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Exendin-4 Induction of Egr-1 Expression in INS-1 β-Cells: Interaction of SRF, not YY1, with SRE Site of Rat Egr-1 Promoter

Myung-Jun Kim,[1] Jung-Hoon Kang,[1] Seo-Yoon Chang,[1] Hyun-Jong Jang,[1] Gyeong Ryul Ryu,[2] Seung Hyun Ko,[2] In-Kyung Jeong,[3] Myung-Suk Kim,[1] and Yang-Hyeok Jo[1]*

[1]Department of Physiology, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea

[2]Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul 137-701, South Korea

[3]Department of Internal Medicine, School of Medicine, Kyung Hee University, Seoul 130-701, South Korea

Abstract Glucagon-like peptide-1 (GLP-1) induces several immediate early response genes such as c-fos, c-jun, and early growth response-1 (Egr-1), which are involved in cell proliferation and differentiation. We recently reported that exendin-4 (EX-4), a potent GLP-1 agonist, upregulated *Egr-1* expression via phosphorylation of CREB, a transcription factor in INS-1 β-cells. This study was designed to investigate the role of another transcription factors, serum response factor (SRF) and Yin Yang-1 (YY1), in EX-4-induced Egr-1 expression. EX-4 significantly increased Egr-1 mRNA and subsequently its protein level. EX-4-induced Egr-1 expression was inhibited by pretreatment with a PKA inhibitor, H-89, and an MEK inhibitor, PD 98059. The siRNA-mediated inhibition of PKA and ERK1 resulted in significant reduction of EX-4-induced Egr-1 expression. Promoter analyses showed that SRE clusters were essential for Egr-1 transcription, and YY1 overexpression did not affect Egr-1 promoter activity. EMSA results demonstrated that EX-4-induced transient increase in DNA–protein complex on SRE site, and that both SRF and phospho-SRF were bound to this site. Treatment of either YY1 consensus oligonucleotide or YY1 antibody did not effect the change of density or migration of the DNA–protein complex. Collectively, EX-4-induced Egr-1 expression is largely dependent on cAMP-mediated extracellular signal-regulated kinase activation, and EX-4 induces Egr-1 transcription via the interaction of SRF and phospho-SRF to SRE sites.
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Key words: exendin-4 (EX-4); Egr-1; INS-1 β-cells; SRE; YY1; cAMP/PKA

Glucagon-like peptide-1 (GLP-1) and its potent agonist exendin-4 (EX-4) have been of much interest due to their insulinotropic and β-cell-proliferating effects [Ørskov, 1992; Egan et al., 2003]. GLP-1 activates multiple signaling pathways such as cAMP/PKA cascade, phosphatidylinositol 3-kinase and mitogen-activated protein kinase, which lead to β-cell maintenance and growth [Jhala et al., 2003; List and Habener, 2004; Stoffers, 2004]. In addition, GLP-1 induces immediate early response genes such as c-fos, c-jun, early growth response-1 (Egr-1), which are involved in β-cell growth and differentiation [Susini et al., 1998; Stoffers, 2004].

Egr-1 is a member of the immediate early gene group of transcription factors [Sukhatme et al., 1988] and the reduction in Egr-1 expression may contribute to decreased β-cell proliferation

Abbreviations used: EX; exendin-4; GLP-1; glucagon-like peptide-1; Egr-1; early growth response-1; SRE; serum response element; SRF; serum response factor; YY1; Yin Yang-1; PKA; protein kinase A; MEK; MAPK/ERK kinase; ERK; extracellular signal-regulated kinase; CRE; cAMP-responsive element; RT-PCR; reverse transcription-polymerase chain reaction; EMSA; electrophoretic mobility shift assay; PMSF; phenylmethylsulfonyl fluoride.

Myung-Jun Kim and Jung-Hoon Kang contributed equally to this study.

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*Correspondence to: Yang-Hyeok Jo, Department of Physiology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, Republic of Korea. E-mail: yhjo@catholic.ac.kr

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and consequent β-cell failure [Garnett et al., 2005].

Recently, we found that binding of phospho-CREB to the proximal cAMP-response element (CRE) site on the Egr-1 promoter is necessary for Egr-1 gene transcription by EX-4 [Kang et al., 2007]. And Egr-1 protein binds to the putative Egr-1 binding site on the cyclin D1 promoter to upregulate the cyclin D1 gene expression in EX-4-treated INS-1 β-cells [Kang et al., 2006]. EX-4 also induces the expression of cyclin D1 gene through binding of CREB to the putative CRE site on the cyclin D1 promoter [Kim et al., 2006]. Thus, it appears that EX-4 simultaneously activates the several genes and recruits even different transcriptional factors to activate one gene in INS-1 β-cells.

Egr-1 promoter possesses *cis*-elements for binding of serum response factor (SRF), CREB, AP1, Ets, and Egr-1 itself [Bernal-Mizrachi et al., 2000; Thiel and Cibelli, 2002; Russell et al., 2003]. Among these *cis*-elements are the proximal and distal clusters of serum response element (SRE) sites. And the SRE sites (consensus sequence: 5′-CC(A/T)$_6$GG-3′) are partly overlapped with the putative binding site (consensus sequence: 5′-CCATNTT-3′) for transcription factor, Yin Yang-1 (YY1). However, it has not been documented well if these sites are involved in the Egr-1 expression.

In the present study, therefore, we investigated the involvement of distal or proximal SRE cluster and the role of YY1 in EX-4-induced Egr-1 expression employing INS-1 β-cells.

MATERIALS AND METHODS

Materials

FBS, RPMI 1640 medium, and OPTI-MEM were purchased from Gibco BRL (Grand Island, NY). Exendin-4 (EX-4) and exendin-(9–39) amide were from Bachem AG (Torrance, CA). Glucose-dependent insulinotropic peptide (GIP) receptor antagonist GIP-(6–30) was kindly provided by Dr. C.M. Isales (Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA). Forskolin, PD98059, and H-89 were from Calbiochem (La Jolla, CA). AccuPrep genomic DNA extraction kit was from Bioneer (Daejon, Korea). Top-Pfu^{TM} DNA polymerase was from Bio-online (Seoul, Korea). Lipofectamine^{TM} 2000 reagent was from Invitrogen (Carlsbad, CA). BCA protein assay kit was from Perbio Science (Erembodegem, Belgium). pGL3 Luciferase Reporter Vector, pSV-β-gal and Sp1 consensus oligonucleotide were from Promega (Madison, WI). Anti-rabbit Egr-1 antibody (C-19), anti-rabbit SRF antibody (H-300), anti-rabbit YY1 antibody (C-20), anti-mouse YYI antibody (H-10) antibody, anti-rabbit Elk-1 antibody (I-20), anti-rabbit Sp1 (H-225) antibody, ERK1 siRNA (sc-29308) and Western blotting Luminol Reagent were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit phospho-SRF (Ser103) antibody was from Cell Signaling Technology, Inc. (Beverly, MA). RNA STAT-60 was from TEL-TEST (Friendswood, TX). First Strand cDNA Synthesis Kit for RT-PCR was from Roche Diagnostics (Mannheim, Germany). PKA siRNA Assay Kit was from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma (St. Louis, MO). EX-4 was prepared in RPMI 1640 containing 0.1% BSA to prevent the peptide from adherence to plastic surfaces. Forskolin was dissolved in ethanol at 100 mM stock solution. PD98059 and H-89 were dissolved in DMSO at 100 mM stock solution and added to the culture medium. The final concentration of vehicles did not exceed 0.1′.

Drugs Treatment

INS-1 cells (kindly provided by Dr. I. Kojima, Institute for Mol & Cell Regulation, Gunma University, Japan, passages 7–12) were cultured in RPMI 1640 containing 10 mM HEPES, 5.6 mM D-glucose, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), and maintained at 37°C in a humidified air containing 5% CO_2 [Kim et al., 2006]. To rule out the effects of high glucose and serum on Egr-1 expression, we used low glucose (5.6 mM)-containing serum-free media in all experiments. The cells were plated at a concentration of 2×10^5/mL except for transient transfection experiment. The cells were incubated with serum-free RPMI medium for 24 h, next treated with EX-4 and forskolin (5 μM) for the indicated times as described in figure legends. Pharmacological inhibitors [H-89 (5 μM): a PKA inhibitor; PD98059 (10 μM): an MEK inhibitor; exendin-(9–39) amide (10 μM): a GLP-1 receptor antagonist; GIP-(6–30) (10 μM): a GIP receptor antagonist] were added 30 min. prior to stimulation of cells

with EX-4 and forskolin. Concentrations of EX-4 used were described in figure legends.

Western Blot Analysis

Following the serum starvation for 24 h, the cells were treated with EX-4 for indicated times to examine the time-dependent expression of Egr-1. The cells were harvested and nuclear extracts were isolated and then Western blot analysis was performed as described previously [Ryu et al., 2004]. Briefly, the cells were suspended in hypotonic buffer A [10 mM KCl, 10 mM HEPES, pH 7.9, 1 mM PMSF, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.2 mM Na_3VO_4, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 0.5% Nonidet P-40]. Following centrifugation, the pellets were suspended in hypertonic buffer B (400 mM NaCl, 20 mM HEPES, pH 7.9, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 μg/mL aprotinin). Twenty micrograms of total protein was separated on a 10% SDS–PAGE and transferred onto nitrocellulose membrane. After transfer, the membrane was stained with Ponceau S to assess transfer efficiency. Following blocking with 5% skimmed milk in TTBS (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), the membrane was incubated at 4°C overnight in blocking buffer containing Egr-1 or YY1 antibody. The membrane was then probed with peroxidase-conjugated anti-rabbit IgG (0.5 μg/mL of TTBS). The signal was visualized by enhanced chemiluminescence system.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis

To examine the mRNA expressions of Egr-1, RT-PCR was performed as described previously [Jeon et al., 2004]. Briefly, total RNA was isolated using STAT-60 reagent according to manufacturer's instruction. RT-PCR was carried out using 1st Strand cDNA Synthesis kit, in which 1 μg of total RNA was used as template. The primers used for the amplification of a 721-bp fragment of rat Egr-1 coding region were designed based on GenBank (accession no: NM_012551) as follows: forward, from +511, 5′-CGGCAGCAGCGCTTTCAATCCTCAAGG-3′; reverse, from +1231, 5′-GCGGCTGGGTTT-GATGAGTTGGGACTG-3′. One-tenth of RT reaction was amplified in a thermal cycler (GeneAmp PCR cycler 9700, PerkinElmer, Norwalk, CT) as following temperature profiles: 5 min. at 95°C; 30 cycles of 30 s at 95°C, 1 min. at 63°C, and 30 s at 72°C; and 7 min. at 72°C. Amplification of a 452-bp fragment of rat GAPDH gene was carried out as an internal control. Based on the result of RT-PCR, INS-1 cells were pretreated with H-89 or PD98059 for 30 min., and then incubated with EX-4 or forskolin for an additional 15 min. Northern blot analysis was performed as described previously [Kim et al., 2004]. Equal amounts of total RNA (10 μg) were electrophoresed through a 1% agarose gel, then transferred to a positively charged nylon membrane by capillary reaction, and secured to the membrane by UV cross-linking. The membrane was hybridized with DIG-labeled Egr-1 cDNA probe. Egr-1 mRNA level was detected by chemiluminescence using CDP-Star as substrate. Equal loading of sample was verified by the ribosomal 18S and 28S bands.

Plasmids

The pSG5 expression vector for YY1 (pSG5-YY1) was kindly provided by Prof. S.J. Um (Sejong University, Korea) [Kang et al., 2004]. Rat Egr-1 promoter ranging from −462 to +27 was generated by PCR amplification method and cloned into pGL3 basic vector, then designated as pEgr-462. Using pEgr-462 as a template, serial deletion constructs (pEgr-324, pEgr-124, pEgr-74, and pEgr-46) were prepared [Kang et al., 2007]. To synthesize pEgr-462 and pEgr-324 devoid of proximal SRE cluster (pEgr-462Δp and pEgr-324Δp, respectively), PCR was performed using pEgr-462 and pEgr-324 as a template and each PCR product was cloned into pEgr-74 (Fig. 3A). The forward primers used were as follows: in pEgr-462D, 5′-TCAGGT-ACCAGGCTCCGGGTTGGGAAC-3′ with *KpnI* site (underlined); in pEgr-324D, 5′-TCAGG-TACCTGCGCTTCCGGCTCTG-3′ with *KpnI* site (underlined). The reverse primer of both constructs was identical as follows: 5′-TCA-GGTACCGGAGTGACGTGAAGACCTC-3′ with *XhoI* site (underlined). These constructs were verified by sequencing using An ABI PRISM 310 genetic analyzer (PerkinElmer Ltd. Co., Seoul, Korea).

Transient Transfection and Luciferase Reporter Assay

Transient transfection was performed by lipofection using Lipofectamine^{TM} 2000 reagent as described previously [Kim et al., 2004].

Briefly, the cells were plated at a density of 4×10^5/well in a 6-well Plate 4 days before the transfection. Cells at 70% confluence were cotransfected with pEgr constructs (pEgr-462, pEgr-324, pEgr-462D, and pEgr-324D, 1 μg) and pSV-β-gal (0.1 μg). Following the serum starvation for 24 h, the cells were next incubated with EX-4 for an additional 10 h. Luciferase and β-galactosidase activities were measured with a luminometer (TD20/20, Turner Designs Instrument, Sunnyvale, CA) and MR700 Microplate Reader (Dynatech Laboratories Inc., Chantilly, VA), respectively. Transfection efficiencies were normalized by a ratio of luciferase activity to β-galactosidase activity obtained from the same sample. To evaluate the effect of YY1 on Egr-1 promoter activity in the basal condition, pEgr-124 (0.5 μg), pSG5-YY1 (0.5 μg), and pSV-β-gal (0.1 μg) were cotransfected into the cells, and then promoter activity was analyzed in the same manner.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Following the serum starvation for 24 h, the cells were treated with EX-4 for indicated times to examine DNA binding activity to SRE site of rat Egr-1 promoter. Nuclear extracts were prepared by the method reported previously [Dignam et al., 1983]. EMSA was performed as described previously [Kim et al., 2006]. The complementary oligonucleotides used as probes or competitors were as follows: wild type (GenBank accession no: AF067056, from −113, 5′-CGGTCCTTCCATATTAGGGCTTCCTGC-3′), consensus SRE (5′-TTGGATGTCCATATTAGGACATCT-3′), mutant SRE (5′-GGTCCTTCCATATTAttGCTTCCTGC-3′, small letter: mutated site), consensus YY1 (5′-CGCTCCCCGGCCATCTTGGCGGCTGGT-3′) from Bioneer (Daejon, Korea). The antibodies against SRF, phospho-SRF, Elk-1, YY1, and Sp1 were used for immune-supershift assay. To verify equal loading of nuclear extract, EMSA was performed using Sp1 consensus oligonucleotide.

siRNA-Mediated Knockdown Study

Both PKA and ERK1 expressions were silenced in MIN6 cells (provided by Dr. Jun-ichi Miyazaki, Osaka University, Japan) using target-specific 20–25 nucleotide siRNA duplexes. The day before transfection, MIN6 cells were seeded in 12-well plates in regular growth medium without antibiotics and grown overnight to reach 50–60% confluence. The day of the experiment, siRNA complexes were prepared and transfection was performed according to the manufacturer's instructions. Concentration of PKA and ERK1 siRNA complexes for MIN6 cell transfection required optimization. The final concentration of siRNA is 50 nM in each well. MIN6 cells were transfected with PKA, ERK1 or control scramble siRNA (which corresponds to a nontargeting 20–25 nucleotide siRNA as a negative control) for 12 h before switching to fresh growth medium, then 48 or 72 h after transfection, cells were stimulated with EX-4 and lysed for Western blot analysis.

Statistical Analysis

The relative band densities were quantified using Scion Imaging software (Scion Corporation, Frederick, MD). All data obtained from each experiment were expressed as mean ± SD. The data were analyzed using one-way ANOVA with Origin 7.0 software (Microcal Software, Northampton, MA). Statistical comparisons among the groups were done by Bonferroni's multiple range t-test after the ANOVA. $P < 0.05$ was accepted as statistically significant.

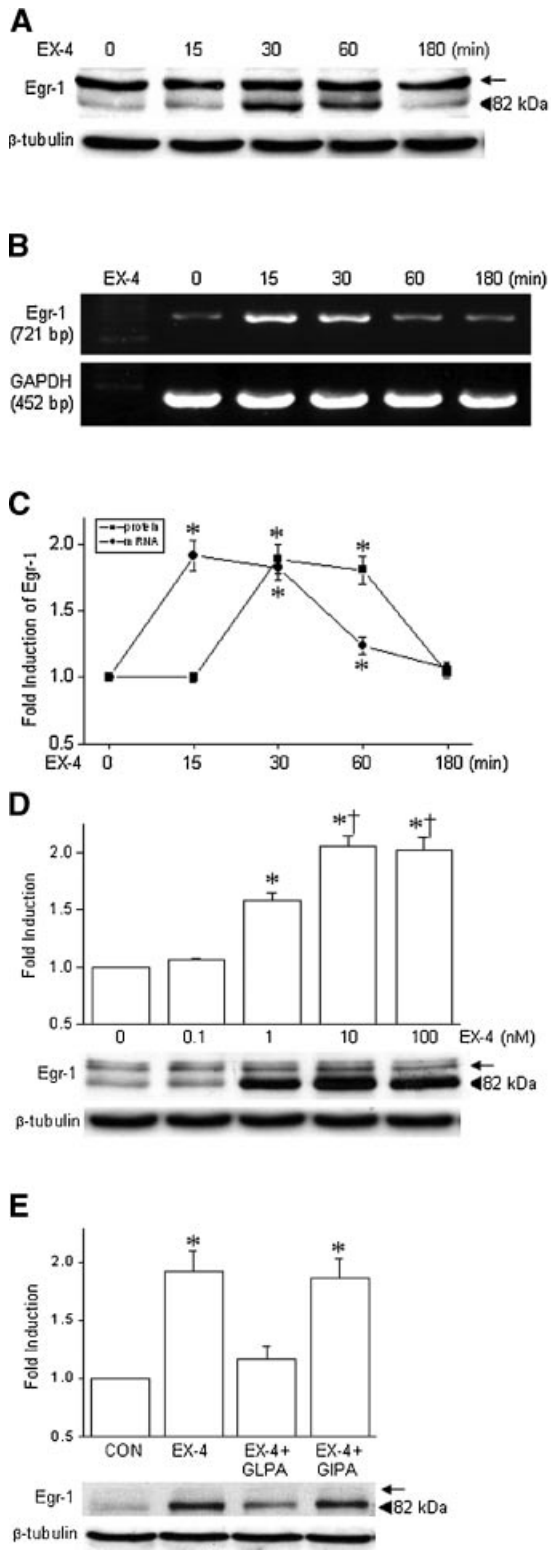
RESULTS

EX-4 Induces Egr-1 mRNA, Subsequently its Protein in INS-1 Cells

Egr-1 protein expression reached a peak 30 min. after EX-4 (10 nM) treatment in INS-1 cells. The expression of Egr-1 gradually declined thereafter, returning to baseline by 3 h post-stimulation (Fig. 1A,C). We next examined the effect of EX-4 on Egr-1 expression at mRNA level. The RT-PCR result showed that Egr-1 mRNA expression was increased between 15 and 30 min. after EX-4 and then returned to basal level by 1 h (Fig. 1B,C). EX-4-induced Egr-1 protein expression was dose-dependent (0.1–10 nM) and was maximally effective at 10 nM (Fig. 1D). To test the possible involvement of GIP receptor in EX-4-induced Egr-1 protein, the cells were pretreated with GIP-(6–30), a GIP receptor antagonist, and Egr-1 expression was not affected by EX-4. However, a GLP-1 receptor antagonist exendin-(9–39) amide significantly inhibited EX-induced Egr-1 expression (Fig. 1E).

The Involvement of cAMP and ERK in Exendin-4-Induced Egr-1 Expression

Based on the previous studies that EX-4 increased intracellular cAMP concentration [Nielsen et al., 2004] and induced extracellular signal-regulated kinase (ERK) phosphorylation in β-cells [Buteau et al., 2001; Arnette et al., 2003; Kim et al., 2006], we investigated the involvements of ERK or cAMP in EX-4-induced Egr-1 expression at both protein and mRNA levels. As shown in Fig. 2A,B, forskolin, a cAMP increasing agent, significantly induced Egr-1 expression compared with EX-4. Also, the induction of Egr-1 by EX-4 was inhibited by both H-89, a PKA inhibitor, and PD 98059, an MEK inhibitor. To further explore the effect of PKA and ERK on Egr-1 expression, the gene silencing study was performed. As shown in Fig. 2C, each transfection of small interfering duplexes of PKA and ERK1 decreased dose-dependently PKA and ERK1 expression, respectively. In PKA-downregulated MIN6 cells, EX-4-induced Egr-1 expression was significantly abolished compared with control siRNA-transfected cells (Fig. 2C1). Meanwhile, EX-4-induced Egr-1 expression was still alive in ERK1-downregulated MIN6 cells, which may result from the action of intact ERK2. However, the Egr-1 expression by EX-4 was significantly reduced compared with control siRNA-transfected cells (Fig. 2C2).

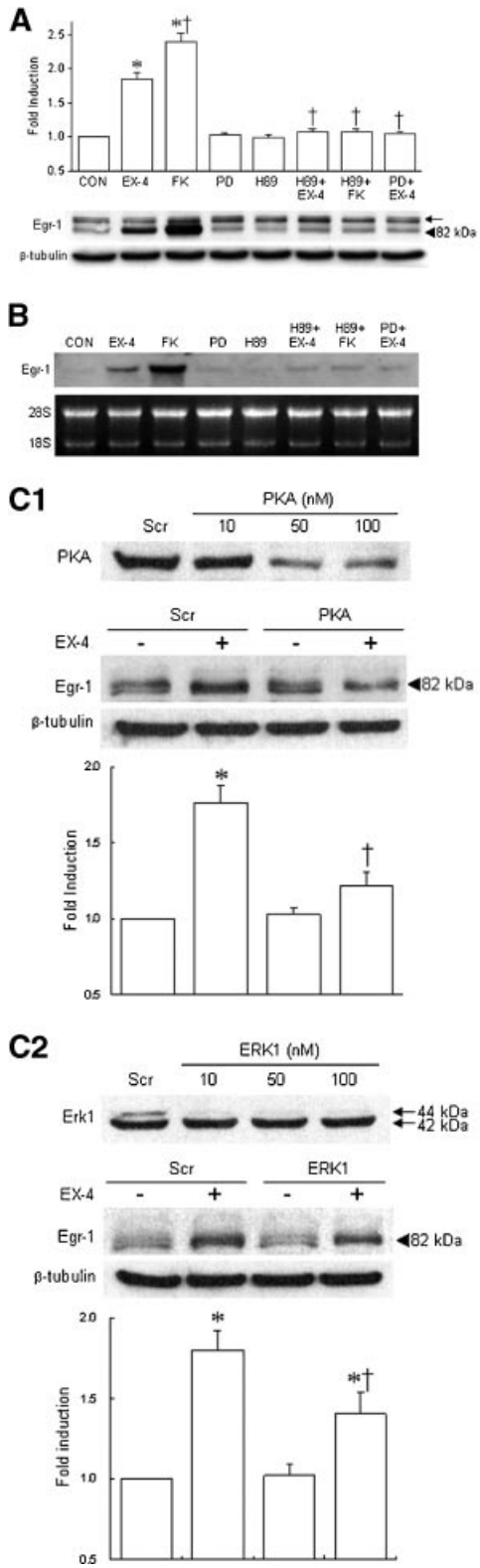


SRE Sites of Rat Egr-1 Promoter are Essential for Egr-1 Gene Expression

To evaluate the involvement of the distal and proximal SRE cluster in Egr-1 induction, Egr-1

Fig. 1. The exendin-4-induced Egr-1 expression levels in INS-1 cells. **A**: Following the serum starvation, INS-1 cells were incubated with exendin-4 (EX-4, 10 nM) for the indicated times. Western blot analysis for Egr-1 was performed. Egr-1 protein level was peak at 30 min. after EX-4 treatment, thereafter gradually decreased. Arrow: cross-reactive molecule. **B**: The cells were treated with EX-4 for indicated times. Egr-1 mRNA levels were measured by RT-PCR. Egr-1 mRNA expression was increased between 15 and 30 min. after EX-4, then returned to basal level by 1 h. **C**: Results of A and B are expressed as mean (±SD) of relative band density from three independent experiments. *, $P < 0.05$ vs. untreated control value. **D**: The cells were treated with indicated doses of EX-4 for 30 min. EX-4 increased Egr-1 protein level dose-dependently, however, a further increase in Egr-1 protein was not observed at 100 nM of EX-4. Arrow: cross-reactive molecule. Data are expressed as mean (±SD) of relative band density from three independent experiments. *, $P < 0.05$ vs. untreated control value; †, $P < 0.05$ vs. 1 nM. **E**: The cells were pretreated with GLP-1 receptor antagonist (GLP1A) or GIP receptor antagonist (GIPA) (both 10 μM) for 30 min. and then treated with EX-4 (10 nM) for 30 min. Arrow: cross-reactive molecule. Data are expressed as mean (±SD) of relative band density from three independent experiments. *, $P < 0.05$ vs. untreated control value.

promoters devoid of SRE cluster were tested for EX-4-responsiveness (Fig. 3A). Compared with wild type pEgr-462, promoter activities of pEgr-324 and pEgr-462Δp (devoid of distal and proximal SRE cluster, respectively) were significantly reduced in both basal and EX-4-stimulated conditions. Additionally, the decreases in promoter activity of pEgr-324Δp (devoid of both SRE clusters) were more augmented, even though the response to EX-4 still remained.



To examine the effect of YYI on Egr-1 induction, pSG5-YY1 and pEgr-124 were cotransfected into the cells. As shown in Fig. 3B, the cells transfected with exogenous YY1 showed no difference in promoter activity compared with the empty vector. To rule out the nonspecific effect of YY1, activity of the promoter cotransfected with pEgr-46 (devoid of SRE site) and YY1 was tested, and no difference was noted compared with pEgr-46 plus empty vector (Fig. 3B).

Binding activity of nuclear protein to the SRE site within the Egr-1 promoter was assessed by EMSA. As shown in Fig. 4A1, ^{32}P-labeled probe containing putative SRE sequence was bound to protein extracted from both control and EX-4-treated nuclei (arrow). This DNA–protein complex appeared to be sequence-specific because a 100-fold molar excess of unlabeled probe abolished the binding activity of the labeled probe by competition (Com). Band intensity of the DNA–protein complex was increased by 10 min. after EX-4 treatment and then rapidly returned to basal level by 30 min (Fig. 4A1,A3). To rule out the possibility that ubiquitous transcription factor bound to SRE site by EX-4, EMSA was performed using Sp1 consensus oligonucleotide. As shown in Fig. 4A2, there was no difference in band density among the experimental groups. To examine the specificity of SRE site, competitor EMSA was performed in basal condition

Fig. 2. The involvement of cAMP and ERK in exendin-4-induced Egr-1 expression. The cells were pretreated with PD98059 (PD, 10 μM) and H-89 (5 μM) for 30 min., next incubated with exendin-4 (EX-4, 10 nM) or forskolin (FK, 10 μM) for an additional 30 min. Egr-1 protein (**A**) and its mRNA (**B**) levels were measured by Western blot and Northern blot analyses, respectively. FK significantly induced Egr-1 expression, and the induction of Egr-1 by EX-4 and FK was blocked by H-89. Arrow: cross-reactive molecule. Each result is representative of three independent experiments. Data are expressed as mean (±SD) of relative Egr-1 protein (A) band density. *, $P < 0.05$ vs. CON; †, $P < 0.05$ vs. EX-4. (**C**) MIN6 cells transfected with scrambled siRNA (Scr) or siRNA directed against PKA or ERK1 were stimulated with EX-4 (10 nM) for 30 min. The nuclear fractions were harvested to analyze the protein expression of Egr-1. Each result is representative of three independent experiments. Data are expressed as mean (±SD) of relative band density from three independent experiments. *, $P < 0.05$ vs. untreated; †, $P < 0.05$ vs. EX-4 in Scr.

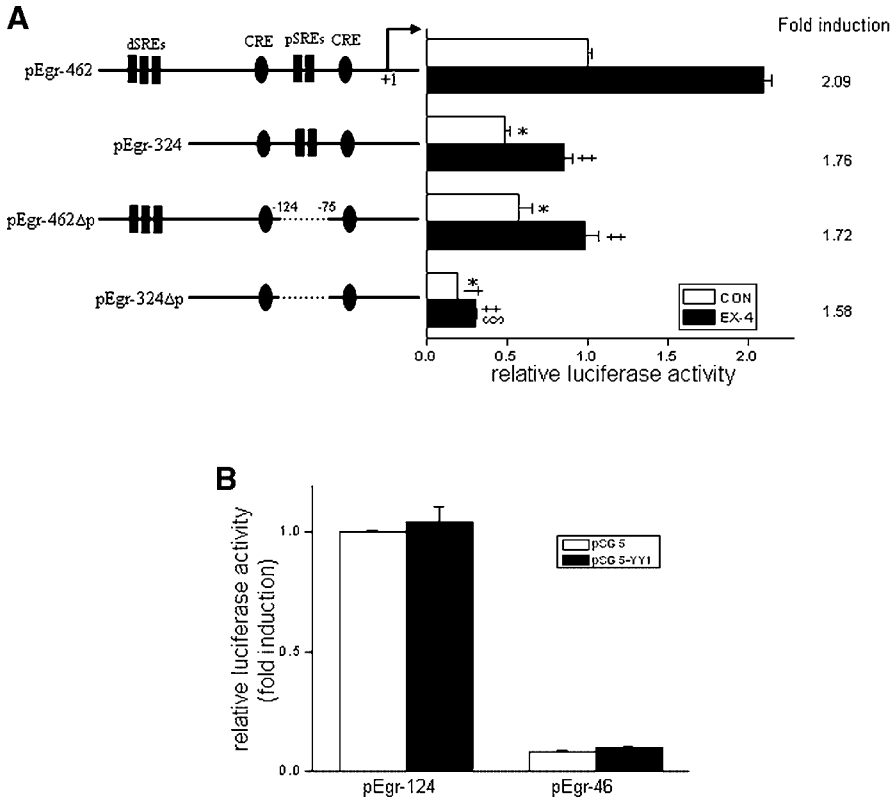


Fig. 3. Identification of SRE clusters implicated in transcriptional regulation of rat Egr-1 gene by exendin-4. **A**: Schematic representation of the wild type or SRE clusters-deleted Egr-1 promoters. CRE: cyclic AMP response element; between −124 and −75: the region of proximal SRE cluster. INS-1 cells were transiently cotransfected with Egr-1 promoters (1 μg) and pSV-β-gal (0.1 μg, as an internal control). Following the serum starvation, the cells were incubated with exendin-4 (EX-4, 10 nM) for 10 h. Cells were then solubilized in lysis buffer and luciferase and β-galactosidase activities were measured. Data are expressed as mean ± SD from three independent experiments and each value is expressed relative to the unstimulated response of pEgr-462. CON: basal response of each pEgr. *, $P < 0.05$ vs. control of pEgr-462; †, $P < 0.05$ vs. control of pEgr-324 and pEgr-462Δp; ‡, $P < 0.05$ vs. EX-4-treated pEgr-462; §, $P < 0.05$ vs. EX-4-treated pEgr-324Δp and pEgr-462Δp. **B**: Cells were cotransfected with pEgr-124 or pEgr-46 (0.5 μg) with or without pSG5-YY1 (0.5 μg), and pSV-β-gal (0.1 μg). Data are expressed as mean (±SD) fold induction relative to pEgr-124 with empty vector (pSG5) from three independent experiments.

(Fig. 4B). The DNA–protein complex completely disappeared in the presence of both excess wild type (WT) and consensus SRE (CONS) oligonucleotides, but not of either mutant SRE (MT) or consensus YY1 oligonucleotide suggesting the site specificity of DNA–protein complex formation. In immune-supershift EMSA, following the incubation of antibodies against SRF or phospho-SRF, supershifted bands were strongly detected (open arrow heads). However, the addition of antibodies against either Elk-1 or YY1 or Sp1 did not affect the mobility and intensity of DNA–protein complex (Fig. 4C).

DISCUSSION

Recently, the function of Egr-1 in pancreatic β-cells has been unraveling through in vivo and in vitro studies. The study using small RNA interference showed that the reduction in Egr-1 expression may contribute to decreased β-cell proliferation and consequent β-cell failure [Garnett et al., 2005]. The present study demonstrates the involvement of SRE clusters in the mechanism of EX-4-induced Egr-1 expression at the transcriptional level.

EX-4-induced rapidly and transiently Egr-1 mRNA, and subsequently its protein expressions. This expression pattern was similar to the results obtained from MIN6 and INS-1 β-cells exposure to high glucose concentration [Josefsen et al., 1999; Bernal-Mizrachi et al., 2000]. EX-4-induction of Egr-1 protein was dose-dependent and was maximally effective at 10 nM (Fig. 1D). This finding is well consistent with other's results in that 10 nM GLP-1 promoted maximum cell growth independent

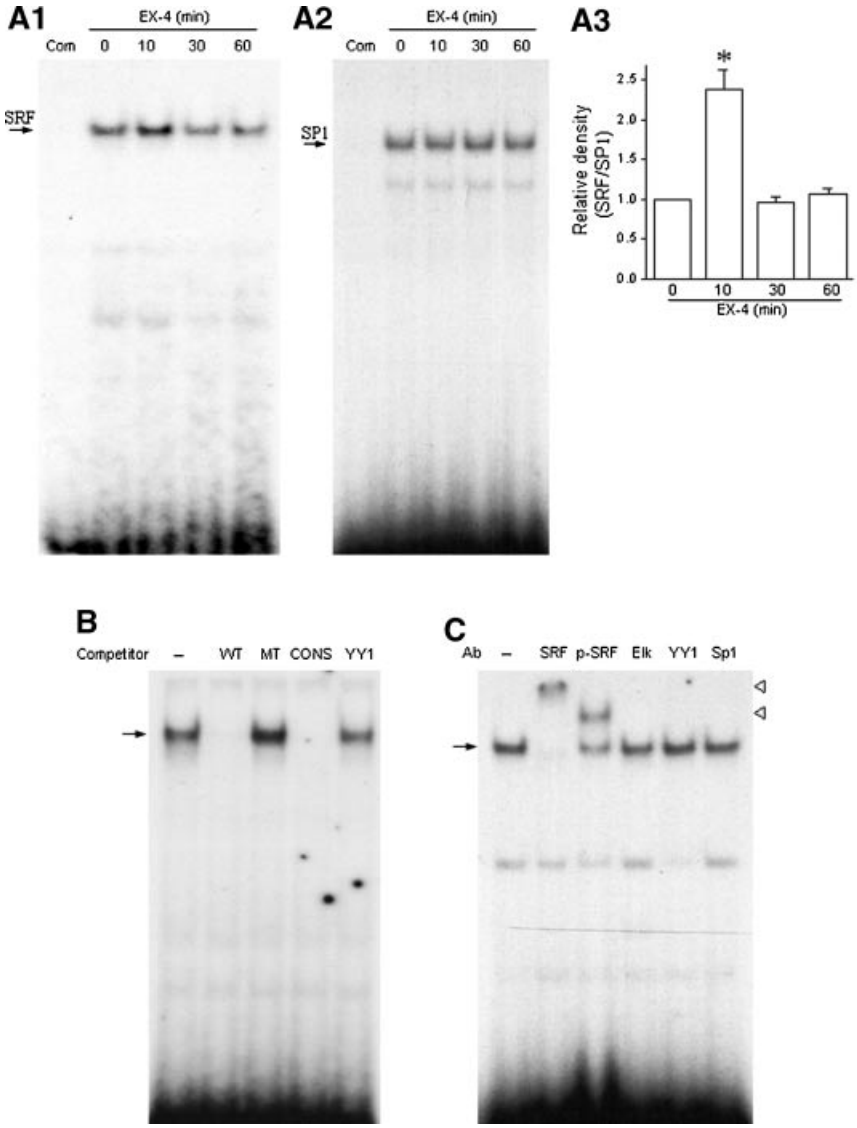


Fig. 4. Gel mobility shift assay of SRE site on rat Egr-1 promoter. **A1**: INS-1 cells were treated with exendin-4 (EX-4, 10 nM) for indicated times. Then, the binding activity of nuclear extracts was analyzed on electrophoretic mobility shift assay (EMSA) using wild type oligonucleotide of Egr-1 promoter (from −113, 5′-CGGTCCTTCCATATTAGGGCTTCCTGC-3′). The arrow indicates the position of major DNA–protein complex. Com: addition of unlabeled wild probe. **A2**: To rule out the possible nonspecific binding of ubiquitous transcription factor, EMSA for Sp1 consensus oligonucleotide was performed employing the same sample in A1. The arrow indicates the position of major DNA–protein complex. **A3**: The band intensity of SRF (A1) was normalized by the band intensity of Sp1 (A2) using densitometry. Data are expressed as mean (±SD) of relative band density from three independent experiments. *, $P < 0.05$ vs. untreated control value. **B**: In basal condition, competition assay was carried out with a 100-fold molar excess of either wild type (WT) or consensus SRE (CONS) or mutant SRE (MT) or consensus YY1 (YY1) oligonucleotides. The arrow indicates the position of major DNA–protein complex. –, no addition of oligonucleotide. **C**: The cells were treated with EX-4 (10 nM) for 10 min. Then, immune-supershift assay was carried out using specific antibody (Ab) against either SRF or phospho-SRF or Elk-1 or YY1. The open arrowheads indicate a supershifted band by the addition of antibody against SRF or phospho-SRF. –, no addition of Ab.

of glucose concentration [Buteau et al., 1999]. However, the higher concentration of EX-4 did not increase a further expression of Egr-1 protein. This result may be due to either low GLP-1 receptor density or receptor desensitization of the cells used [Widmann et al., 1994, 1996].

EX-4 increased intracellular cAMP concentration via activation of adenylyl cyclase [Nielsen et al., 2004] and induced ERK phosphorylation in β-cells [Buteau et al., 2001; Arnette et al., 2003; Kim et al., 2006]. Therefore, we examined the implications of cAMP or ERK in Egr-1 expression at protein and mRNA levels.

Our data showed that forskolin significantly induced Egr-1 expression compared with EX-4 and that EX-4- and forskolin-induced Egr-1 expression was blocked by H-89. Additionally, gene silencing of PKA significantly inhibited EX-4-induction of Egr-1, which suggests an involvement of cAMP in EX-4-induced Egr-1 expression. This result was verified in different cells by studies that pharmacological or hormonal activation of cAMP/PKA cascade upregulated Egr-1 expression [Bernal-Mizrachi et al., 2000; Espey et al., 2000; Tai et al., 2001]. Also, EX-4-induced Egr-1 expression was inhibited by both PD 98059 and ERK1 gene silencing, which suggests the involvement of ERK signaling. This finding is consistent well with others' results in that Egr-1 biosynthesis is strongly promoted by activation of ERK upon mitogenic stimuli [Kaufmann et al., 2001; Thiel and Cibelli, 2002].

These two signaling molecules, that is, cAMP and ERK may separately function in Egr-1 expression. However, high glucose activated ERK2 pathway in a cAMP-dependent manner [Frödin et al., 1995] and EX-4-induced-ERK phosphorylation was dependent on intracellular cAMP level [Kim et al., 2006] in INS-1 β-cells. Furthermore, induction of early response genes *Egr-1*, *jun*B, and *nur*77 was reported to be correlated with MAP kinase activated by CPT-cAMP, a membrane permeable cAMP analogue [Frödin et al., 1995]. Therefore, the mechanism of cAMP-mediated ERK activation appears to be involved in EX-4-induced Egr-1 expression at least in INS-1 β-cells.

Using deletion constructs, our promoter analyses showed that both proximal and distal SRE clusters were implicated in both basal and EX-4-induced transcriptional activation of Egr-1 gene. Such an involvement of the SRE sites in Egr-1 expression was also widely demonstrated in different cells using different stimuli. The SRE sites of rat Egr-1 promoter were implicated in forskolin- or KCl-stimulated induction of Egr-1 in MIN6 cells [Bernal-Mizrachi et al., 2000]. The SRE sites in upper region of human Egr-1 promoter were responsible for nitric oxide-induced Egr-1 synthesis in SH-SY5Y neuroblastoma cells [Cibelli et al., 2002]. Egr-1 was induced by 17β-estradiol in MCF-7 cells via the activation of SRE sites of human Egr-1 promoter [Chen et al., 2004]. In the present study, we found that in INS-1 β-cells also, SRE site is essential in expression of the Egr-1 gene transactivated by EX-4. Meanwhile, the proximal CRE site previously reported may be implicated in the intact response to EX-4 of the promoter construct of which both SRE clusters are deleted [Kang et al., 2007].

YY1 is a zinc finger protein that can regulate transcriptional activity as an activator or a repressor in different promoter context [Shi et al., 1991]. For example, YY1 and SRE bind to SRE site of skeletal α-actin promoter in a mutually exclusive manner [Lee et al., 1991, 1992]. Normally, YY1 blocks access of SRF and prevents activation of the promoter. However, during myogenic differentiation, YY1's DNA binding activity decreases and then SRF easily binds to the promoter leading to the transcriptional activation. Therefore, we tested the effect of YY1 on Egr-1 transcriptional activity using pSG5-YY1 and pEgr-124 (possessing YY1 consensus sequence). The overexpression of exogenous YY1 had no effect on Egr-1 promoter activity. Furthermore, competition EMSA showed that the addition of wild type oligonucleotide comprising YY1 consensus sequence did not affect the band density of DNA–protein complex. In addition, there was no supershifted band by the preincubation with YY1 antibody in basal condition (data not shown). Therefore, YY1 does not appear to bind to the SRE site of Egr-1 promoter in INS-1 cells.

The SRE site is continuously occupied in vivo by SRF and Ets proteins of the ternary complex factors (TCF) Elk-1, Net, and Sap-1 [Treisman, 1992]. We evaluated nuclear factor binding activity to the SRE site using EMSA. Our data demonstrated the rapid and transient formation of DNA–protein complex induced by EX-4 was similar to the temporal kinetics of the EX-4-induced Egr-1 mRNA expression (Fig. 4A vs. Fig. 1B). These findings suggest that the activation of SRE site on the Egr-1 promoter was accompanied by the synthesis of Egr-1 mRNA. As a consequence, activated SRF induces transcription by binding to SREs and by recruiting these TCF to the SRE site. Additionally, phosphorylation of SRF at Ser^{103} augments the binding potential of SRF to SRE site [Rivera et al., 1993]. As expected, according to our immune-supershift assay, both SRF and phospho-SRF were bound to SRE site of Egr-1 promoter supporting that SRF tightly bound to the SRE site leading to the transcriptional activation [Treisman, 1992]. Meanwhile, Elk-1, one of TCF, did not bind to SRE site of Egr-1

promoter in basal and EX-4-stimulated conditions. The SRE site is known to be constitutively bound by SRF but not by TCF in preadipocyte cells [Clarkson et al., 1999]. In primary human endothelial cells also, the Elk-1 and Sap-1 did not bind to SRE site of Egr-1 promoter [Wu et al., 2002]. Therefore, SRE clusters are likely to transduce EX-4 signal by SRF-dependent, but not TCF-dependent mechanism in INS-1 β-cells.

Collectively, EX-4-induced Egr-1 expression appears to be largely dependent on cAMP/PKA signaling pathway in INS-1 β-cells. And the interaction of both SRF and phospho-SRF with SRE site of the promoter may be implicated in both basal and EX-4-induced Egr-1 transcription.

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REFERENCES

Arnette D, Gibson TB, Lawrence MC, January B, Khoo S, McGlynn K, Vanderbilt CA, Cobb MH. 2003. Regulation of ERK1 and ERK2 by glucose and peptide hormone in pancreatic β cells. J Biol Chem 278:32517–32525.

Bernal-Mizrachi E, Wice B, Inoue H, Permutt MA. 2000. Activation of serum response factor in the depolarization induction of Egr-1 transcription in pancreatic islet β-cells. J Biol Chem 275:25681–25689.

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 beta (INS-1)-cells. Diabetologia 42:856–864.

Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ, Prentki M. 2001. Protein kinase Cζ activation mediates glucagon-like peptide-1-induced pancreatic β-cell proliferation. Diabetes 50:2237–2243.

Chen CC, Lee WR, Safe S. 2004. Egr-1 is activated by 17β-estradiol in MCF-7 cells by mitogen-activated protein kinase-dependent phosphorylation of elk-1. J Cell Biochem 93:1063–1074.

Cibelli G, Policastro V, Rössler OG, Thiel G. 2002. Nitric oxide-induced programmed cell death in human neuroblastoma cells is accompanied by the synthesis of Egr-1, a zinc finger transcription factor. J Neurosci Res 67:450–460.

Clarkson RW, Shang CA, Levitt LK, Howard T, Waters MJ. 1999. Ternary complex factors Elk-1 and Sap-1a mediate growth hormone-induced transcription of egr-1 (early growth response factor-1) in 3T3-F442A preadipocytes. Mol Endocrinol 13:619–631.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Egan JM, Bulotta A, Hui H, Perfetti R. 2003. GLP-1 receptor agonists are growth and differentiation factors for pancreatic islet beta cells. Diabetes Metab Res Rev 19:115–123.

Espey LL, Ujioka T, Russell DL, Skelsey M, Vladu B, Robker RL, Okamura H, Richards JS. 2000. Induction of early growth response protein-1 gene expression in the rat ovary in response to an ovulatory dose of human chorionic gonadotropin. Endocrinology 141:2385–2391.

Frödin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB, Van Obberghen E. 1995. Glucose, other secretagogue, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting β-cell line. J Biol Chem 270:7882–7889.

Garnett KE, Chapman P, Chambers JA, Waddell ID, Boam DS. 2005. Differential gene expression between Zucker Fatty rats and Zucker Diabetic Fatty rats: A potential role for the immediate-early gene Egr-1 in regulation of beta cell proliferation. J Mol Endocrinol 35:13–25.

Jeon SY, Baek K-H, Kim YS, Park CG, Kwon HS, Ko SH, Song KH, Yoo SJ, Son HS, Cha BY, Lee KW, Son HY, Kang SK, Yoon KH. 2004. Differentially up-regulated genes in proliferating porcine neonatal pancreas cells caused by epidermal growth factor. J Cell Biochem 91: 354–364.

Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M. 2003. cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS. Genes Dev 17:1575–1580.

Josefsen K, Sørensen LR, Buschard K, Birkenbach M. 1999. Glucose induces early growth response gene (Egr-1) expression in pancreatic beta cells. Diabetologia 42:195–203.

Kang JH, Chang SY, Yeom DH, Kim SA, Um SJ, Hong KJ. 2004. Weakening of the repressive YY-1 site on the thrombospondin-1 promoter via c-Jun/YY-1 interaction. Exp Mol Med 36:300–310.

Kang JH, Kim MJ, Ko SH, Jeong IK, Koh KH, Rhie DJ, Yoon SH, Hahn SJ, Kim MS, Jo YH. 2006. Upregulation of rat CCND1 gene by exendin-4 in pancreatic beta-cell line INS-1: Interaction of early growth response-1 (Egr-1) with *cis*-regulatory element. Diabetologia 49:969–979.

Kang JH, Kim MJ, Jang HI, Koh KH, Yeom KS, Rhie DJ, Yoon SH, Hahn SJ, Kim MS, Jo YH. 2007. Proximal cyclic AMP response element is essential for exendin-4 induction of rat *EGR-1* gene. Am J Physiol Endocrinol Metab 292:E215–E222.

Kaufmann K, Bach K, Thiel G. 2001. Extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1. Biol Chem 382:1077–1081.

Kim MJ, Ryu GR, Kang JH, Sim SS, Min DS, Rhie DJ, Yoon SH, Hahn SJ, Jeong IK, Hong KJ, Kim MS, Jo YH. 2004.

Inhibitory effects of epicatechin on interleukin-1-induced inducible nitric oxide synthase expression in RINm5F cells and rat pancreatic islets by down-regulation of NF-κB activation. Biochem Pharmacol 68:1775–1785.

Kim MJ, Kang JH, Park YG, Ryu GR, Ko SH, Jeong IK, Koh KH, Rhie DJ, Yoon SH, Hahn SJ, Kim MS, Jo YH. 2006. Exendin-4 induction of cyclin D1 expression in INS-1 beta-cells Involvement of cAMP response-like element. J Endocrinol 188:623–633.

Lee TC, Chow KL, Fang P, Schwartz RJ. 1991. Activation of skeletal alpha-actin gene transcription: The cooperative formation of serum response factor-binding complexes over positive *cis*-acting promoter serum response elements displaces a negative-acting nuclear factor enriched in replicating myoblasts and nonmyogenic cells. Mol Cell Biol 11:5090–5100.

Lee TC, Shi Y, Schwartz RJ. 1992. Displacement of BrdUrd-induced YY1 by serum response factor activates skeletal alpha-actin transcription in embryonic myoblasts. Proc Natl Acad Sci USA 89:9814–9818.

List JF, Habener JF. 2004. Glucagon-like peptide-1 agonists and the development and growth of pancreatic β-cells. Am J Physiol Endocrinol Metab 286:E875–E881.

Nielsen LL, Young AA, Parkes DG. 2004. Pharmacology of exenatide (synthetic exendin-4): A potential therapeutic for improved glycemic control of type 2 diabetes. Regul Pept 117:77–88.

Ørskov C. 1992. Glucagon-like peptide-1, a new hormone of the entero-insular axis. Diabetologia 35:701–711.

Rivera VM, Miranti CK, Misra RP, Ginty DD, Chen RH, Blenis J, Greenberg ME. 1993. A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. Mol Cell Biol 13:6260–6273.

Russell DL, Doyle KM, Gonzales-Robayna I, Pipaon C, Richards JS. 2003. Egr-1 induction in rat granulose cells by follicle-stimulating hormone and leutenizing hormone: Combinatorial regulation by transcription factors cyclic adenosine 3′,5′-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1. Mol Endocrinol 17:520–533.

Ryu GR, Sung CH, Kim MJ, Sung JH, Lee KH, Park DW, Sim SS, Min DS, Rhie DJ, Yoon SH, Hahn SJ, Kim MS, Jo YH. 2004. Changes in IP3 receptor are associated with altered calcium response to cholecystokinin in diabetic rat pancreatic acini. Pancreas 22:47–52.

Shi Y, Seto E, Chang LS, Shenk T. 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. Cell 67:377–388.

Stoffers DA. 2004. The development of beta-cell mass: Recent progress and potential role of GLP-1. Horm Metab Res 36:811–821.

Sukhatme VP, Cao XM, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, Cohen DR, Edwards SA, Shows RB, Curran T. 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 53:37–43.

Susini S, Roche E, Prentki M, Schlegel W. 1998. Glucose and glucoincretin peptides synergize to induce c-*fos*, c-*jun*, *junB*, *zif-268*, and *nur-77* gene expression in pancreatic β (INS-1) cells. FASEB J 12:1173–1182.

Tai TC, Morita K, Wong DL. 2001. Role of Egr-1 in cAMP-dependent protein kinase regulation of the phenylethanolamine *N*-methyltransferase gene. J Neurochem 76: 1851–1859.

Thiel G, Cibelli G. 2002. Regulation of life and death by the zinc finger transcription factor Egr-1. J Cell Physiol 193:287–292.

Treisman R. 1992. The serum response element. Trends Biochem Sci 17:423–426.

Widmann C, Bürki E, Dolci W, Thorens B. 1994. Signal transduction by the cloned glucagons-like peptide-1 receptor: Comparison with signaling by the endogenous receptors of β cell lines. Mol Pharmacol 45:1029–1035.

Widmann C, Dolci W, Thorens B. 1996. Desensitization and phosphorylation of the glucagons-like peptide-1 (GLP-1) receptor by GLP-1 and 4-phorbol 12-myristate 13-acetate. Mol Endocrinol 10:62–75.

Wu SQ, Minami T, Donovan D, Aird WC. 2002. The proximal serum response element in the Egr-1 promoter mediates response to thrombin in human endothelial cells. Blood 100:4454–4461.