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Upregulation of rat *Ccnd1* gene by exendin-4 in pancreatic beta cell line INS-1: interaction of early growth response-1 with *cis*-regulatory element

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Abstract *Aims/hypothesis:* The aim of this study was to investigate the effect of exendin-4 on the expression of cyclin D1 gene (*Ccnd1*), which is critical in regulating the progression of the cell cycle in INS-1 cells. *Materials and methods:* INS-1 cells were stimulated with exendin-4 (10 nmol/l). Transient transfection and luciferase reporter assays were performed to measure promoter activities of rat *Ccnd1*. Electrophoretic mobility shift and chromatin immunoprecipitation assays were used to examine the binding of transcription factors to sites responsive to exendin-4 in vitro and in vivo, respectively. *Results:* Exendin-4 increased both *Ccnd1* mRNA and its protein levels in a time-dependent manner. The region from -174 to +130 of the promoter was found to contain *cis*-regulatory elements responsible for exendin-4-mediated gene induction. Early growth response-1 (EGR1) protein was bound to the region from -153 to -134, which includes the putative EGR1 binding site (5'-CACCCCCG-3'). Moreover, exendin-4 recruited

EGR1 protein to the promoter in vivo. *Conclusions/interpretation:* These findings suggest that exendin-4 activates *Ccnd1* transcription through induction of EGR1 binding to a *cis*-regulatory element between -153 and -134 on the rat *Ccnd1* promoter. These results provide an important indication that exendin-4 is a growth factor regulating beta cell proliferation.

Keywords CCND1 · Cyclin D1 · Early growth response-1 · Exendin-4 · INS-1 cell

Abbreviations AP2: activating protein 2 · ATF: activating transcription factor · CCND1: cyclin D1 · ChIP: chromatin immunoprecipitation (assay) · CREB: cyclic AMP-responsive element binding protein · EGR1: early growth response 1 protein · GLP-1: glucagon-like peptide-1 · IEGs: immediate early genes · SP1: specific protein 1 · STAT5: signal transducer and activator transcription 5

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Introduction

Glucagon-like peptide-1 (GLP-1) is a potent glucorecretin hormone secreted from the intestinal L cells [1, 2]. It displays pleiotropic effects including beta cell proliferation and insulinotropic action [3–6]. Several immediate early genes (IEGs), which are implicated in the regulation of cell replication, are induced by GLP-1 [7]. The IEGs include the transcription factors *c-fos*, *c-Jun*, Jun B and Zif268. In pancreatic beta cells, the activator protein-1 (AP1) transcription factor consists of *c-fos* and *c-Jun* and is involved in the mitogenic signal for insulin receptor substrate [8]. Zif268, also known as EGR1, has been suggested to mediate beta cell growth as well as adaptive responses to sustained glucose stimulation [9]. Moreover, GLP-1 activates PI3-K and extracellular regulated kinase to induce target genes such as *Fos* (*c-fos*) *Jun* (*c-Jun*) and *Egr1* (*zif268*), leading to beta cell proliferation [6, 10]. Thus, it is conceivable that GLP-1 might activate the cell cycles through induction of these transcription factors for proliferation of beta cells.

The mammalian cell cycle is mainly regulated by cyclins that are associated with cyclin-dependent kinases [11, 12]. In particular, cyclin D1 (CCND1) plays a key role in progression of the cell cycle from the G1 to the S phase. *Ccnd1* expression appears to be predominantly regulated at the transcriptional level, although post-transcriptional mechanisms are also involved [13, 14]. The promoter region of *Ccnd1* contains multiple potential *cis*-regulatory elements, including binding sites for AP1, signal transducer and activator transcription 5 (STAT5), EGR1, specific protein 1 (SP1) and activating transcription factor (ATF)/cyclic AMP-responsive element binding protein (CREB), which are important for the transcriptional activation of *Ccnd1* [15–17]. The AP1 binding site has been shown to be involved in angiotensin II-induced activation of the *Ccnd1* promoter in human adrenal cells [15]. The STAT5 binding site has been implicated in cytokine-dependent proliferation of haematopoietic cells [16]. Oestrogen-induced activation of *Ccnd1* was shown to be mediated by the ATF/CREB binding site, in which ATF2 and *c-Jun* formed heterodimers [17]. These studies suggest that the transcriptional regulation of *Ccnd1* may occur in a cell- and stimulus-specific manner. However, little is known about the transcriptional regulation of *Ccnd1* in pancreatic beta cells in response to GLP-1.

We examined the effect of exendin-4, a potent GLP-1 agonist, using INS-1 cells as a model for pancreatic beta cells, on the expression of *Ccnd1*, and on the *cis*-regulatory regions and the *trans*-acting factors involved in the transcriptional activation of the gene.

Materials and methods

Materials

Cell culture media, exendin-4, poly(dI-dC) and cycloheximide were purchased from Sigma-Aldrich (St Louis, MO, USA). pGL3 basic, pSV40- β -galactosidase control vector and luciferase assay reagent were from Promega (Madison, WI, USA). pCR3.1 vector and Lipofectamine-Plus reagent were from Invitrogen (Carlsbad, CA, USA). Anti-mouse CCND1 (556470) was from Cell Signaling (Beverly, MA, USA). Polyclonal anti-SP1 (1C6), anti-EGR1 (C-19), anti-AP2 (C-18), anti-tubulin (T 4026) antibodies, and EGR1, SP1 and AP2 consensus sequences were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Quikchange Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA, USA).

Isolation of pancreatic islets and culture of INS-1 cells

Primary pancreatic islets were isolated from Sprague–Dawley rats (Daehan Biolink Company, Chungbuk, Korea) using collagenase digestion and Ficoll gradient separation, as described previously [18]. INS-1 cells (between passages 10 and 19) were cultured in RPMI 1640 medium supplemented with 5.6 mmol/l D-glucose, 10% FBS,

2 mmol/l L-glutamine, 50 μ mol/l 2-mercaptoethanol, 1 mmol/l pyruvate, streptomycin (100 U/ml), and penicillin (100 U/ml) at 37°C under a 5% CO₂ atmosphere.

Western blot analysis

Western blot analysis was done as described previously [18]. Briefly, 30 μ g of total protein was separated on a 10% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membrane was blocked. The blots were then immunoblotted with anti-CCND1, anti-tubulin or anti-EGR1 antibodies. Following incubation with the secondary antibodies, the membrane was visualised with a chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA).

Northern blot analysis

Northern blot analysis was performed as described previously [18]. Briefly, total RNA was extracted using STAT60 Reagent (TelTest, Friendswood, TX, USA) according to the manufacturer's instructions. Samples (15 μ g) were fractionated in a 1% formaldehyde agarose gel and transferred onto a nylon membrane (Schleicher & Schuell). Hybridisation was performed in a rapid hybridisation buffer (Amersham biosciences) with a rat *Ccnd1* riboprobe labelled with [³²P]-dCTP (specific activity 111 TBq/mmol; PerkinElmer Life Science, Boston, MA, USA). The blot was washed under high-stringency conditions and was subjected to autoradiography.

Construction of serial-deletion rat *Ccnd1* promoters

A 827-bp fragment of rat *Ccnd1* promoter was amplified by PCR using 1 μ g of the genomic DNA from INS-1 cells. The PCR primers used were as follows: sense primer with the *KpnI* site (underlined), 5'-TGGGTACC GCCAGGATAAACCGGTCACTGTAAGAAG-3', antisense primer with *XhoI* (underlined), 5'-GGTAAGCTT GTGGGCTGTGGTCCGGGTGTTGGGTC-3'. The amplified product was cloned into pGL3-basic vector (pCD1-827). To prepare serial deletion constructs, PCR was performed using pCD1-827 construct as a template. The sense primers with *KpnI* sites were as follows: pCD1-625, sense primer (5'-TGGGTACCCCTTTGCAACCACCC CAGTGC-3'); pCD1-446, sense primer (5'-TGGGTACC GAAACGCGCCCATCTCTCTCC-3'); pCD1-174, sense primer (5'-TGGGTACCGGCAGGCTTTGGGTTTGTCC-3'); pCD1-73, sense primer (5'-TGGGTACCGGCTTT GATCTCTGCTTAACAAC-3'); pCD1-34, sense primer (5'-TGGGTACCCTACGGAGTTTTGTTGAAGTTG-3'). The antisense primer was the same as that used for the construction of the pCD1-827.

Site-directed mutagenesis

The pCD1mEgr-827 reporter plasmid was prepared using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol. The top strand of the primer set was as follows: 5'-GTTTGTCCCCCCCCACCC TTGCCGCTCACTGCTC-3'. The two underlined nucleotides in the high GC box were changed from C (wild type) to T. The mutation was verified by DNA sequence analysis.

Transient transfection and luciferase activity assays

Constructs of the rat *Ccnd1* promoter (1 µg/well) were transiently transfected into INS-1 cells using Lipofectamine-Plus reagent. After 6 h of transfection, media were replaced with normal culture media and incubated for an additional 24 h. Transfected cells were serum-starved for 24 h and then stimulated with exendin-4 for 18 h. To control transfection efficiency, cells were cotransfected with 100 ng of the internal control vector (pSV40-gal). Luciferase activities were normalised to β-galactosidase activity and data were expressed relative to the unstimulated value of the pCD1-827 construct.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed with a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Grand Island, NY, USA) according to the manufacturer's instructions. The conditions for PCR were as follows: 30 s at 95°C, 30 s at 55°C and 30 s at 72°C for 25 cycles, with final extension for 10 min at 72°C. Primers for ChIP were as follows: forward, 5'-CGGCGATTTGCATATCTACGAAGG-3'; reverse, 5'-AAGCCGGGCAGAGAAAAGGAG-3'.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Preparation of nuclear proteins from INS-1 cells and electrophoretic mobility shift assays (EMSAs) was performed as described previously [15]. In supershift studies, 2 µg of each antibody was preincubated with the nuclear extract for 10 min at 4°C before addition of the labelled probe. In competition experiments, the nuclear extract was incubated with a 100-fold molar excess of the appropriate unlabelled competitor oligonucleotides. Oligonucleotides used were as follows: oligo 3' (-114 to -87), 5'-TGCGCCCGCCTCGGCCGCCCCCTCC-3'; oligo 5' (-153 to -134), 5'-CCCACCCCGCCGCTCACTG-3'; oligo 5' EGR1 mutant, 5'-CCCACCCtGCCGCTCAC TG-3'; EGR1 consensus, 5'-GGATCCAGCGGGGCGC GAGCGGGGGGCGA-3'; SP1 consensus, 5'-TGAAGCC CCGCCCCAACGGA-3'; and AP2 consensus, 5'-GATC GAACTGACCGCCGCGGCCGT-3'.

Statistical analysis

The relative band densities were quantified using Scion Imaging software (Scion Corporation, Frederick, MD, USA). Data were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA and Student's *t*-test. Statistical significance was accepted at $p < 0.05$.

Results

Effect of exendin-4 on *Ccnd1* expression

INS-1 cells were serum-starved for 24 h and treated with exendin-4 (10 nmol/l). Total cellular RNA or protein was extracted at different periods. Exendin-4 significantly increased *Ccnd1* expression. *Ccnd1* mRNA level reached

Fig. 1 Induction of *Ccnd1* expression. **a** Northern blot analysis. Data are expressed as mean and SD density ratio of *Ccnd1* mRNA to 28S RNA from three independent experiments. * $p < 0.05$ vs unstimulated control. **b** Western blot analysis. Data are expressed as mean and SD density ratio of CCND1 to tubulin from three independent experiments. * $p < 0.05$ vs unstimulated control

its maximum at 3 h and returned to control level by 12 h (Fig. 1a). CCND1 protein level peaked at 6 h and had declined by 12 h (Fig. 1b).

Mapping the *cis*-regulatory elements responsible for exendin-4-induced transcriptional activation on the rat *Ccnd1* promoter

To evaluate the transcriptional activation of rat *Ccnd1* by exendin-4, the promoter activities were examined. Putative binding sites for *trans*-acting factors on the promoter are shown (Fig. 2a). Results of the transient transfection assay revealed that exendin-4 increased the activities of the pCD1-827, -625, -446 and -174 constructs approximately 2.6-fold. However, the stimulatory effect of exendin-4 was reduced to about 1.6-fold in pCD1-73 and -34 (Fig. 2b), suggesting the location of the *cis*-regulatory elements, which are mainly involved in exendin-4-induced transcriptional activation of *Ccnd1*, between -174 and -73.

Exendin-4 induction of specific interaction of nuclear protein with the region between -153 and -134 on the rat *Ccnd1* promoter

The proximal region of rat *Ccnd1* promoter contains several important *cis*-regulatory elements, such as NFκB, SP1, CREB and two putative EGR1 binding sites (Fig. 3). Since the two EGR1 sites (-151 to -143 and -108 to -100) are located between the sequences of -174 and -73, it is necessary to examine the involvement of these EGR1 binding sites of rat in exendin-4-induced *Ccnd1* activation. The interaction of these EGR1 binding sites with nuclear transcriptional factors was tested by EMSAs. Two radioactively labelled synthetic oligonucleotide probes (-153 to -134, oligo 5' and -114 to -87, oligo 3') bearing each sequence of the putative EGR1 binding sites were prepared. Incubation of the oligo 3' probe with nuclear extract of the INS-1 cells resulted in the binding of the oligonucleotide to multiple nuclear protein complexes. The binding activities were not influenced by exendin-4 treatment (Fig. 4a). However, when oligo 5' was used as a probe, one protein complex was induced within 30 min, continued until 1 h, and then declined progressively up to 4 h (Fig. 4b, arrow), suggesting the involvement of the putative EGR1 binding site of -151 to -143 in the

Fig. 2 The region responsible for induction of the rat *Ccnd1* promoter. **a** Schematic diagram of the full-length and 5' serially deleted promoter-luciferase constructs. Numbers at the top indicate the positions of the putative binding sites for regulatory factors

starting from the transcription initiation site. **b** Promoter analysis of *Ccnd1* deletion constructs. Data are mean and SD from three independent experiments. Filled bars, stimulated with exendin-4; open bars, unstimulated. * $p < 0.05$ vs unstimulated self control

Fig. 3 Sequence comparison of the region from -174 to $+130$ in the rat *Ccnd1* promoter with the corresponding sequence in human *CCND1*. The putative *cis*-regulatory elements are indicated by boxes. Alignment of the sequences was based on the transcription

start site and generated using the BioEdit program (version 7.0.5.2). The accession numbers of the genes are as follows: human, Z29078; rat, AB042564

interaction with nuclear protein to activate the *Ccnd1* promoter in response to extendin-4.

Characterisation of the nuclear protein interacting with the region between -153 and -134 on the rat *Ccnd1* promoter

To characterise the nuclear proteins that were bound to the oligo 5' probe, a supershift assay was performed. Because the oligo 5' sequence is G+C-rich and contains putative EGR1 and SP1 binding sites, antibodies against EGR1 and SP1 were used. Antibody against AP2 transcription factor was used as control. Extendin-4-induced nuclear protein complex was completely abolished by preincubation with anti-EGR1 antibody but not with anti-SP1 or anti-AP2 antibodies (Fig. 5a). The competition assay also confirmed the binding specificity of EGR1 to the oligo 5' probe. As shown in Fig. 5b, a 100-fold molar excess of unlabelled oligo 5' entirely competed away the binding activities of all complexes (lane 2). The extendin-4-induced complex was specifically competed away with a 100-fold molar excess of unlabelled oligonucleotide bearing EGR1 consensus sequence. The oligonucleotides bearing a mutant oligo 5', consensus AP2 or SP1 sequence were without effect. Thus,

it appears that the nuclear protein that was bound to the region between -153 and -134 of oligo 5' in response to extendin-4 is EGR1.

Functional relevance of the EGR1 binding site in the rat *Ccnd1* promoter activities

To determine whether the putative EGR1 site of the rat *Ccnd1* described earlier was responsible for transducing the extendin-4 signal, the putative EGR1 binding sequence (-151 CACCCCGC -143) within the *Ccnd1* promoter construct (pCD1 -827) was mutated to CACCCTTGC by site-directed mutagenesis. The wild-type or mutant EGR1 construct was transiently transfected into INS-1 cells and the promoter activity of each construct was analysed. As shown in Fig. 6, the mutant EGR1 construct showed a 34% decrease in luciferase activity compared with the wild-type construct, indicating the involvement of an intact EGR1-binding site on the proximal *Ccnd1* promoter sequence in stimulating the expression of the rat *Ccnd1* promoter by extendin-4.

Fig. 4 Interaction of nuclear proteins with 5' putative EGR1 binding site. Nuclear extracts were prepared after incubation of INS-1 cells in the absence (zero time) or presence of exendin-4 (10 nmol/l) for the indicated time points. End-radiolabelled oligonucleotides

containing the 3' (a) or 5' (b) putative EGR1 binding site were incubated with the nuclear extracts. Experiments were repeated independently four times

Recruitment of EGR1 to rat *Ccnd1* promoter in vivo by exendin-4

The effect of exendin-4 in inducing the recruitment of EGR1 protein to the rat *Ccnd1* promoter to activate the gene was investigated in vivo by ChIP assay. Purified DNAs were amplified by standard PCR using a pair of primers flanking the putative EGR1 binding region (–213 to –69) of the rat *Ccnd1* promoter (Fig. 7a). As shown in Fig. 7b, in vivo binding of EGR1 to the *Ccnd1* promoter was clearly increased in response to exendin-4, while the binding of SP1 was not changed, indicating the recruitment of EGR1 to the chromatin around the exendin-4-responsive region.

Effect of exendin-4 on EGR1 production in INS-1 cells and primary pancreatic islets

We determined the kinetics of protein production. Production of EGR1 protein was induced maximally between 30 and 60 min and had declined rapidly by 240 min in response to exendin-4 (Fig. 8a). EGR1 is an immediate-early response protein that is newly synthesised upon stimulation [19, 20]. Thus, for the interaction of EGR1 with the *Ccnd1* promoter, de novo EGR1 protein synthesis is required. Serum-starved INS-1 cells pretreated with cycloheximide (10 µg/ml), a protein synthesis inhibitor, for 1 h and exposed to exendin-4 for an additional 1 h failed to induce the endogenous EGR1 protein and to form the EGR1/DNA complex (Fig. 8b, arrow), suggesting the requirement of the newly synthesised EGR1 protein for the formation of the EGR1/DNA complex. Next, we examined the effect of newly synthesised transcription factors on the production of CCND1 protein. Pretreatment with cyclo-

Fig. 5 Characterisation of the binding activity of 5' putative EGR1 binding site. **a** A supershift assay was performed with anti-EGR1, anti-SP1 and anti-AP2 antibodies. **b** A competition experiment was performed using a 100× molar excess of unlabelled oligonucleotides

containing the wild-type, mutant EGR1 or three consensus EGR1, AP2 or SP1. Nuclear extracts were prepared from the exendin-4-stimulated INS-1 cells for 1 h. Experiments were repeated independently three times

heximide inhibited CCND1 production induced by exendin-4 below the unstimulated level (Fig. 8c). The inductive response to exendin-4 also occurred in primary pancreatic islets. As shown in Fig. 8d, the islets significantly induced the EGR1 and CCND1 proteins.

Discussion

Exendin-4, a potential therapeutic agent in the treatment of type 2 diabetes, has been known as a growth factor in beta cells as well as in islets [21, 22]. The molecular mechanisms governing the regulation of beta cell proliferation by exendin-4 are not well understood. Therefore, the aim of this study was to investigate the transcriptional

regulation of *Ccnd1* by exendin-4 in INS-1 cells employing serial deletion constructs of rat *Ccnd1* promoter.

In this study, we found that exendin-4 induced *Ccnd1* mRNA and subsequent protein expression in INS-1 cells. The involvement of CCND1 protein in beta cell proliferation has recently been verified by others: overexpression of either *Ccnd1* alone or in combination with *Cdk4*-induced beta cell proliferation in rat and human pancreatic islets [23], and CCND1 protein was shown to be essential for normal postnatal pancreatic beta cell growth [24]. These studies, together with our results, implicate CCND1 protein in beta cell proliferation, although CCND2 was also reported to trigger the progression of beta cell replication in the early postnatal period [24, 25].

Fig. 6 Effect of mutation of the putative EGR1 site on the induction of the reporter gene. Luciferase activity induced by the wild-type (pCD1-827) and mutant EGR1 (pCD1mEgr-827) constructs. Data are mean and SD from five independent experiments. * $p < 0.05$ vs unstimulated self-control. # $p < 0.05$ vs pCD1-827 construct

We clearly demonstrated that the induction of *Ccnd1* expression by exendin-4 occurred predominantly at the

transcriptional level. Results of the promoter analysis revealed that the region between -174 and -73 was largely involved in *Ccnd1* promoter activity, implicating this region as a major exendin-4-responsive element. The importance of this region has also been emphasised in human *Ccnd1* promoters of oesophageal squamous carcinoma [26] and of Chinese hamster ovary cells stably overexpressing angiotensin II type 1A receptor [27]. Meanwhile, other *cis*-regulatory elements located between -73 and +130 of the promoter also appear to be involved in promoter activities in response to exendin-4. In fact, the CRE (cyclic AMP-responsive element) and c-Ets sites, located at -48 and -16, respectively, have been demonstrated to be involved in induction of the transcriptional activity of human *Ccnd1* [28, 29]. In particular, promotion of beta cell survival by GLP1 via the second messenger cyclic AMP was significantly reduced in mice deficient in CREB activity, leading to diabetes [30]. Thus, the increased activities of the -73 and -34 constructs in our promoter assay can be explained by the presence of these sites, which are conserved in both human and rat promoters (Fig. 3).

Our EMSAs demonstrated that the transcriptional activation of *Ccnd1* was mediated by binding of EGR1 to the

Fig. 7 Recruitment of EGR1 to the *Ccnd1* promoter in vivo.

a The amplified region of rat *Ccnd1* promoter and primers used in the chromatin immunoprecipitation assay. **b** PCR was performed to amplify the region from -213 to -69. The levels of binding of SP1 and EGR1 to the *Ccnd1* promoter region were expressed relative to the band intensity of the unstimulated control. *Input samples* represent 0.05, 0.01 and 0.002% of the total DNA, whereas the PCRs of the immunoprecipitations include 5% of the resuspended DNA. Data are expressed as mean ($\pm$ SD) from three independent experiments. * $p < 0.01$ vs unstimulated self control

Fig. 8 Effects of exendin-4 on EGR1 expression in INS-1 cells and in isolated rat pancreatic islets. Western blot analysis (**a**) was performed to determine the time course of EGR1 expression. **b** Nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) was performed with radiolabelled oligo 5' probe containing the putative EGR1 binding site. Western blot analysis of EGR1 (*below*) was performed with the same samples used in EMSA. Experiments were repeated independently three times. **c** Inhibition of exendin-4 induction of CCND1 protein by cycloheximide (CHX). * $p < 0.01$ vs unstimulated control, CHX or CHX plus exendin-4; # $p < 0.05$ vs unstimulated control. **d** Induction of EGR1 and CCND1 proteins by exendin-4 in rat pancreatic islets. The islets were treated the same way as INS-1 cells. Data are mean and SD density ratio of CCND1 to tubulin from three independent experiments. * $p < 0.05$ vs unstimulated control

site (−153 to −134) on rat *Ccnd1* promoter. The antisense strand (5'-GCGGGGGTG-3') of the putative EGR1 sequence perfectly matches a consensus EGR1 binding sequence, 5'-GCG(T/G)(G/A)GG(C/A/T)G-3' [31]. Specific EGR1 binding to this putative binding site was confirmed by immune-supershift and competition assays. Moreover, we showed that the rat *Ccnd1* promoter functionally interacted with EGR1 by site-directed mutation at the putative EGR1 site. These findings were confirmed by the ChIP assay, demonstrating a clear increase in EGR1 binding to the *Ccnd1* promoter by exendin-4 in vivo.

However, the oligo 3' probe that included another putative EGR1 binding site (−108 to −100) failed to form a complex with EGR1. In humans, this EGR1 binding site, overlapping two SP1 binding sites, corresponds to the consensus EGR1 site and is essential for the binding of EGR1 in order to activate *Ccnd1* transcription in response to growth factors [26, 27]. Comparing the sequences of the rat (5'-CGCCTCGGC-3') and human (5'-CGCCCCCGC-3'), the thymine and guanine at −104 and −102 in rats are replaced with cytosine in humans. Specifically, the cytosine residue at −102 has been described as a critical base for EGR1 binding [31]. Thus, the presence of a guanine residue at position −102 of the rat promoter may be unfavourable for EGR1 binding in our EMSA study.

The *cis*-regulatory sequence required for EGR1/SP1 binding is represented by the G+C-rich region of the promoter, in which EGR1 and SP1 binding sites generally overlap [26, 32, 33]. Therefore, a dual interplay between EGR1 and SP1 has been demonstrated for a number of gene promoters. In our EMSA using the oligo 5' probe, which contains potential SP1 binding sites overlapping the EGR1 site (Fig. 3), SP1 binding activity was not observed in the supershift and competition assays (Fig. 5). However, SP1 was constitutively bound to the promoter irrespective of exendin-4 treatment in the ChIP experiments. These results could have occurred because the amplified region contains consensus SP1 sequence (5'-CCCGCCCC-3', −100 to −93). Meanwhile, the oligo 3' probe containing the binding site of the consensus SP1 was supershifted by anti-SP1 antibody but not by anti-EGR1 or anti-AP2 antibodies (data not shown). These results are consistent with previous studies showing binding of EGR1 to an overlapping EGR1/SP1 site without displacement of SP1 in the human *Ccnd1* promoter [26, 27].

The EGR1 transcription factor, also known as Krox24, Zif268 or Tis8, is a zinc-finger protein that is a critical upstream regulator of growth, proliferation and differentiation. Expression of *Egr1* is rapidly induced by mitogens, insulin and glucose in different cells, including beta cells [19, 20, 34, 35]. In our experiments, the rapid and transient induction of EGR1 protein in response to exendin-4 (Fig. 8a) was consistent with previous studies [9, 34]. In addition, the time course of EGR1 protein induction was consistent with that of EGR1 binding to the radiolabelled oligo 5' probe (Fig. 4b). Moreover, the requirement for de novo EGR1 protein synthesis strongly supports the validity of our EMSA data in the experiment employing cyclohex-

imide. The results of the study on the newly synthesised transcription factors using cycloheximide indicate that constitutively expressed transcription factors, as well as inducible transcription factors such as EGR1, may contribute to the production of CCND1 protein.

In summary, we found that exendin-4 rapidly induced the EGR1 transcription factor and that EGR1 was bound to its putative binding site between −151 and −143 on the rat *Ccnd1* promoter, which is different from the functional EGR1 binding site (between −108 and −100) of humans. Through the EGR1-mediated induction of CCND1 protein, exendin-4 may contribute to beta cell proliferation.

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References

1. Deacon CF (2004) Therapeutic strategies based on glucagon-like peptide 1. *Diabetes* 53:2181–2189
2. Vilsboll T, Holst JJ (2004) Incretins, insulin secretion and type 2 diabetes mellitus. *Diabetologia* 47:357–366
3. Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL (2004) Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. *Diabetologia* 47:478–487
4. Stoffers DA, Kieffer TJ, Hussain MA et al (2000) Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes* 49:741–748
5. Buteau J, Foisy S, Joly E, Prentki M (2003) Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor. *Diabetes* 52:124–132
6. 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
7. Susini S, Roche E, Prentki M, Schlegel W (1998) Glucose and glucagon-like peptides synergize to induce *c-fos*, *c-Jun*, *junB*, *zif-268*, and *nur-77* gene expression in pancreatic beta(INS-1) cells. *FASEB J* 12:1173–1182
8. Schuppin GT, Pons S, Hugl S et al (1998) A specific increased expression of insulin receptor substrate 2 in pancreatic beta-cell lines is involved in mediating serum-stimulated beta-cell growth. *Diabetes* 47:1074–1085
9. Josefsen K, Sorensen LR, Buschard K, Birkenbach M (1999) Glucose induces early growth response gene (*Egr-1*) expression in pancreatic beta cells. *Diabetologia* 42:195–203
10. Montrose-Rafizadeh C, Avdonin P, Garant MJ et al (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
11. Hunter T, Pines J (1994) Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell* 79:573–582
12. Sherr CJ (1994) G1 phase progression: cycling on cue. *Cell* 79:551–555
13. Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S (1998) Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J Biol Chem* 273:14424–14429

14. Lin S, Wang W, Wilson GM et al (2000) Down-regulation of cyclin D1 expression by prostaglandin A (2) is mediated by enhanced cyclin D1 mRNA turnover. *Mol Cell Biol* 20: 7903–7913
15. Shiozawa T, Miyamoto T, Kashima H, Nakayama K, Nikaido T, Konishi I (2004) Estrogen-induced proliferation of normal endometrial glandular cells is initiated by transcriptional activation of cyclin D1 via binding of *c-Jun* to an AP-1 sequence. *Oncogene* 23:8603–8610
16. Matsumura I, Kitamura T, Wakao H et al (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J* 18:1367–1377
17. Lee RJ, Albanese C, Stenger RJ et al (1999) pp60 (v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60 (v-src) signaling in breast cancer cells. *J Biol Chem* 274: 7341–7350
18. Kim MJ, Ryu GR, Kang JH et al (2004) Inhibitory effects of epicatechin on interleukin-1 β -induced inducible nitric oxide synthase expression in RINm5F cells and rat pancreatic islets by down-regulation of NF- κ B activation. *Biochem Pharmacol* 68:1775–1785
19. Christy BA, Lau LF, Nathans D (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with 'zinc finger' sequences. *Proc Natl Acad Sci USA* 85:7857–7861
20. Sukhatme VP, Cao XM, Chang LC et al (1988) A zinc finger-encoding gene coregulated with *c-fos* during growth and differentiation, and after cellular depolarization. *Cell* 53:37–43
21. Gedulin BR, Nikoulina SE, Smith PA et al (2005) Exenatide (exendin-4) improves insulin sensitivity and β -cell mass in insulin-resistant obese fa/fa Zucker rats independent of glycemia and body weight. *Endocrinology* 146:2069–2076
22. 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
23. Cozar-Castellano I, Takane KK, Bottino R, Balamurugan AN, Stewart AF (2004) Induction of β -cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. *Diabetes* 53:149–159
24. Kushner JA, Ciemerych MA, Sicinska E et al (2005) Cyclins D2 and D1 are essential for postnatal pancreatic β -cell growth. *Mol Cell Biol* 25:3752–3762
25. Georgia S, Bhushan A (2004) β cell replication is the primary mechanism for maintaining postnatal β cell mass. *J Clin Invest* 114:963–968
26. Yan YX, Nakagawa H, Lee MH, Rustgi AK (1997) Transforming growth factor- α enhances cyclin D1 transcription through the binding of early growth response protein to a *cis*-regulatory element in the cyclin D1 promoter. *J Biol Chem* 272:33181–33190
27. Guillemot L, Levy A, Raymondjean M, Rothhut B (2001) Angiotensin II-induced transcriptional activation of the cyclin D1 gene is mediated by Egr-1 in CHO-AT (1A) cells. *J Biol Chem* 276:39394–39403
28. Nagata D, Suzuki E, Nishimatsu H et al (2001) Transcriptional activation of the cyclin D1 gene is mediated by multiple *cis*-elements, including SP1 sites and a cAMP-responsive element in vascular endothelial cells. *J Biol Chem* 276:662–669
29. Albanese C, Johnson J, Watanabe G et al (1995) Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* 270:23589–23597
30. Jhala US, Canettier G, Screaton RA et al (2003) cAMP promotes pancreatic β -cell survival via CREB-mediated induction of IRS2. *Genes Dev* 17:1575–1580
31. Swirnoff AH, Milbrandt J (1995) DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol Cell Biol* 15:2275–2287
32. Bahouth SW, Beauchamp MJ, Vu KN (2002) Reciprocal regulation of β (1)-adrenergic receptor gene transcription by Sp1 and early growth response gene 1: induction of EGR-1 inhibits the expression of the β (1)-adrenergic receptor gene. *Mol Pharmacol* 61:379–390
33. Khachigian LM, Williams AJ, Collins T (1995) Interplay of Sp1 and Egr-1 in the proximal platelet-derived growth factor A-chain promoter in cultured vascular endothelial cells. *J Biol Chem* 270:27679–27686
34. Keeton AB, Bortoff KD, Bennett WL, Franklin JL, Venable DY, Messina JL (2003) Insulin-regulated expression of Egr-1 and Krox20: dependence on ERK1/2 and interaction with p38 and PI3-kinase pathways. *Endocrinology* 144:5402–5410
35. Silverman ES, Collins T (1999) Pathways of Egr-1-mediated gene transcription in vascular biology. *Am J Pathol* 154: 665–670