ESSENTIAL INTERACTION OF EGR-1 AT AN ISLET SPECIFIC RESPONSE ELEMENT FOR BASAL AND GASTRIN-DEPENDENT GLUCAGON GENE TRANSACTIVATION IN PANCREATIC ALPHA CELLS

Stéphane Leung-Theung-Long, Emmanuelle Roulet, Pascal Clerc, Chantal Escrieut, Sophie Marchal-Victorion, Beate Ritz-Laser, Jacques Philippe, Lucien Pradayrol, Catherine Seva, Daniel Fourmy, Marlène Dufresne.

1 Inserm U531, IFR31, Hospital Rangueil, Toulouse, France
2 Diabetes Unit, University Hospital Geneva, Geneva, Switzerland

Running title: Gastrin stimulates glucagon gene expression

Address correspondence to: Marlène Dufresne, Inserm U531, IFR31, Hospital Rangueil, TSA 50032, 31059 Toulouse cedex 9, France, Tel. +33 5 61 32 24 05; Fax. +33 5 61 32 24 03; E-mail: dufresne@toulouse.inserm.fr

The peptide hormone gastrin is secreted from G cells of the gastric antrum and is the main inducer of gastric acid secretion via activation of its receptor the CCK2 receptor. Both gastrin and CCK2 receptors are also transiently detected in the fetal pancreas and believed to exert growth/differentiation effects during endocrine pancreatic development. We previously demonstrated that, while gastrin expression is extinguished in adult pancreas, CCK2 receptors are present in human glucagon-producing cells where their activation stimulates glucagon secretion. Based on these findings, we investigate, in the present study, whether gastrin regulates glucagon gene expression. To this aim, the CCK2 receptor was stably expressed into a glucagon-producing pancreatic islet cell line and a glucagon-reporter fusion gene was transiently transfected in this new cellular model. We report that gastrin stimulates glucagon gene expression in glucagon-producing pancreatic cells. By using 5' progressively increased sequences of the glucagon gene, gastrin responsiveness was located within the minimal promoter. Moreover, we clearly identified Egr-1 as an essential transcription factor interacting with the islet cell-specific G4 element. Egr-1 was shown to be essential for basal and gastrin-dependent glucagon gene transactivation. Furthermore, our results demonstrate that the MEK1/ERK1/2 pathway couples the CCK2 receptor to nuclearization and DNA binding of Egr-1. In conclusion, our data bring new informations concerning the transcriptional regulation of glucagon gene. Moreover they open new working hypothesis with reference to a potential role of gastrin in glucagon-producing pancreatic cells.

Gastrin, one important gastrointestinal regulatory peptide, was initially recognized as the main inducer of gastric acid secretion. Gastrin is secreted from endocrine G cells of the gastric antrum during post-natal and adult life in response to a meal (1). The CCK2 receptor, a G protein-coupled receptor, specifically mediates actions of gastrin on target cells via activation of multiple signaling enzymes including the phospholipase C with subsequent phosphoinositide breakdown, intracellular calcium mobilization and protein kinase C stimulation (2), but also intracellular mediators classically described in the regulation of mitogenesis and cellular adhesion by growth factors. The mitogen-activated protein kinases, ERK1/2, Jun kinase and p38MAPK, as well as the phosphatidylinositol 3-kinase are known targets of gastrin. Several groups, including ours, have documented the contribution of Src and Fak families tyrosine kinases upstream the activation of these pathways (3-6).

Besides a role in the stomach, several lines of evidence, including our data, raise the possibility that the endocrine pancreas is also a potential physiological target of gastrin. Indeed, owing to the presence of responsive elements that account for islet expression on its gene promoter, the major site of expression of amidated gastrin is the pancreas of mammals during fetal life (7,8). After birth, gastrin ceases to be expressed in the pancreas supporting a specific role played in this organ during the fetal period (9). We confirmed the presence of amidated gastrin in human fetal pancreas together with localization of CCK2 receptors.

Copyright 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
partly in undifferentiated cells and in glucagon-producing cells (10).

Additionally, and further supporting a role in the endocrine pancreas, we previously demonstrated that gastrin may contribute to glucose homeostasis in adult via stimulation of glucagon secretion. Indeed, expression of CCK2 receptor on adult pancreatic glucagon cells was linked to a physiological secretion of glucagon from isolated human islets in response to gastrin (10). Of importance, the essential role of gastrin in the normal islet glucagon counterregulatory response to hypoglycemia has been recently confirmed in mice knockout for its gene (11).

Although glucagon is the second major hormone in the control of glucose homeostasis, acting as a counterregulator to insulin, the regulation of its secretion, biosynthesis and gene expression by nutrients and other factors has not been as intensively investigated as in the case of insulin. However, the metabolic consequences of abnormal α cells function are well defined. Indeed, chronic hyperglucagonemia is a common feature of type II diabetic patients and is partially responsible of hyperglycemia (12). Conversely, loss of glucagon response impairs recovery from hypoglycemia in non-diabetic but is also a major factor in the susceptibility of patients with type I diabetes to prolonged and severe hypoglycemia (13). There is evidence that increased glucagon gene expression accompanies hyperglucagonemia but also that regulation of glucagon gene expression may differ from that of glucagon secretion (14). Effects of gastrin on the expression of glucagon are therefore important to consider.

In the present study, we investigated whether gastrin regulates glucagon gene expression. To this aim, and to characterize the molecular mechanisms involved in this effect, we engineered a new cellular model derived from the glucagonoma cell line InR1G9 (15) stably expressing the human CCK2 receptor. We now report that gastrin stimulates glucagon gene expression and identify Egr-1 as an essential transcription factor for basal and gastrin-stimulated glucagon gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** The glucagon-producing hamster InR1G9 cell line was stably transfected with a vector (PRFEneo) expressing the human CCK2 receptor using Fugene™ 6 reagent (Roche) as previously described (6) and grown at 37°C in a 5% CO₂ humidified atmosphere, in RPMI 1640 with Glutamax-I (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Biowhitaker), 50 units/ml of penicillin (Invitrogen), 50 μg/ml of streptomycin (Invitrogen) and 200 μg/ml of genetin (Sigma).

**Receptor binding assay.** Approximately 24 h after their transfer to 24-well plates (20,000 cells/well), cells were washed with phosphate-buffered saline (PBS), pH 6.95, 0.1% BSA and then incubated for 60 min at 37°C in 0.5 ml of RPMI, 0.1% BSA with 60 pM sulfated 125I-CCK9 in the presence of competing CCK9. Cells were washed twice with cold PBS, pH 6.95, containing 2% BSA, and cell-associated radioligand was collected with 0.1 N NaOH added to each well. The radioactivity was counted in a γ counter (Auto-Gamma, Packard, Downers Grove, IL).

**CCK2-receptor fluorescent probe.** The [Thr28,Ahx31]CCK-(25-33) peptide (CCK9) was derivatized with fluorescein isothiocyanate (FITC) (Sigma). The peptide was dissolved in 0.1 M borate buffer pH 9 and reacted overnight with an equimolar quantity of fluorescent label. The fluorescent probe was purified by reverse phase HPLC.

**CCK2 receptor localization.** Cells were cultured on glass slides overnight at 37°C. Cells were washed twice with PBS containing 0.2% BSA and incubated with 50 nM FITC-coupled CCK9 for 1 h at 4°C. Cells were then fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and slides were mounted.

**Glucagon secretion and radioimmunoassay.** Cells were cultured for 1 h in serum-free medium, containing 0.1% BSA, in 24-well plates (50,000 cells/well). Cells were incubated in 1 ml of RPMI, 0.1% BSA, containing CCK or gastrin at 37°C for 1 h. Media were then collected and stored at -20°C until use. Secretion of glucagon was quantified on 50 μl of media by radioimmunoassay using 16 pM 125I-glucagon, and an antibody raised against the carboxy terminus of glucagon (Gan8, 1:75,000, generously provided by Dr. D. Bataille, Inserm U376, Montpellier, France). Free glucagon was separated from antibody-bound glucagon adding 4 mg/ml activated charcoal and 5 mg/ml dextran T70 for 15 min at 4°C. After centrifugation at 2,000 g during 20 min, supernatants, containing glucagon-antibody complexes, were collected and radioactivity was counted in a γ counter (Auto-Gamma, Packard, Downers Grove, IL).
Northern blot analysis. Cells (1.5x10^6) were cultured in 60 mm-culture dishes overnight in serum-free medium, containing 0.1% BSA, for 24 h at 37°C before stimulation with gastrin (1 µM). Total cellular RNA was extracted by Trizol® reagent (Invitrogen). RNA samples (10 µg/lane) were separated on formaldehyde-containing 1% agarose gel and blotted onto nylon membrane. The blot was hybridized with glucagon cDNA probe labeled with [α-32P]dCTP by random primer extension. Specific hybridization was visualized after exposure to PhosphorImager (Molecular Dynamics). To ensure RNA integrity and to confirm equal loading between lanes, membrane was rehybridized with a probe for 18S rRNA.

Glucagon mRNA stability. Glucagon mRNA half-life was determined from an actinomycin D decay curve. Actinomycin D (5 µg/ml) (Sigma) was added to the medium of both control and gastrin-stimulated cells. Total cellular RNA was extracted 4, 8 and 12 h after addition of gastrin. Levels of glucagon mRNA were then analyzed by Northern blot.

DNA transfection. Cells (4x10^6) were plated in 100 mm-culture dishes 24 h before co-transfection. They were transfected using the DEAE-dextran method, with 3 µg of reporter gene plasmid (pOCAT) and 1 µg of pRSV-Luc, a transfection control plasmid. Reporter gene plasmid consisted of either rat wild-type glucagon gene -2.5 kb fragment (nucleotides -2.5 kb to +58) or 292-bp fragment (nucleotides -292 to +58), 169-bp fragment (nucleotides -169 to +58) or 138-bp fragment (nucleotides -138 to +58) of the 5'-flanking sequence of the rat glucagon gene, linked to the reporter gene CAT (-2.5 kb CAT, -292 CAT, -169 CAT and -138 CAT respectively). When indicated, -138 CAT was mutated at nucleotides -135/-130, -120/-115, -107/-102, -89/-88, -72/-71 or -56/-55 (called E47/β2 mut, 120 mut, βTF1 mut, Pax6 mut, Cdx2/3 mut, and Isl1 mut respectively).

Chloramphenicol acetyl transferase assay. 24 h after transfection, cells were transferred into 60 mm-culture dishes and cultured in serum-free medium with 0.1% BSA overnight at 37°C. Cells were then stimulated or not with gastrin for 24 h in RPMI 1640, 0.1% BSA. When indicated, inhibition of MEK1 was achieved incubating serum-starved cells with the specific inhibitor of MEK1, PD98059 (50 µM) for 1 h at 37°C before stimulation with gastrin. Cells were washed with cold PBS, trypsinized and centrifuged 5 min at 100 g at 4°C. Cells were washed twice with cold PBS, resuspended carefully in 400 µl of a 10 mM HEPES buffer pH 7.9 containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM DTT and 1.2 mM PMSF, and incubated for 15 min on ice. 30 µl of 10% Nonidet P-40 was added, mixture was vortexed and centrifuged 1 min at 4°C at 10,000 g. Pellets were incubated and shaken at 4°C for 15 min with 60 µl of a 20 mM HEPES buffer pH 7.9 containing 25% glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM DTT and 1.2 mM PMSF, centrifuged for 10 min at 15,000 g at 4°C and supernatants were stored at -80°C until use.

Electrophoretic mobility shift assays (EMSAs). Double-stranded oligonucleotide probe 120 corresponding to the nucleotides 129/-108 (5'-AGCACAGATGGGGCGGTGGAATTT-3') of the glucagon gene was 5' end-labeled with [32P]dATP using T4 Kinase (Invitrogen). Binding reactions were performed at room temperature for 20 min in a final volume of 20 µl binding buffer containing 20 mM HEPES pH 7.9, 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 50 mM KCl, 50 µg/ml BSA, 10 mM DTT, 10 µM ZnSO₄, 1 µg of polydeoxyinosinic-deoxyctydilic acid, 10 fmol of oligonucleotide probe and 10 µg of nuclear proteins. For
supershift experiments, 1 µg of a rabbit anti-Egr-1 antibody (sc-110 X, Santa Cruz Biotechnology Inc., Heidelberg, Germany) was incubated with nuclear proteins for 20 min at room temperature followed by an incubation period of 10 min on ice before addition of the radiolabeled probe. For competition experiments, 50-, 100-, or 200-fold excess of non-radioactive probe 120 mutated (5’-AGCAGAGTTGATAGTGAAT-3’) or not, or non-radioactive Egr-1 consensus site probe (5’-GGATCCAGCGGGGGCGAGCGGGGGCGAG-3’), was incubated with nuclear proteins 10 min at room temperature before addition of the radiolabeled probe 120. DNA-protein complexes were separated on a 6% nondenaturing polyacrylamide gel containing 0.1 M Tris, 90 mM boric acid and 1 mM EDTA at 150 V for 6 h at 4°C, dried and analyzed with PhosphorImager scanning (Molecular Dynamics).

Cloning of glucagon gene promoter from InR1G9-CCK2 cells. InR1G9-CCK2 genomic DNA was purified according the manufacturer's procedures (QIAamp DNA mini kit, Qiagen). A primer pair for PCR amplification (5’-ATCAAGGGATAAGACCCTCAAATGA-3’ as the forward and 5’-GGGAACCTTGAGTGTTCTTGCG-3’ as the reverse) was developed from conserved structures in the DNA sequences of rat, mouse and human glucagon gene promoters in databases. PCR was carried out in a total volume of 100 µl according to the manufacturer's procedures (Invitrogen). The amplification reaction involved denaturation at 95°C for 5 min, followed by 30 cycles as follows : 95°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1.5 min. After cycling, a terminal elongation of 10 min at 72°C was performed, and PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen). Plasmids DNA were transferred into E. coli and isolated by mini-prep (Macherey-Nagel). Both strands of PCR products were sequenced using M13 reverse and M13 forward primers (Applied Biosystem). The partial hamster glucagon gene promoter sequence is available under the GenBank accession number AY842856.

Chromatin immunoprecipitation assays (ChIP). Cells (20x10^6) were plated in 150 mm culture dishes. Before stimulation with gastrin (1 µM) for 1 h, cells were cultured overnight in serum-free medium with 0.1% BSA. Chromatin cross-linking was performed by adding 1% formaldehyde to the InR1G9-CCK2 cells at room temperature for 8 minutes. The reaction was stopped by adding glycerol to a final concentration of 0.125 M. Cells were then washed twice with ice-cold PBS, collected in 5 ml PBS, and harvested by brief centrifugation. Cells were resuspended in ice-cold cell lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% NP-40, protease inhibitors: 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM benzamidine), incubated on ice for 5 minutes, and briefly centrifuged. Cells were then resuspended in nuclear lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% sarcosyl, 0.5 M NaCl, protease inhibitors). Sonication of the cells was performed for 10 seconds in 100 µl of TEN buffer (10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl) with a Branson 250 sonifier. Chromatin were immunoprecipitated with 10 µg of rabbit polyclonal anti-Egr-1 (sc-110 X, Santa Cruz Biotechnology Inc.). A normal rabbit IgG (sc-2027, Santa Cruz Biotechnology Inc.) was used as the non-specific antibody control. After incubation with 12 µl protein A sepharose (Amersham Bioscience, Uppsala, Sweden) for 1.5 hour at room temperature, the beads were washed 7 times in RIPA buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 0.5 M LiCl, 0.7% sodium deoxycholate, 1% NP-40), and once in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and extracted twice with 100 µl of elution buffer (100 mM Tris-HCl pH 8.0, 1% SDS). Eluates were pooled, treated with proteinase K (100 µg/ml) for 1 hour at 42°C, heated at 65°C overnight to reverse formaldehyde cross-linking and purified using a QIAquick PCR purification kit (Qiagen). Purified immunoprecipitated DNA was analyzed by real-time PCR using a LightCycler (Roche Diagnostics). Primers used for the analysis of the hamster glucagon promoter were: 5’-CAAAGCGAGTGGGTGAGTG-3’ and 5’-GCCACGCAGATATTACGGTG-3’ yielding an amplification product of 133 bp. The amount of PCR product was calculated from standard curves obtained from PCR with the same primers and serially diluted total DNA. Data were expressed as fold-differences relative to control conditions, in which normal rabbit serum was used instead of specific anti-Egr-1 antibody.

Western blot analysis. For study of nuclear Egr-1 accumulation, 20 µg of nuclear protein extracts were separated by SDS-PAGE and blotted on polyvinylidene difluoride.
membrane (PerkinElmer). Western blot analysis was performed using a rabbit anti-Egr-1 antibody (1:10,000, Santa Cruz Biotechnology Inc., Heidelberg, Germany). To demonstrate equivalent protein loading, membranes were reprobed with a mouse anti-GAPDH antibody (1:500, Chemicon International Inc., Temecula, CA). For study of ERK1/2 activation, 50 µg of cytoplasmic protein extracted as previously described (5) were separated by SDS-PAGE and blotted on membranes. Western blot analysis was performed using a goat anti-ERK1/2 Mitogen Activated Protein Kinases (MAPKs) (1:10,000, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and a mouse antibody that specifically recognized the phosphorylated forms of ERK (1:10,000, Cell Signaling Technology, Inc., Beverly, MA). Membranes were incubated with peroxidase-coupled secondary antibodies (1:10,000, Pierce, Bezons, France) and proteins were detected using the enhanced chemiluminescence system (ECL, Amersham Biosciences).

Phosphorylation. Cells (4x10^6) were plated in 100 mm-culture dishes overnight at 37°C in serum-free RPMI 1640 medium without phosphate (ICN), containing 0.1% BSA. 200 µCi of [32P]inorganic phosphate (PerkinElmer) was added to medium 2 hours before stimulation with gastrin (1 µM). After 1 h of stimulation with gastrin, whole nuclear extracts were prepared and were subjected to immunoprecipitation overnight at 4°C, as described previously (3) using a rabbit anti-Egr-1 antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) preadsorbed on protein A-Sepharose. Whole immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (PerkinElmer), that was then exposed to PhosphorImager. To confirm the identity of the immunoprecipitated radiolabeled proteins, the membrane was blotted with a rabbit anti-Egr-1 antibody (1:10,000, Santa Cruz Biotechnology Inc., Heidelberg, Germany) and revealed using the ECL system.

Immunocytochemistry. Cells were cultured on glass slides. Before stimulation with gastrin (1 µM) for 1 h, cells were cultured in serum-free medium with 0.1% BSA overnight at 37°C and preincubated with the MEK1 inhibitor, PD98059, when indicated. After stimulation, cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.2% Triton in PBS for 5 min at room temperature. After blocking non-specific background staining (Protein Block, DAKO S.A., Trappes, France) for 10 min at room temperature, cells were incubated with anti-Egr-1 antibody (1:5,000) in PBS overnight at 4°C. Egr-1 was localized using a Cy2-coupled anti-rabbit secondary antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For visualizing nuclei, cells were incubated with DAPI (4',6-diamidino-2-phenylindole) (0.4 µg/ml, Sigma Aldrich) in PBS for 2 min at room temperature before mounting slides.

Data analysis. Data are presented as means ± S.E.M. and statistical significance was determined using the Student’s t test. The threshold for statistical significance was a p value of less than 0.05.

RESULTS

Characteristics of InR1G9-CCK2 cells.

Studies that determined the factors important for the regulation of glucagon gene expression mostly used the InR1G9 cells. Indeed, this cell line that has similar characteristics of native islet alpha cells, producing stable and high level of glucagon, possesses the cellular machinery controlling glucagon gene expression. Therefore, to assess whether CCK2 receptor regulates glucagon gene in pancreatic alpha cells, we stably transfected the InR1G9 cells with a plasmid encoding the human CCK2 receptor cDNA. Selection of InR1G9 cell clones was based on their capacity to secrete glucagon in response to the CCK2 receptor agonists, CCK and gastrin, but also on the biphasic dose-response pattern of secretion as previously observed on isolated human islets of Langerhans (10). One clone was selected, InR1G9-CCK2, that responded dose-dependently to gastrin and CCK, with glucagon secretion being detectable at 10 pM of agonists, and reaching a maximum at 1 nM (18.1 ± 4.4 pmol/10^6 cells/h and 18.9 ± 4.7 pmol/10^6 cells/h for CCK and gastrin respectively) (Fig. 1A). Concentrations of CCK and gastrin eliciting half-maximal level of glucagon secretion were identical (0.09 ± 0.01 nM and 0.14 ± 0.01 nM for CCK and gastrin respectively). At supramaximal concentrations of agonists, glucagon secretion decreased to nearly 50% of maximal secretion for both agonists. Non transfected InR1G9 cells did not respond to
gastrin or CCK (data not shown) in accordance with the absence of CCK2 receptor expression (Fig. 1B). In agreement to expression of a CCK2 receptor with typical binding characteristics in InR1G9-CCK2 cells, the binding affinity of sulfated CCK was in the nanomolar range ($K_d = 3.1 \pm 0.9$ nM). The maximum number of CCK2 receptor binding sites seen by CCK was $2.1 \pm 0.4$ pmol/10$^6$ cells. Taken together, these results show that the selected InR1G9-CCK2 cells provides a new suitable experimental model for the study of the regulation of the glucagon gene by gastrin in alpha pancreatic cells.

**Gastrin stimulates glucagon gene expression.**

We first determined whether the level of glucagon mRNA varied in response to CCK2 receptor activation by performing Northern blot analysis. Glucagon mRNA and 18S rRNA were quantified from InR1G9-CCK2 cells stimulated with gastrin for up to 24 h. Gastrin increased glucagon mRNA abundance in a dose- and time-dependent manner. Indeed, stimulation was detectable with 1 nM gastrin from 6 h after induction (data not shown). Maximum stimulation (2.9-fold compared with non-stimulated cells) was observed at 24 h after stimulation with 1 µM of gastrin (Fig. 2A).

To eliminate the possibility that the increased level of glucagon mRNA resulted from a gastrin-dependent glucagon mRNA stabilization, we compared glucagon mRNA half-life in both control and gastrin-stimulated cells. For this purpose, actinomycin D, which completely blocks RNA synthesis, was added to InR1G9-CCK2 cells before incubation in the presence or not of gastrin for 4, 8 and 12 h. In these conditions, half-life of glucagon mRNA was identical in both control and gastrin-treated cells. For this purpose, actinomycin D, which completely blocks RNA synthesis, was added to InR1G9-CCK2 cells before incubation in the presence or not of gastrin for 4, 8 and 12 h. In these conditions, half-life of glucagon mRNA was identical in both control and gastrin-treated cells. Indeed, stimulation was detectable with 1 nM gastrin from 6 h after induction (data not shown). Maximum stimulation (2.9-fold compared with non-stimulated cells) was observed at 24 h after stimulation with 1 µM of gastrin (Fig. 2A).

To eliminate the possibility that the increased level of glucagon mRNA resulted from a gastrin-dependent glucagon mRNA stabilization, we compared glucagon mRNA half-life in both control and gastrin-stimulated cells. For this purpose, actinomycin D, which completely blocks RNA synthesis, was added to InR1G9-CCK2 cells before incubation in the presence or not of gastrin for 4, 8 and 12 h. In these conditions, half-life of glucagon mRNA was identical in both control and gastrin-treated cells. For this purpose, actinomycin D, which completely blocks RNA synthesis, was added to InR1G9-CCK2 cells before incubation in the presence or not of gastrin for 4, 8 and 12 h. In these conditions, half-life of glucagon mRNA was identical in both control and gastrin-treated cells. Indeed, stimulation was detectable with 1 nM gastrin from 6 h after induction (data not shown). Maximum stimulation (2.9-fold compared with non-stimulated cells) was observed at 24 h after stimulation with 1 µM of gastrin (Fig. 2A).

Identification of a gastrin response element on the G4 element.

To more precisely map the gastrin-responsive region of the glucagon gene promoter, we measured the transcriptional activity after mutation of the nucleotide sequences required for binding of transcription factors known to interact with this region (Fig. 4A). Isl-1, Cdx-2/3 and Pax-6 have been characterized for their ability to bind the G1 element whereas IEF1 or the E47/β2 heterodimer and the uncharacterized βTF1 were identified to interact with G4 element (Fig. 3A). Furthermore, it has been described that the G4 element possesses, in addition to E47/β2 and βTF1 binding sites, a cis-acting sequence, (region -120) interacting with an as yet unidentified transcription factor (Fig. 4A) (17). Results obtained after transfection of the different mutant plasmids into InR1G9-CCK2 cells show that whereas basal transcriptional relative activity was unchanged following mutations of Isl1 or Cdx2/3 binding sequences, it decreased from 5.64 ± 0.66 to 1.04 ± 0.20 when the Pax6 binding sequence was mutated, confirming that Pax6 is an essential transcription factor for the glucagon gene (Table 1). Mutations on the βTF1 or E47/β2 binding sequences did not affect basal transcriptional relative activity either. Noteworthy, the mutation at position -120 (120mut) dramatically decreased the basal promoter transcriptional relative
activity from 5.64 ± 0.66 to 1.80 ± 0.64 (Table 1).

Stimulation with gastrin following transfection of the different mutants shows that glucagon gene transactivation by gastrin was not modified by mutations preventing binding of either Isl1, Cdx2/3, Pax6, βTF1 or E47/β2 on the promoter (Table 1, Fig. 4B). Remarkably, the mutation at position -120 reduced significantly the effect of gastrin on glucagon promoter transcriptional activity by 60% when compared with wild-type promoter (1.65 ± 0.22 and 2.66 ± 0.25 fold basal respectively) (Table 1, Fig. 4B), suggesting that this sequence contains a response element to gastrin. Taken together, these data demonstrate that gastrin stimulation of glucagon gene expression involves an unidentified critical trans-acting transcription factor. This factor binds to the nucleotide region -120 of the G4 element of the glucagon gene and is essential for basal and gastrin-stimulated glucagon gene expression.

Egr-1 interacts with the G4 element of the glucagon gene promoter following gastrin stimulation of nuclear accumulation.

Molecular mechanisms of glucagon gene transactivation by gastrin were next investigated. First, to identify the transcription factors that interact with the G4 element and mediates the gastrin-stimulated glucagon gene transactivation, we performed electrophoretic mobility shift assays using the 120 probe, corresponding to a partial sequence of the G4 element (nucleotides -129/-108). Formation of a major DNA-proteins complex was observed following incubation of the 32P-labeled sequence with nuclear extracts prepared from InR1G9-CCK2 cells treated with gastrin for up to 24 h (Fig. 5A). Time-course analysis showed that gastrin-dependent formation of this complex was transient, with a binding peak after 1 h of agonist stimulation, decreased between 1 and 3 h of stimulation, whereas the complex disappeared after 6 h of treatment.

To characterize the specificity of the complex of the -120 region DNA sequence, competition studies were performed with increasing concentrations of unlabeled 120 probe. Intensity of the radioactive signal diminished progressively until complete extinction with 200-fold molar excess indicating that the proteins complex specifically interacted with the 120 probe (Fig. 5B). We next assessed whether mutations on the nucleotide region -120 that altered gastrin stimulation of the transcriptional activity also prevented formation of the DNA-proteins complex. Results presented in figure 5B show that an excess of unlabeled 120 mut probe did not affect the signal intensity, thus indicating that gastrin responsiveness is linked to the formation of a specific DNA-proteins interaction in this region of the promoter.

Analysis of the nucleotide region -120 on the G4 element shows that it contains a GC-rich motif, GAGTGGGCG, that constitutes a putative binding domain for the early growth response protein 1, Egr-1. Indeed, Egr-1 protein is a zinc-finger-containing transcription factor that specifically binds the DNA sequence GCG(G/T)GGGCG (18). Because of its unknown importance we first investigated whether the DNA-proteins complex contained the transcription factor Egr-1 performing EMSA competition experiments with an oligonucleotide containing the Egr-1 binding consensus site (Fig. 5C). Formation of the complex disappeared completely with an excess of this unlabeled oligonucleotide, suggesting that Egr-1 is present in this complex. To further confirm the presence of Egr-1, we carried out EMSA supershift experiments employing an anti-Egr-1 antibody (Fig. 5D). The complex was supershifted demonstrating that Egr-1 binds to G4 element of the glucagon gene promoter. Moreover, we also assessed whether this complex contains other transcription factors in addition to Egr-1 performing EMSA supershift experiments using anti-Sp1, anti-E47, anti-BETA2 or anti-p300 antibodies but none of these antibodies supershifted the complex (data not shown).

We also determined whether gastrin regulates nuclear translocation of Egr-1 in InR1G9-CCK2 cells by Western blot analysis of its nuclear abundance in response to gastrin stimulation for up to 24 h. Results of these experiments show a time-dependent increase of Egr-1 in the nucleus, being maximum after 1 h and reaching basal level after 6 h of gastrin stimulation (Fig. 6A), that is identical to the time-course of association of Egr-1 to G4 element that was observed with EMSA experiments. Given that the phosphorylated form of Egr-1 binds to DNA more efficiently than non-phosphorylated forms suggesting that its phosphorylation is critical for its transcriptional activity (19), we determined whether Egr-1 is phosphorylated in the nuclei of InR1G9-CCK2 cells after stimulation with gastrin. Results of
radiolabeling experiment followed by Egr-1 immunoprecipitation show an accumulation of 32P-labeled Egr-1 in the nuclei of gastrin-stimulated InR1G9-CCK2 cells (Fig. 6B) demonstrating that gastrin phosphorylates Egr-1, in addition to its nuclearization.

The proximal glucagon gene promoter of InR1G9-CCK2 cells interacts with Egr-1 in a gastrin-dependent manner.

In order to demonstrate that Egr-1 proteins are indeed recruited to the endogenous proximal glucagon gene promoter of InR1G9-CCK2 cells in response to gastrin, we performed a chromatin immunoprecipitation assay (ChIP). However, given that nucleotide sequence of the hamster glucagon gene promoter was unknown, prior cloning of this sequence from InR1G9-CCK2 cells was necessary and a partial sequence is shown in figure 7A. Following stimulation by gastrin, cross-linked DNA-proteins complexes were immunoprecipitated with the specific antibody recognizing Egr-1. The DNA fragments containing the G4 element were amplified by real-time PCR using primers flanking the G4 element of the hamster glucagon gene promoter. As shown in figure 7B, there was a 2.9-fold enrichment of immunoprecipitated glucagon gene promoter in the gastrin-stimulated InR1G9-CCK2 cells compared with the untreated cells thus demonstrating gastrin-dependent binding of Egr-1 to this sequence.

The MEK1/ERK1/2 pathway is critical for gastrin-dependent glucagon gene transactivation.

Egr-1 binding to DNA is significantly increased by inhibitors of protein serine/threonine phosphatases, suggesting that its activation is under the control of protein kinases and/or phosphatases (20). Among multiple protein kinases, proteins that belong to mitogen-activated protein kinase (MAPK) family could be involved in the regulation of many genes. Since the MAPK signaling pathway is activated by gastrin (21), we first tested whether it contributed to the CCK2 receptor signaling cascade in the InR1G9-CCK2 cells. Results of Western blot analysis verified that gastrin increases ERK1/2 phosphorylation in a time-dependent manner with a peak of stimulation at 5 min in InR1G9-CCK2 cells (Fig. 8A). ERK proteins phosphorylation was maintained at least during 1 h of gastrin stimulation and inhibited to nearly basal level with the specific inhibitor of MEK1 signaling pathway, PD98059 (Fig. 8A). The importance of the MEK/ERK cascade in the gastrin-induced glucagon gene transactivation, was then investigated. Figure 8B shows that PD98059 reduced partially but significantly, more than 50% of the gastrin-stimulated glucagon transcriptional activity of the proximal promoter when compared with PD98059-non treated cells (1.69 ± 0.16 and 2.55 ± 0.29-fold basal respectively).

We further explored whether gastrin-induced binding of Egr-1 on the glucagon gene promoter involved MAPK pathway comparing EMSA analysis with nuclear extracts from gastrin-stimulated InR1G9-CCK2 cells treated or not with PD98059. Binding of Egr-1 was markedly decreased in the presence of the MEK1 specific inhibitor strongly suggesting that ERK1/2 activation by gastrin contributes to Egr-1 activation (Fig. 8C).

We next investigated whether Egr-1 nuclearization induced by gastrin stimulation was affected by treatment with PD98059. Addition of the MEK1 specific inhibitor dramatically prevented gastrin-induced nuclear accumulation of Egr-1 (Fig. 8D), indicating that ERK1/2 activation is an essential step for nuclearization of the transcription factor. These results were further confirmed performing an immunocytochemical analysis of gastrin- and PD98059-treated cells. As shown in figure 9, Egr-1 nuclear accumulation increased after 1 h of gastrin stimulation in InR1G9-CCK2 cells (Fig. 9A and 9B) whereas Egr-1 nuclearization in response to gastrin was strongly decreased in the presence of PD98059 (Fig. 9C and 9D).

Taken together, our results demonstrate that gastrin-stimulated glucagon gene expression is dependent on the MAPK signaling pathway in InR1G9-CCK2 cells via nuclearization and DNA binding of Egr-1.

DISCUSSION

The results of the current study highlight a new role for the gastrointestinal regulatory peptide gastrin in the pancreas. Indeed, using a new cell line expressing the CCK2 receptor, we determined that gastrin is an effective stimulant of glucagon RNA expression. This finding provides the first evidence that gastrin directly regulates the expression of a pancreatic endocrine hormone further supporting a role of gastrin in glucose homeostasis. This together with the previously reported role of gastrin in the
While our report provides additional evidences involved the MEK1/ERK1/2 signaling cascade. disappearing after a maximum at 1 h, and to CgA promoter was rapid, transient, our results, gastrin-dependent binding of Egr-1 gene in gastric epithelial cells (25). Similarly to was reported for the chromogranin A (CgA) gastrin-regulated transactivation of promoter activity of Egr-1 (19). Participation of Egr-1 to stimulation consistent with DNA-binding show phosphorylation of Egr-1 following gastrin gene in pancreatic alpha cells. Furthermore, we in gastrin-dependent regulation of the glucagon expression, its binding to the IS is a critical step function of Egr-1 for basal glucagon gene expression, the trans-acting nuclear proteins that bind IS remained to be defined. In agreement with the known consensus DNA binding site GCG(G/T)GGCG (18), we now show that the zinc-finger transcription factor Egr-1 recognizes the GC-rich domain contained in the intervening sequence. Egr-1 is encoded by an immediate-early gene and consequently rapidly induced and activated by many environmental signals including growth factors, stress or hormones (22,23). Thus activation of Egr-1 may also adapt to rapid elevated glucagon production in stressful conditions such as hypoglycemia. While induction of Egr-1 gene together with other immediate-early response genes has been linked to long-term pleiotropic effects of glucose and secretagogues in pancreatic beta-cells (24), this study is the first to report activation of Egr-1 in pancreatic alpha cells.

We also demonstrate that besides a key function of Egr-1 for basal glucagon gene expression, its binding to the IS is a critical step in gastrin-dependent regulation of the glucagon gene in pancreatic alpha cells. Furthermore, we show phosphorylation of Egr-1 following gastrin stimulation consistent with DNA-binding activity of Egr-1 (19). Participation of Egr-1 to gastrin-regulated transactivation of promoter was reported for the chromogranin A (CgA) gene in gastric epithelial cells (25). Similarly to our results, gastrin-dependent binding of Egr-1 to CgA promoter was rapid, transient, disappearing after a maximum at 1 h, and involved the MEK1/ERK1/2 signaling cascade. While our report provides additional evidences that Egr-1 is one of the gastrin-regulated transcription factors essential differences exist according to the regulated promoter. First, the fact that Egr-1 controls the basal activity of the glucagon gene whereas it is not the case for basal transactivation of the CgA gene, demonstrates that the functional relevance of the transcription factor is different in the two situations. Moreover, an interplay of Egr-1 and Sp1, resulting of overlapping consensus motifs (26), is crucial for gastrin regulation of the CgA promoter. In the context of the glucagon gene promoter, Sp1 is absent of the DNA-proteins complex induced by gastrin indicating no functional interaction between these two factors.

Finally, besides supporting an essential role for gastrin in glucose homeostasis via regulation of islet glucagon cell function, results of the present study are also of particular interest considering our previous data demonstrating the presence of the CCK2 receptor and of amidated gastrin, its specific ligand in 17 weeks human fetal pancreas (10). Full understanding of the functions of gastrin in human pancreas during the embryonic period would deserve investigations especially at period of development earlier than 17 weeks of gestation but these remain limited because of scarce material. However, the fact that gastrin stimulates glucagon gene expression via activation of its receptor present in glucagon cells open new interesting prospects concerning its contribution to pancreatic development. Indeed, glucagon is the earliest peptide hormone that is expressed in the developing pancreas and recent data have proposed a key role for glucagon in the early phase of insulin cells differentiation (27). This suggests that gastrin could actively contribute to the paracrine induction of differentiation of pancreatic cells during fetal life by inducing or maintaining the alpha cell phenotype via induction of Egr-1. Indeed, during mouse embryogenesis, Egr-1 is present in different sites where epithelial mesenchymal interactions are important such as the developing tooth germ, the nasal and salivary glands (28). Induction of its expression, following FGF receptors activation has been reported in rat embryonic pancreatic rudiments suggesting that it may play a role in the promotion of proliferation of embryonic pancreatic epithelial cells (29). Therefore the hypothesis that it is one of the mediator of the developmental effects of gastrin can be raised from now on.
REFERENCES

1. Dockray, G. J. (1999) J Physiol 518(Pt 2), 315-24.
2. Silvente-Poirot, S., Dufresne, M., Vaysse, N., and Formy, D. (1993) Eur J Biochem 215(3), 513-29.
3. Kowalski-Chauvel, A., Pradayrol, L., Vaysse, N., and Seva, C. (1996) J Biol Chem 271(42), 26356-61.
4. Daulhac, L., Kowalski-Chauvel, A., Pradayrol, L., Vaysse, N., and Seva, C. (1999) J Biol Chem 274(29), 20657-63.
5. Dehez, S., Bierkamp, C., Kowalski-Chauvel, A., Daulhac, L., Escrieut, C., Susini, C., Pradayrol, L., Fourmy, D., and Seva, C. (2002) Cell Growth Differ, 13(8), 375-85.
6. Bierkamp, C., Kowalski-Chauvel, A., Dehez, S., Fourmy, D., Pradayrol, L., and Seva, C. (2002) Oncogene 21(50), 7656-70.
7. Bardram, L., Hilsted, L., and Rehfeld, J. F. (1990) Proc Natl Acad Sci U S A 87(1), 298-302.
8. Wang, T. C., and Dockray, G. J. (1999) Am J Physiol 277(1 Pt 1), G6-11.
9. Brand, S. J., Andersen, B. N., and Rehfeld, J. F. (1984) Nature 309(5967), 456-8.
10. Saillan-Barreau, C., Dufresne, M., Clerc, P., Sanchez, D., Corominola, H., Moriscot, C., Guy-Crotte, O., Escrieut, C., Vaysse, N., Gomis, R., Tarasova, N., and Fourmy, D. (1999) Diabetes 48(10), 2015-21.
11. Boushey, R. P., Abadir, A., Flamez, D., Baggio, L. L., Li, Y., Berger, V., Marshall, B. A., Finegood, D., Wang, T. C., Schuit, F., and Drucker, D. J. (2003) Gastroenterology 125(4), 1164-74.
12. Jiang, G., and Zhang, B. B. (2003) Am J Physiol Endocrinol Metab 284(4), E671-8.
13. Taborsky, G. J., Jr., Ahren, B., and Havel, P. J. (1998) Diabetes 47(7), 995-1005.
14. Dumonteil, E., Magnan, C., Ritz-Laser, B., Meda, P., Dussoix, P., Gilbert, M., Ktorza, A., and Philippe, J. (1998) Endocrinology 139(11), 4540-6.
15. Takaki, R., Ono, J., Nakamura, M., Yokogawa, Y., Kuma, S., Hiraoka, T., Yamaguchi, K., Hamaguchi, K., and Uchida, S. (1986) In Vitro Cell Dev Biol 22(3 Pt 1), 120-6.
16. Philippe, J., Drucker, D. J., Knepel, W., Jepeal, L., Misulovin, Z., and Habener, J. F. (1988) Mol Cell Biol 8(11), 4877-88.
17. Cordier-Bussat, M., Morel, C., and Philippe, J. (1995) Mol Cell Biol 15(7), 3904-16.
18. Swirnoff, A. H., and Milbrandt, J. (1995) Mol Cell Biol 15(4), 2275-87.
19. Huang, R. P., and Adamson, E. D. (1994) Biochem Biophys Res Commun 200(3), 1271-6.
20. Cao, X., Mahendran, R., Guy, G. R., and Tan, Y. H. (1993) J Biol Chem 268(23), 16949-57.
21. Seva, C., Kowalski-Chauvel, A., Blanchet, J. S., Vaysse, N., and Pradayrol, L. (1996) FEBS Lett 378(1), 74-8.
22. Silverman, E. S., and Collins, T. (1999) Am J Pathol 154(3), 665-70.
23. Thiel, G., and Cibelli, G. (2002) J Cell Physiol 193(3), 287-92.
24. Susini, S., Roche, E., Pretki, M., and Schlegel, W. (1998) Faseb J 12(12), 1173-82.
25. Raychowdhury, R., Schafer, G., Fleming, J., Rosewicz, S., Wiedenmann, B., Wang, T. C., and Hocker, M. (2002) Mol Endocrinol 16(12), 2802-18.
26. Marco, E., Garcia-Nieto, R., and Gago, F. (2003) J Mol Biol 328(1), 9-32.
27. Prasad, K., Daume, E., Preuett, B., Spilde, T., Bhatia, A., Kobayashi, H., Hembree, M., Manna, P., and Gitesc, G. K. (2002) Diabetes 51(11), 3229-36.
28. McMahon, A. P., Champion, J. E., McMahon, J. A., and Sukhatme, V. P. (1990) Development 108(2), 281-7.
29. Le Bras, S., Miralles, F., Basmaciogullari, A., Czernichow, P., and Scharfmann, R. (1998) Diabetes 47(8), 1236-42.

ACKNOWLEDGEMENTS

This study was supported by grants from the Association pour la Recherche sur le Cancer (n°4430 and n°4514), from the Génopôle of Toulouse (n°ur531-257-8RB06). Stéphane Leung-Theung-Long is a recipient from the Ligue contre le Cancer (région Midi-Pyrénées), University of Toulouse (ATUPS)
FIGURES LEGENDS

FIG. 1. Characteristics of InR1G9 cells expressing the CCK2 receptor. A, Glucagon secretion studies from InR1G9-CCK2 cells. Cells were stimulated for 1 h with increasing concentrations of either CCK (●●●●●●) or gastrin (-----) and amounts of secreted glucagon were measured by radioimmunoassay as described in Experimental Procedures. Results are expressed as percentage of maximal secretion with basal secretion subtracted (6.4 ± 2.3 pmol/10⁶ cells/h). Values are mean ± S.E.M. from 4 separate experiments. Each experiment was realized in triplicate. *, p < 0.05, **, p < 0.01, ***, p < 0.001 compared with maximal secretion. B, Localization of CCK2 receptor on InR1G9 cells expressing the CCK2 receptor (InR1G9-CCK2) or not (InR1G9) using a FITC-coupled CCK9 ligand.

FIG. 2. Gastrin stimulates glucagon gene expression. A, Effect of gastrin on glucagon mRNA level. Cells were stimulated 24 h with or without gastrin (1 μM) and total RNA was extracted for Northern blot analysis as described in Experimental Procedures. Blots were quantified by PhosphorImager scanning. Values are mean ± S.E.M. from 3 separate experiments. Each experiment was realized in duplicate. **, p < 0.01. B, Effect of gastrin on glucagon mRNA half-life. Actinomycin D (5 μg/ml) was added to control and gastrin-treated cells, and RNA was extracted after 4, 8 and 12 h. Glucagon mRNA was quantified by Northern blot analysis, using the zero time of actinomycin treatment as a reference for 100% survival. Values are mean ± S.E.M. from 3 separate experiments. Each experiment was realized in duplicate.

FIG. 3. Identification of the minimal sequence of the glucagon gene responding to gastrin. A, Schematic representation of control elements and transcription factors regulating the glucagon gene. CRE, cAMP response element; CREB, CRE binding protein; WiHe, winged helix family protein; HNF-3β, hepatocyte nuclear factor-3β; Pbx, PBC homeoprotein; Prep1, homeodomain protein; E47/β2, basic helix-loop-helix family protein; βTF1, β cell transcription factor 1; Cdx-2/3, caudal-type homeobox protein 2/3; Ets, Ets family protein; Isl-1, insulin factor 1; Pax, paired homeobox protein. B, Minimal glucagon promoter analysis. The indicated constructs, containing increasing 5’-flanking sequence of the glucagon gene linked to CAT, and a plasmid encoding luciferase were co-transfected into InR1G9-CCK2 cells, and cells treated (black bars) or not (white bars) with gastrin (1 μM) for 24 h. After protein extraction, CAT and luciferase activities were measured. Results represent relative CAT/luciferase activities over control. Values are mean ± S.E.M. from 3 separate experiments. Each experiment was realized in duplicate. *, p < 0.05, **, p < 0.01 compared with non-treated cells.

FIG. 4. Mapping of a gastrin cis-regulating sequence on the glucagon proximal promoter. A, Wild-type (WT) and mutated (mut) nucleotide sequences of binding domains for transcription factors known to interact with the glucagon gene promoter. Binding domains are shown in grey box and mutated nucleotides abolishing binding of transcription factor are underlined. A putative binding domain in the 120 region for an unknown factor is also indicated. B, Effect of mutations within binding domains on gastrin-stimulated glucagon gene expression. The wild-type minimal promoter, containing the first 138 bp of the promoter (G1 and G4 elements), or indicated mutated promoter were co-transfected with a plasmid encoding luciferase into InR1G9-CCK2 cells. Cells were treated (black bars) or not (white bars) with gastrin (1 μM) for 24 h. After protein extraction, CAT and luciferase activities were measured. Results represent relative CAT/luciferase activities over basal for each construct. Values are mean ± S.E.M. from 3 separate experiments. Each experiment was realized in duplicate. *, p < 0.05, **, p < 0.01, ***, p < 0.001 compared with control.

FIG. 5. Egr-1 binds to G4 element of the glucagon gene promoter. A, Electrophoretic mobility shift assays (EMSA) were performed with 10 μg of nuclear extracts from InR1G9-CCK2 cells, stimulated or not with gastrin (1 μM) for the times indicated and incubated with the 32P-labeled oligonucleotide.
encoding partially the G4 element (sequence of the 120 probe is shown in Experimental Procedures). B, EMSA competition studies were carried out with 10 µg of nuclear extracts from 1 h gastrin-stimulated InR1G9-CCK2 cells using 50-, 100-, and 200-fold molar excess of unlabeled 120 probe or mutated 120 probe (sequence is shown in Experimental Procedures) as indicated. C, 50-, 100-, and 200-fold molar excess of unlabeled Egr-1 consensus binding site oligonucleotide (sequence is shown in Experimental Procedures) were used for EMSA competition experiment. D, For supershift assays, nuclear extracts were preincubated with an anti-Egr-1 antibody before addition of the radiolabeled probe. The data shown are representative of three independent experiments.

FIG. 6. Gastrin stimulates nuclear accumulation and phosphorylation of Egr-1. A, Nuclear extracts were prepared from InR1G9-CCK2 cells stimulated or not with gastrin (1 µM) for the indicated time periods, and Western blot studies were performed using Egr-1 antibody. To demonstrate equivalent protein loading, the blot was reprobed with an anti-GAPDH antibody. The data shown represent typical results of three independent experiments. B, InR1G9-CCK2 cells were metabolically labeled with [32Pi], and nuclear extracts were prepared after stimulation or not with gastrin (1 µM) for 1 h, analyzed on a SDS-PAGE and radioactivity revealed by PhosphorImager (upper panel). The membrane was immunoblotted (IB) with an anti-Egr-1 antibody to demonstrate that the radiolabeled immunoprecipitated proteins are Egr-1 (lower panel).

FIG. 7. Egr-1 binds to the endogenous glucagon gene promoter in InR1G9-CCK2 cells. A, Partial sequence of the glucagon gene promoter of InR1G9-CCK2 cells. Conserved DNA sequences that interact with Isl-1, Cdx-2/3, Pax-6, β-TF1 and E47/β2 transcription factors are shown in grey boxes. Putative nucleotide sequence interacting with Egr-1 is shown in blank box. B, Results of real-time PCR following chromatin immunoprecipitation. The data shown are derivated from quantitative real-time PCR analysis of the glucagon gene promoter after ChIP with the specific anti-Egr-1 antibody. Data are expressed as fold-differences between gastrin-stimulated cells (black bar) versus gastrin-unstimulated cells (white bar). Values are mean ± S.E.M. from three independent experiments performed in duplicate. **, p < 0.01 compared with non-treated cells.

FIG. 8. The MAP kinase pathway is critical for Egr-1-dependent gastrin-stimulated glucagon gene expression. A, Cells were stimulated with gastrin (1 µM) for the indicated time periods in presence or absence of PD98059. ERK1/2 phosphorylation was then analyzed by Western blot assays (upper panel). As a loading control, the blot was stripped and reprobed with non-phospho-specific anti-ERK1/2 antibody (lower panel). The data shown represent typical results of a series of three independent experiments. B, Reporter plasmid containing G1 and G4 elements (-138 bp) of the glucagon gene promoter linked to CAT and plasmid expressing luciferase were co-transfected into InR1G9-CCK2 cells. Cells were pretreated or not with the specific inhibitor of the MEK1, PD98059 (50 µM), and stimulated (black bars) or not (white bars) with gastrin (1 µM) for 24 h. After protein extraction, CAT and luciferase activities were measured. Results represent relative CAT/luciferase activities over non-treated cells. Values are mean ± S.E.M. from 3 separate experiments. Each experiment was realized in duplicate. *, p < 0.05, **, p < 0.01 compared with control. C, InR1G9-CCK2 nuclear extracts were prepared after stimulation or not with gastrin for 1 h in presence or absence of PD98059 (50 µM) and analyzed in EMSA studies using 120 probe. The data shown represent typical results of three independent experiments. D, Nuclear extracts were prepared after stimulation of InR1G9-CCK2 cells with gastrin (1 µM) for 1 h, with or without PD98059 (50 µM), and Egr-1 nuclear abundance was assayed by Western blot studies. As a loading control, the blot was reprobed with an anti-GAPDH antibody. The data shown represent typical results of three independent experiments.

FIG. 9. Egr-1 immunocytochemistry. Cells were stimulated (B and D) or not (A and C) with gastrin (1 µM) for 1 h in the presence (C and D) or not (A and B) of PD98059 (50 µM). Then, immunocytochemical studies were performed in order to determine Egr-1 localization using an anti-Egr-1 antibody. To visualize nuclei, cells were incubated with DAPI.
TABLE 1. Comparative effects of transcription factors binding site mutations on transcriptional activity of the glucagon gene proximal promoter.

| Glucagon proximal promoter | Glucagon gene expression Relative activities |
|----------------------------|---------------------------------------------|
|                            | Basal | Gastrin-stimulated | Gastrin-stimulated (fold basal) |
| WT                         | 5.64 ± 0.66 | 14.73 ± 1.08 ** | 2.66 ± 0.25 ** |
| Isl1 mut                   | 6.42 ± 1.45 | 16.49 ± 2.66 ** | 2.64 ± 0.17 ** |
| Cdx2/3 mut                 | 3.14 ± 0.91 | 9.42 ± 2.21 ** | 3.11 ± 0.19 ** |
| Pax6 mut                   | 1.04 ± 0.20 †† | 3.60 ± 0.58 *** | 3.51 ± 0.20 *** |
| βTF1 mut                   | 3.40 ± 1.24 | 9.97 ± 2.90 *   | 3.11 ± 0.29 *   |
| 120 mut                    | 1.80 ± 0.64 † | 2.70 ± 0.82     | 1.65 ± 0.22     |
| E47/β2 mut                 | 3.39 ± 1.11 | 9.26 ± 2.49 *   | 2.82 ± 0.25 *   |

Basal and gastrin-stimulated transcriptional relative activities of either wild-type (WT) proximal glucagon gene promoter plasmid or plasmids mutated (mut) on the nucleotide sequences binding the different transcription factors interacting with this region. Results represent basal relative CAT/luciferase activities (basal), gastrin-stimulated relative CAT/luciferase activities (gastrin-stimulated) or gastrin-stimulated relative CAT/luciferase activities over basal (gastrin-stimulated fold basal) following gastrin stimulation for each construct. Values are means ± S.E.M from 3 separate experiments. Each experiment was realized in duplicate. †, p < 0.05, ††, p < 0.01 compared with basal relative activity of WT glucagon proximal promoter. *, p < 0.05, **, p < 0.01, ***, p < 0.001 compared with unstimulated relative activity for each construct.
Figure 1.

A. Glucagon secretion (% of max) versus log (M) for lnR1G9-CCK2.

B. Immunofluorescence images of lnR1G9 and lnR1G9-CCK2.
Figure 2.

A. Gastrin - +

Glucagon mRNA

18 S

Glucagon mRNA (fold basal)

B.

Gastrin mRNA (%)

time, h

Control

Gastrin
Figure 3.

A.

B.
Figure 4.

A.

|        | E47/β2            | βTF1            |
|--------|-------------------|-----------------|
| WT     | 5’-aggagcagactaga| 5’-aggagcagacta|
| mut    | -140              | -100            |

|        | Pax-6  | Cdx-2/3 | Isl-1 |
|--------|--------|---------|-------|
| WT     | ccccattatat   | -90     | -70   |
| mut    | ...cagcatattt  | ...cagcatat  | cagcatatg...|
|        | ...caaatattt  | ...caaatatt  | ...caaatattgcaat |

|        | WT     | mut    |
|--------|--------|--------|
| WT     | ggctaaacagcttgagactatatatttaagcacagcacc | ggctaaacagcttgagactatatatttaagcagcacc |
| mut    | -40    | -10    |

B.

![Bar graph showing CAT/Luc (fold basal) for WT, Isl1 mut, Cdx2/3 mut, Pax6 mut, βTF1 mut, 120 mut, and E47/β2 mut.](http://www.jbc.org/)
Figure 5.

|        | A.         | B.          | C.         | D.         |
|--------|------------|-------------|------------|------------|
| Gastrin| - - + + + + + + + | + + + + + + + + + | + + + + | - - + + + |
| Time, h| 0.5 1 2 3 6 24 | 120 - 50 100 200 | 0 1 0 1  | 0 1 |
| Proteins| - + + + + + + + | - 50 100 200 | - - + + | - - + + |

Complex

---

Complex
Figure 6.

A.

| Gastrin | - | + | + | + | + | + | + |
|---------|---|---|---|---|---|---|---|
| Time, h | 0.5 | 1 | 2 | 3 | 6 | 24 |   |

Egr-1
GAPDH

B.

Gastrin | - | + |
|---------|---|---|

$^{32}$P Egr-1
IB : Egr-1
Figure 7.

A.

gactcggtctatttgactgtaattcatttcagggatgtaagtgttttt
tcattgctgtgatatacagctatccactgcattcccatttccaaacagaaagg
cacaagagaatataaaagtttccggtctctgagggtctcaaccccgggatc
gacacagaatgcagagcaagcctctgtgaatctttgacac
E47/β2      Egr-1      β-TF1

agcaggaatgcagagcaagcctctgtgaatctttgacac
Pax-6      Cdx-2/3      Isl-1

aaacccctatttacagatgagaatttatatttcacgctaatatctg
cgtgctaaccaggctggaggatatatataaaagcagcgcaccctgtgtgcga
agtcagagctttggg

B.

Relative enrichment (fold basal)

**
Figure 8.

A. 

| Gastrin       | - | + | - | + | + | + | + | + |
|---------------|---|---|---|---|---|---|---|---|
| Time, min     | 5 | 1 | 3 | 5 | 15| 30| 60|   |
| PD98059       | + | - | - | - | - | - | - | - |

B. 

![Graph showing CAT/Luc expression](image)

C. 

| Gastrin | - | + | - | + |
|---------|---|---|---|---|
| Time, h | 1 | 1 |   |   |
| PD98059 | - | - | + | + |

D. 

| Gastrin       | - | + | - | + |
|---------------|---|---|---|---|
| Time, h       | 1 | 1 |   |   |
| PD98059       | - | - | + | + |

[p-ERK1/2, ERK1/2](image)

Egr-1

[GAPDH](image)
Figure 9.
Essential interaction of Egr-1 at an islet specific response element for basal and gastrin-dependent glucagon gene transactivation in pancreatic alpha cells
Stéphane Leung-Theung-Long, Emmanuelle Roulet, Pascal Clerc, Chantal Escrieut, Sophie Marchal-Victorion, Beate Ritz-Laser, Jacques Philippe, Lucien Pradayrol, Catherine Seva, Daniel Fourmy and Marlène Dufresne

J. Biol. Chem. published online December 14, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407485200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts