. *P < 0.05 vs. untreated control or DMSO; †P < 0.05 vs. exendin-4 only. B: INS-1 cells serum starved for 24 h were incubated for 1 h in the presence of H-89 (10 μM) or PD-98059 (50 μM) and treated with exendin-4 for 10 min and for 1 h to determine levels of phospho-CREB and EGR-1, respectively. Similar results were obtained from 4 independent experiments.
mutation of the distal CRE of rat EGR-1 promoter resulted in a significant decrease in the exendin-4 induction of EGR-1 gene, although less than that of the proximal CRE (mdCRE-462 vs. mpCRE-462, Fig. 3). Interestingly, the deletion effect of the distal CRE at −139 on induction of EGR-1 promoter activation was negligible in our deletion analysis study (p-159 vs. p-124, Fig. 2). The discrepancy between the two studies is not clear; however, the strong stimulatory effect of the proximal CRE might have masked the effect of the distal CRE. Likewise, in human EGR-1 promoter, the importance of distal CRE was revealed only by mutation assay but not by deletion analysis (6).

CREB, a ubiquitously expressed transcription factor, plays a critical role in mediating many cellular responses to various physiological stimuli (16, 17). CREB activation is generally mediated through phosphorylation on the critical residue Ser133 within its kinase-inducible domain (16). The phosphorylation of CREB facilitates its association with the transcriptional coactivators such as CREB-binding protein, leading to promote the expression of CREB target genes. In our supershift and ChIP assays using an antibody against a phosphorylated CREB on Ser133, the binding activity of phospho-CREB was remarkably increased following treatment with exendin-4 in vitro and in vivo. In addition, cotransfection data also showed that overexpression of mutant CREB vectors resulted in a significant reduction of the activity of EGR-1 promoter. The involvement of CREB phosphorylation in our study is very well consistent with human EGR-1 studies showing that rapid phosphorylation of CREB mediated the activation of the promoter via binding to the proximal CRE site (14). These and our findings strongly indicate that exendin-4-induced phosphorylation of CREB is an important step in the transcriptional activation of EGR-1 gene.

Among multiple signaling pathways, PKA and ERK pathways have been well known to mediate EGR-1 expression in response to extracellular mitogens (1–3, 10, 24). In pancreatic β-cells, cAMP/PKA/CREB pathway induced by GLP-1 or exendin-4 is believed to be a critical signaling pathway for cell survival and growth (12). In addition, the PKA signaling pathway was shown to mediate the induction of EGR-1 in insulinoma cell line MIN6 under high glucose concentration (2). In other cell types, the cAMP/PKA pathway has been suggested to cross-talk with Rap1/Raf/MEK to activate ERK (9). According to our observation employing pharmacological inhibitors, H-89 was more potent in suppressing the exendin-4-activated EGR-1 gene expression as well as pCREB induction than PD-98059, suggesting that the PKA pathway is mainly involved in exendin-4-induced EGR-1 expression. However, further experiments are required to clarify the exact signaling pathway for EGR-1 transcriptional activation by exendin-4 in β-cells.

In conclusion, this study represents an important mechanism by which exendin-4 modulates the transcription of EGR-1 gene, the regulation of which is necessary for pancreatic β-cell growth.

GRANTS

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REFERENCES


