Stimulation of pancreatic β -cell replication by incretins involves transcriptional induction of cyclin D1 via multiple signalling pathways

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Abstract

The incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP), have been suggested to act as β -cell growth factors and may therefore be of critical importance for the maintenance of a proper β -cell mass. We have investigated the molecular mechanism of incretin-induced β -cell replication in primary monolayer cultures of newborn rat islet cells. GLP-1, GIP and the long-acting GLP-1 derivative, liraglutide, increased β -cell replication 50–80% at 10–100 nM upon a 24 h stimulus, whereas glucagon at a similar concentration had no significant effect. The stimulatory effect of GLP-1 and GIP was efficiently mimicked by the adenylate cyclase activator, forskolin, at 10 nM ($\sim 90\%$ increase) and was additive (~ 170 – 250% increase) with the growth response to human growth hormone (hGH), indicating the use of distinct intracellular signalling pathways leading to mitosis by incretins and cytokines, respectively. The response to both GLP-1 and GIP was completely blocked by the protein kinase A (PKA) inhibitor, H89. In addition, the phosphoinositol 3-kinase (PI3K) inhibitor wortmannin and the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059, both inhibited GLP-1- and GIP-stimulated proliferation. The p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580, had no inhibitory effect on either GLP-1

or GIP stimulated proliferation. Cyclin Ds act as molecular switches for the G0/G1-S phase transition in many cell types and we have previously demonstrated hGH-induced cyclin D2 expression in the insulinoma cell line, INS-1. GLP-1 time-dependently induced the cyclin D1 mRNA and protein levels in INS-1E, whereas the cyclin D2 levels were unaffected. However, minor effect of GLP-1 stimulation was observed on the cyclin D3 mRNA levels. Transient transfection of a cyclin D1 promoter-luciferase reporter construct into islet monolayer cells or INS-1 cells revealed approximately a 2–3 fold increase of transcriptional activity in response to GLP-1 and GIP, and a 4–7 fold increase in response to forskolin. However, treatment of either cell type with hGH had no effect on cyclin D1 promoter activity. The stimulation of the cyclin D1 promoter by GLP-1 was inhibited by H89, wortmannin, and PD98059. We conclude that incretin-induced β -cell replication is dependent on cAMP/PKA, p42 MAPK and PI3K activities, which may involve transcriptional induction of cyclin D1. GLP-1, GIP and liraglutide may have the potential to increase β -cell replication in humans which would have significant impact on long-term diabetes treatment.

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Introduction

The intestinal incretin hormones, glucagon-like peptide-1 (7–36) amide (GLP-1) and glucose-dependent insulintropic peptide (GIP) are released in response to fat and carbohydrate ingestion. Both peptides act to augment glucose-induced insulin secretion from the pancreatic β -cells and are thus thought to be important for the

maintenance of normal glucose tolerance (Holst *et al.* 1997, Holst 1999). Patients with type 2 diabetes appear to have an impaired incretin effect, and long-acting derivatives of GLP-1 are in clinical development for the treatment of this syndrome (Holst 1999). Further potential advantages of GLP-1 for the treatment of type 2 diabetes are the stimulatory actions on insulin gene expression (Skoglund *et al.* 2000) and biosynthesis (Fehmann & Habener 1992),

and the promotion of β -cell neogenesis and replication both *in vivo* and *in vitro* (Buteau *et al.* 1999, 2001, Xu *et al.* 1999, Stoffers *et al.* 2000, Nielsen *et al.* 2001). In addition, GIP was also described as a growth factor for the insulinoma cell line, INS-1 (Trümper *et al.* 2001). The role of incretins as regulators of β -cell replication (Farilla *et al.* 2003) and anti-apoptosis that have been recently described (Hui *et al.* 2003, Urusova *et al.* 2004, Bregenholt *et al.* 2005) may be of particular importance since functional β -cells are progressively lost during development of type 2 diabetes (Bonner-Weir & Weir 2001).

Both glucagon, GLP-1, and GIP receptors are expressed on pancreatic β -cells (Moens *et al.* 1996). The response to these hormones leads to the activation of the adenylate cyclase and the cAMP/protein kinase A (PKA) signalling pathway. Furthermore, GLP-1 and GIP have been reported to stimulate phosphoinositol 3-kinase (PI3K) and p42 mitogen-activated protein kinase (MAPK) activities in insulinoma cells (Frödin *et al.* 1995, Buteau *et al.* 1999, Trümper *et al.* 2001), and up-regulation of the DNA-binding activity of the transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1), has been suggested to play a role in GLP-1 induced DNA-synthesis and regulation of both the insulin gene and genes encoding metabolic enzymes (Buteau *et al.* 1999). In addition, involvement of p38 MAPK and an atypical protein kinase C isoform, PKC β , was demonstrated in GLP-1-induced replication of INS-1 cells (Buteau *et al.* 2001). Recent experiments indicate that the GLP-1 response in pancreatic β -cells also involves activation of the epidermal growth factor receptor (EGFR) (Buteau *et al.* 2003). Thus extensive cross-talk between the G-protein coupled receptor and tyrosine kinase coupled receptor signalling pathways in β -cells is emerging, as reviewed recently by Brubaker & Drucker (2004). Therefore, we found it of interest to compare the effects of incretin and growth hormone/prolactin on β -cell proliferation.

So far most studies on incretin signalling have been conducted with insulinoma cell lines, in particular the INS-1 cell line, which display many features of primary β -cells, e.g. glucose-dependent insulin secretion (Asfari *et al.* 1995). However, tumour cell lines are perturbed in their growth regulation and clear interpretations in studies of growth control using these are difficult. In the present study, we have evaluated the contribution of several signalling pathways potentially involved in GLP-1 and GIP-induced β -cell replication using primary rat β -cells in monolayer cultures, and compared the effect of the potent β -cell growth factor, human growth hormone (hGH), acting via the Janus kinase 2/signal transducer and activator of transcription 5 (STAT5) pathway (Friedrichsen *et al.* 2001). Furthermore, in both primary β -cells and INS-1E cells, we have investigated the role of GLP-1 and GIP in the regulation of the cell cycle regulatory factors, Cyclin Ds, which are critical for G1 phase progression and S-phase entry in most cell types.

Materials and Methods

Reagents

INS-1E cells, kindly provided by Dr C B Wollheim, Geneva, Switzerland, were cultured in RPMI 1640 with Glutamax (Gibco/Invitrogen) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 50 μ M β -mercaptoethanol at 37 °C in a humidified atmosphere containing 5% CO₂. Cell passages 42–65 were used. GLP-1, liraglutide, exendin (9–39), and hGH (3 IU/mg) were obtained from Novo Nordisk, Bagsværd, Denmark. Glucagon was purchased from Bachem (Bubendorf, Switzerland). Wortmannin, PD98059, SB203580, and H89 were purchased from Calbiochem (La Jolla, CA, USA), and forskolin and bromodeoxyuridine (BrdU) from Sigma-Aldrich. All inhibitors were dissolved in dimethylsulfoxide (Sigma). A monoclonal antibody (M744) to BrdU was obtained from DAKO (Glostrup, Denmark). Guinea pig anti-insulin antiserum was provided by Novo Nordisk. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Islet isolation and culture

Pancreatic islets were isolated from 3–5 day-old Wistar rats (Møllegaard, Lille Skensved, Denmark) by the collagenase digestion method (Brunstedt *et al.* 1984). The islets were pre-cultured at 37 °C for 5–7 days in bacteriological dishes in RPMI 1640 containing 20 mM HEPES, 100 units/ml penicillin, 100 mg/ml streptomycin and 0.5% normal human serum (HS). The islets were collected by centrifugation and dispersed by trypsin-EDTA treatment, as previously described in Nielsen *et al.* (1989). Dispersed islet cells were plated in either 9 cm² slide flasks (Nunc, Roskilde, Denmark), approximately 100 000 cells/flask, for BrdU incorporation assay or in 24-well dishes, approximately 100 000 cells/well for transient transfection (see below) in RPMI 1640 supplemented with 2% HS and 500 ng/ml hGH. The cells were allowed to attach and spread for 5–7 days before experimentation.

BrdU incorporation assay

The islet cell monolayers were washed twice in RPMI 1640 containing 2% HS and pre-cultured for 24 h. The medium was replaced with RPMI 1640 containing 2% HS and 10 μ M BrdU, peptides and compounds were added as indicated. Cells were pre-incubated with protein kinase inhibitors for 15 min prior to hormone addition and the incubation continued for 24 h, after which the slides were washed twice in serum-free medium and the cells fixed in 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The slides were exposed to 1.5 M HCl for 1 h and washed to neutrality. Incubation with BrdU and

insulin antibodies was carried out in PBS containing 0.3% Triton X-100 and 0.1% human serum albumin for 1.5 h at room temperature. The antibodies were visualized using Texas-red conjugated goat anti-mouse immunoglobulin (IgG) and fluorescein isothiocyanate-conjugated goat antequinea pig IgG, respectively. Double-positive cells as a percentage (mitotic index) of the total number of insulin-positive cells counted (1000–2000/slide) were determined by counting under the microscope.

RNA extraction and quantitative RT-PCR

Islets were cultured in suspension in RPMI 1640 containing 0.5% HS and stimulated for 24 h. Total RNA was isolated using RNeasy (Qiagen) as described by the protocol of the manufacturer, cDNA was synthesized from 1 μ g of RNA as previously described (Friedrichsen *et al.* 2001). Primer sets complementary to cyclin Ds and internal control genes and conditions of RT-PCR were carried out as described previously (Friedrichsen *et al.* 2001).

INS-1E cells were treated with or without GLP-1 (100 nM) (Novo Nordisk) or forskolin (10 μ M) (Sigma) for 6 and 12 h. Total RNA was isolated and purified from INS-1E by Trizol reagent (Invitrogen). For the first strand cDNA synthesis, RNA (0.75 μ g) was reversed-transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, St. Leon-Rot, Germany) with oligo (dT)18 according to manufacturer's instructions. The first strand cDNA obtained was diluted 1:2 and stored at -20°C until use. Quantitative PCR was carried out using the LightCycler (LC) (Roche) with LC FastStart DNA Master^{PLUS} SYBR Green I (Roche) in a total reaction volume of 10 μ l, containing 2 μ l of the diluted first strand cDNA. The real-time PCR runs were all performed using the same reaction conditions for denaturation, amplification, and extension (initial activation, 10 min at 95°C ; followed by 10 s denaturation at 95°C , 5 s annealing at 55°C , 20 s extension at 72°C ; for 35 to 40 cycles). Gel electrophoresis and/or melting curve analysis were used to confirm the fidelity of the LC PCR products. The oligonucleotide primers used in the PCR were synthesized by Sigma-Genosys Ltd. The primer pairs for cyclin D1, 2, 3 and Ribosomal Protein L13 (RPL13) (the reference gene) were designed based on rat and mouse sequence information. The primers were: cyclin D1 sense TGGCCTCTAAGATGAAGGAGA, antisense AGGAA GTGTTTCGATGAAATCGT, (170 bp); cyclin D2 sense CTGCAGAACCTGTTGACTATCG, antisense TAATT CATGGCCAGAGGAAAG, (171 bp); cyclin D3 ATGT CTTCCCTCTGGCTATGAA, antisense GGTCCGT ATAGATGCAGAGCTT, (162 bp); and RPL13 sense CCACCCTATGACAAGAAAAAGC; antisense ACATT CTTTCTGCCTGTTTCC, (227 bp).

To create the calibration standards for the real-time PCR, PCR fragments generated with the

above-mentioned primer pairs using rat islet cDNA as a template together with PCR master mix (Roche) were sub-cloned into pSTBlue-1 vector (pSTBlue-1 Acceptor Vector kit; Novagen, Germany), and identity was confirmed by sequencing. These respective individual PCR fragments containing plasmids were linearised by HindIII, serial-diluted by a factor of 10, to create a series of diluted PCR fragments bearing plasmid solutions. They were employed as the external calibration standards in real-time PCR, and RPL13 expression was used as the internal reference control for the quantification of relative cyclin D1, 2 and 3 gene expression.

Western blotting

INS-1E cells were seeded in 30 mm cell culture dishes (1×10^6 cells per dish) and cultured for two days. Prior to stimulation, the cells were incubated in RPMI 1640 containing 0.25% bovine serum albumin (BSA) overnight and the medium was changed next day to stimulate the cells with 10 nM GLP-1 for 1.5, 3, 6 and 12 h. Samples were harvested by the addition of RIPA lysis buffer (1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM AEBSF, 1 mM orthovanadate, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin in PBS) and the lysate was transferred into eppendorf tubes. The lysate was kept on ice for 30 min to ensure total lysis of the cells and the samples were frozen in liquid nitrogen. The lysate was centrifuged for 3 min at 1100 g and the supernatant used to determine total protein content (Bio-Rad protein assay dye reagent). Equal amounts of total protein (40 μ g/lane) were separated on a SDS-PAGE (NuPAGE 12% Bis-Tris Gel; Invitrogen) in running buffer (NuPAGE MOPS-SDS). Prior to separation, $4 \times$ sample buffer (NuPAGE LDS sample buffer and NuPAGE sample reduction agent; Invitrogen) was added to the samples and denatured by 70°C for 10 min. The proteins were transferred to a Invitrolon PVDF membrane using the Novex electrophoresis and blotting system (Invitrogen). The PVDF membrane was blocked by incubation in 5% skimmed milk in TBS-T (Tris-HCl buffered saline containing 0.1% Tween-20) and protein expression was detected by incubation for 90 min at RT with the primary antibody (mouse monoclonal anti-cyclin D1, D2 and D3 antibody (1:200); Lab Vision Corporation, USA and rabbit polyclonal anti-Cdk4 (1:500); Santa Cruz Biotechnology, CA, USA) followed by a 1 h incubation at room temperature with the HRP-conjugated secondary antibody (rabbit anti-mouse IgG, 1:1000; Dako and swine anti-rabbit IgG, 1:1000, Dako; Denmark). Proteins were visualized using enhanced chemoluminescence (ECL) plus Western blotting detection reagents (RPN2132; Amersham Biosciences). The blot was stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA).

Transient transfection

A firefly luciferase reporter construct containing 2.4 kb of the mouse cyclin D1 promoter (kindly provided by Dr Martin Eilers, University of Marburg, Marburg, Germany) along with the internal control plasmid TK-renilla (Promega) were transiently transfected into islet monolayer or INS-1E cells using LipofectAMINE 2000 (Invitrogen Life Technologies) as previously described (Friedrichsen *et al.* 2003) using 2 μ l Lipofect AMINE 2000/well. Islet and INS-1E cells were incubated overnight with DNA-lipofectamine-Optimem mix in RPMI 1640 containing 2% HS and Optimem, respectively. The media were replaced for islet cells with RPMI 1640 containing 2% HS and for INS-1E cells with RPMI 1640 containing 0.25% BSA and 50 μ M β -mercaptoethanol, incubation continued for 24 h in the presence of compounds as indicated. Luciferase reporter assay was performed as previously described (Galsgaard *et al.* 1999, Friedrichsen *et al.* 2003).

Results

Effect of GLP-1, liraglutide, GIP and glucagon on β -cell replication in vitro

Monolayer cultures of pancreatic islet cells were stimulated with GLP-1, liraglutide, GIP and glucagon in a concentration range of 0.1–100 nM for 24 h. DNA synthesis of the β -cells measured by incorporation of BrdU followed by immunocytochemical staining for insulin and BrdU was carried out as previously described (Nielsen *et al.* 1989). GLP-1, liraglutide and GIP significantly increased the mitotic index from a basal level of approximately 3–4% to 5–7% fold at a maximal stimulatory concentration of 10–100 nM (Fig. 1). Co-incubation of GLP-1 and liraglutide with the GLP-1 receptor antagonist, exendin (9–39) completely inhibited the effect of both peptides, indicating that the effect of liraglutide as expected is mediated by the GLP-1 receptor. Incubation with 0.1–100 nM of glucagon had no significant effect on replication of the β -cells in these cultures. Co-incubation of GLP-1 (100 nM) or GIP (100 nM) with hGH (0.5 μ g/ml) for 24 h had an additive/synergistic effect on β -cell replication (Fig. 2A). Treatment with hGH led to an approximate 120% increase of mitotic activity after 24 h, while GLP-1 and GIP instigated an approximate 50% increase. Combination of hGH and GLP-1 led to a 170% increase of BrdU-positive β -cells (indicating an additive effect), while co-incubation of GIP with hGH produced an approximate 250% increase (suggesting a synergistic effect). The effect of GIP and hGH in combination produced a mitotic index of up to approximately 20% (Fig. 2B).

Figure 1 Effect of glucagon, GLP-1, GIP and liraglutide, on β -cell replication. Dispersed islet cells in monolayer culture were stimulated with peptides, as indicated, for 24 h in the presence of 10 μ M BrdU. BrdU-positive β -cells were visualized by double-immunostaining for insulin and BrdU and quantified by counting under the microscope. The mitotic index was calculated as the fraction of BrdU-positive β -cells in percent of the total number of β -cells counted (1000–2000/slide). Data represented are means $\pm$ S.E.M. ($n=3-6$). Asterisks indicate statistically significant differences (t-test, unpaired) between cells cultured in the absence and presence of the indicated peptides. * $P<0.05$; ** $P<0.01$. Ex, exendin (9–39).

Effect of protein kinase inhibitors on GLP-1 and GIP-induced β -cell replication

The islet cell monolayer cultures were incubated with GLP-1 (100 nM) or GIP (100 nM) in the presence of the PKA inhibitor, H89 (10 μ M), the PI3K inhibitor, wortmannin (10 nM), the MEK inhibitor, PD98059 (20 μ M), and the p38 MAPK inhibitor, SB203580 (10 μ M) and the effect of these kinase inhibitors on β -cell replication was measured by BrdU incorporation assay (Fig. 3). The β -cell mitotic response to both GLP-1 and GIP was completely inhibited by H89. In addition, GLP-1-stimulated proliferation was completely inhibited by wortmannin and GIP-stimulated DNA synthesis was blocked by PD98059. A tendency for the inhibition of GLP-1-stimulated DNA-synthesis by PD98059 and GIP-stimulated DNA-synthesis by wortmannin was also observed. No inhibitory activity was observed by co-incubation with SB203580 on either GLP-1 or GIP-stimulated activity. As previously reported, wortmannin and PD98059 have no inhibitory effect on hGH-stimulated β -cell replication in this assay and neither of the various inhibitors significantly affected the basal mitotic rate at the concentrations used (data not shown, described previously in Friedrichsen *et al.* 2001). The adenylate cyclase activator forskolin (10 μ M) instigated an increase of β -cell replication similar to GLP-1 and GIP, indicating that elevation of cAMP is a sufficient stimulus to initiate mitosis of pancreatic β -cells.

Analysis of GLP-1 induced cyclin D expression

To determine whether induction of the cell cycle regulatory factors, cyclin Ds, may be involved in the mitotic

Figure 2 Effect of combinatorial treatment with hGH, GLP-1, and GIP on β -cell replication. Islet cell monolayer cultures were stimulated with peptides as indicated and β -cell replication was measured as described in Fig. 1. The basal BrdU incorporation range was 3.8 ± 0.1 percent BrdU-positive beta-cells. (A) Quantified data representing means $\pm$ S.E.M. of 3–5 independent experiments. (B) A representative of the observed effect of the combination of hGH and GIP.

response to GLP-1, newborn rat islets were stimulated for 24 h with hGH, GLP-1 and forskolin and the mRNA levels of cyclin D1, D2, and D3 were determined by quantitative RT-PCR (Fig. 4). As previously demonstrated in INS-1 cells, we observed in primary islets a significant increase (approximately 80%) of cyclin D2 mRNA levels in response to hGH treatment whereas hGH had no effect on cyclin D1 and D3 mRNA levels. In contrast, we found a statistically significant effect of forskolin on cyclin D1 expression and a ~ 1.4 fold increase of cyclin D1 expression in response to GLP-1 stimulation, which was not found to be statistically significant. GLP-1 is a weaker growth stimulus islet cells compared with hGH. Forskolin appears, based on the reporter assay analysis, to be a more potent inducer of the cyclin D1 promoter than GLP-1. We would therefore anticipate a smaller induction of cyclin D1 mRNA expression by GLP-1 than forskolin.

Additionally, INS-1E cells were stimulated for 6 and 12 h with GLP-1 and the mRNA levels of cyclin D1, D2,

and D3 were determined by quantitative RT-PCR (Fig. 5A, left panel). The cyclin D1 mRNA level was significantly increased by GLP-1 with an increase of 100 and 37% over their respective basal levels at 6 and 12 h. GLP-1 had a minor but significant effect on cyclin D3 expression at these time points, 20 and 28% over their basal levels, respectively. The cyclin D2 level was not affected by GLP-1 stimulation. The same effects were obtained with forskolin stimulation, however the inductions were more marked (Fig. 5A, right panel). Cyclin D1 expression was induced 900 and 400% over their respective controls at 6 and 12 h, respectively and cyclin D3 was induced 150 and 110%, respectively, at these two time points. Forskolin had no effect on cyclin D2. At the protein level, cyclin D1 was induced by GLP-1 in a time-dependent manner with maximal induction after 12 h (Fig. 5B). However, the cyclin D2 and D3 levels were not affected by GLP-1 at any time points. Furthermore, we investigated the catalytic subunit of cyclin D, CDK4, and found that it was neither affected by GLP-1.

Figure 3 Effect of protein kinase inhibitors on GLP-1 and GIP induced β -cell replication. Islet cell monolayer cultures were pre-treated with protein kinase inhibitors for 10–15 min, stimulated for 24 h with GLP-1 (100 nM) or GIP (100 nM) in the presence of BrdU, and BrdU incorporation measured as described in Fig. 1. Quantified data represent the means $\pm$ S.E.M. of 3–7 independent experiments. H89 (10 μ M), PD98059 (20 μ M), SB203580 (10 μ M), wortmannin (10 nM), forskolin (10 μ M). Asterisks indicate statistically significant differences (*t*-test, unpaired) between cells cultured in the absence and presence of the indicated inhibitors. **P*<0.05; ***P*<0.01.

Transactivation of the cyclin D1 promoter by GLP-1

To further address the role of GLP-1 and potentially other cAMP-elevating hormones in the transcriptional control of the cyclin D1 gene in pancreatic β -cells, we performed reporter gene analysis using a 2.4 kb promoter fragment of the cyclin D1 gene coupled to a cDNA encoding firefly luciferase. Upon stimulation of islet cells, transiently transfected with this construct, we observed a 2.5-fold increase of promoter activity in response to GLP-1 treatment and a 6–8-fold increase of promoter activity in response to forskolin (Fig. 6A). Stimulation with hGH had no effect on cyclin D1 promoter activity, and neither GLP-1, hGH nor forskolin affected the activity of the promoter-less reporter vector, pGL2-b. Effects on cyclin D1 promoter activity similar to those produced by GLP-1 have been observed with GIP in these islet cell cultures (data not shown). Transient transfection of the reporter construct into INS-1E cells followed by stimulation with

Figure 4 Effect of GLP-1 on cyclin D mRNA in newborn rats. Islets were cultured in suspension in the absence or presence of GLP-1, hGH, and forskolin, as indicated, for 24 h. The islets were harvested, RNA extracted and quantitative RT-PCR analysis performed using primer sets specific for cyclin D1, D2, and D3, respectively, and using primer sets specific for tata-binding protein (TBP) and glucose 6-phosphate dehydrogenase (G6 PDH) as internal controls. Data represented (means $\pm$ S.D. of 3 independent cDNA preparations) shows the calculated relative mRNA levels where the basal expression is assigned 1.0. Asterisks indicate statistically significant differences (*t*-test, unpaired) between cells cultured in the absence and presence of the indicated treatment. **P*<0.05.

hGH, GLP-1, GIP or forskolin produced similar effects as in islet cells. Thus, GLP-1, GIP and forskolin specifically increased cyclin D1 promoter activity approximately 1.5, 2, and 4-fold respectively, whereas no effect of hGH was observed (Fig. 6B).

Effect of protein kinase inhibitors on GLP-1-induced cyclin D1 promoter activity

In order to initially characterize the signalling pathways and transcription factors that may be involved in GLP-1- and cAMP-induced transcriptional activation of the cyclin D1 promoter, protein kinase inhibitors were co-incubated with GLP-1 and forskolin in the cyclin D1 promoter-reporter assay (Fig. 7). The PKA inhibitor, H89, the PI3K inhibitor, wortmannin, and the MEK inhibitor, PD98059, all inhibited GLP-1-induced activation of the cyclin D1 promoter, whereas the p38 MAPK inhibitor, SB203580 had no effect. The same effects of the inhibitors were observed for forskolin-induced cyclin D1 promoter activity (data not shown).

Figure 5 Effect of GLP-1 on cyclin D mRNA and protein levels in INS-1E cells. (A) INS-1E cells were grown as monolayer culture in the absence or presence of GLP-1 for 6 and 12 h. The INS-1E cells were harvested, RNA extracted and quantitative PCR analyses of the relative expression of cyclin D1, D2, and D3 mRNA were performed using primer sets specific for cyclin D1, D2, and D3, respectively. The respective mRNA expression levels were relative to RPL13 expression level in the same sample at 6 and 12 h. Data represent mean $\pm$ S.E.M. of 6 independent cDNA preparations done in duplicate in the individual PCR run. The cDNA preparations were done in duplicate in the individual PCR run. Open bar is control, filled bar is 100 nM GLP-1 (7–36) and dotted bar is 10 μ M forskolin (Fsk). The statistical analysis was assessed first by one-way ANOVA, followed by Tukey's multiple comparison test. *****P*<0.001; ****P*<0.01; ***P*<0.05. (B) INS-1E were stimulated with 10 nM GLP-1 and harvested at the indicated timepoints. Total protein was extracted and Western blot performed using specific antibodies for cyclin D1–D3 and CDK4. Data illustrate a representative of 2 independent experiments.

Figure 6 Effect of GLP-1 and GIP on cyclin D1 promoter activity in insulin producing cells. (A) Islet cell monolayer cultures were prepared in 24-well dishes and transfected overnight using LipofectAMINE 2000 with cyclin D1-promoter-luciferase construct or promoter-less luciferase vector (pGL2-b). The transfected cells were stimulated with peptide as indicated for 24 h and the cells extracted and subjected to analysis of firefly luciferase activity. Data (means $\pm$ S.E.M. of 4–7 independent experiments) are represented as fold induction where the basal activity is assigned a value of 1.0. (B) INS-1E cells transfected and subjected to analysis of peptide-induced cyclin D1 promoter activity as above. Data represented are means $\pm$ S.E.M. of 3–4 independent experiments. Asterisks indicate statistically significant differences (*t*-test, unpaired) between cells cultured in the absence and presence of the indicated treatment. **P*<0.05. The internal control plasmid produced in the islet cells activities in the range of 15 000–50 000 RLU/10 s per 20 μ l extract, whereas in the INS-1 cells the values ranged from 100 000–800 000 RLU/10 s per 20 μ l extract.

Discussion

The differentiated pancreatic β -cell has generally been considered ‘post-mitotic’ with very little potential for replication left in the adult organism. However, several

Figure 7 Effect of protein kinase inhibitors on GLP-1 induced cyclin D1 promoter activity. INS-1E cells were transiently transfected as described in Fig. 5 with cyclin D1 promoter-luciferase construct and treated with GLP-1 for 24 h in the absence or presence of protein kinase inhibitors as indicated. Cells were subjected to analysis of luciferase activity and the depicted data (means $\pm$ S.E.M.) shows a single experiment performed with triplicate incubates, representative of 3 independent experiments. Asterisks indicate statistically significant differences (*t*-test, unpaired) between cells cultured in the absence and presence of the indicated treatment. ***P*<0.01.

recent studies indicate that the β -cell mass is far more plastic than originally thought and that β -cell replication may occur in response to metabolic demand and certain growth factors and hormones (Eisenbarth & Stegall 1996, Bonner-Weir & Weir 2001, Nielsen *et al.* 2001). In the present study we have shown that GLP-1, GIP, and the long-acting GLP-1 derivative, liraglutide, stimulate β -cell proliferation *in vitro*. As expected, the effects of GLP-1 and liraglutide were dependent on the GLP-1 receptor, since a molar excess of exendin 9–39 was able to block the activity of both peptides. In addition, forskolin had a mitogenic effect on the β -cells indicating that elevation of cAMP levels is sufficient to drive proliferation in these cells in accordance with previous findings (Frödin *et al.* 1995). Surprisingly, glucagon failed to stimulate β -cell proliferation in our system. Glucagon receptors have been found to be expressed in rat β -cells but the activity to stimulate cAMP formation was 45-fold less than that of GLP-1 and GIP receptors (Moens *et al.* 1996). High concentrations of glucagon will activate GLP-1 receptors (Moens *et al.* 1998). Interestingly, glucagon but neither GLP-1 nor pituitary adenylyl cyclase-activating peptide were found to stimulate the expression of the transcriptional repressor inducible cAMP early repressor in pancreatic β -cells (Hussain *et al.* 2000).

The effects of GLP-1 and GIP on proliferation of the primary β -cells were found to be dependent on cAMP/PKA, p42 MAPK and PI3K signalling pathways according to inhibitor experiments, similar to previous studies in insulinoma cell lines (Buteau *et al.* 1999, Trümper *et al.* 2001). Our studies indicated some differences in GLP-1 and GIP signalling; GLP-1 signalling seems to be more

dependent on PI3K activity and this may rely on the recent findings showing that GLP-1 induces transactivation of the EGFR leading to PI3K activation (Buteau *et al.* 2003). GIP on the other hand appears mostly dependent on p42 MAPK activity, but further studies are needed to clarify this phenomenon. Inhibition of p38 MAPK using SB203580, had no effect on the activities of GLP-1 and GIP, in contrast to a recent study in INS-1 cells (Buteau *et al.* 2001). Whether this represents a difference between the tumour cell line and primary cells, which we have also observed in relation to growth hormone and prolactin signalling (Friedrichsen *et al.* 2001), or differences of experimental protocols, awaits to be determined. However, our finding of opposing roles of p42/44 MAPK and p38 MAPK in cell proliferation is similar to findings in other cell types (Lavoie *et al.* 1996). The stimulatory effect of incretins and hGH on β -cell replication appeared to be additive in the case of GLP-1 and even synergistic in the case of GIP that also indicates a slight difference in signalling between the two incretins. Although most studies including the knockout of one or both receptors for GLP-1 and GIP suggest a similar mode of action of the two hormones in β -cells (Preitner *et al.* 2004) however, subtle differences have been reported. Thus GLP-1 receptors seem to be more prone to desensitization than GIP receptors (Delmeire *et al.* 2004) and in type 2 diabetes the β -cell response to GIP is reduced, whereas the response to GLP-1 is retained (Holst & Gromada 2004).

Exit from the G0 phase of the cell cycle and progression through the so-called restriction point of the G1 phase appears to rely, in most cell types, on transcriptional induction of the cell cycle regulatory factors, cyclin Ds (for review see Reetz *et al.* 1991, Lania *et al.* 1999, McDonald & El Deiry 2000, Garrett 2001). Three cyclin D isoforms exist (D1, D2, D3) encoded by separate genes and with both redundant and non-redundant functions (Fantl *et al.* 1995, Sicinski *et al.* 1996, Lahti *et al.* 1997). Cyclin Ds act as co-factors for cyclin-dependent kinases (cdk) 4 and 6, which are responsible for phosphorylation of retinoblastoma protein and the release of E2F transcription factors, critical for transcriptional induction of many S-phase genes. An important role of these proteins in β -cell mass regulation is suggested by the finding that Cdk-4 knockout mice carry a diabetic phenotype, whereas knockin of a constitutive active Cdk-4 mutant is associated with β -cell hyperplasia (Rane *et al.* 1999).

We have previously demonstrated that hGH, which is a potent growth factor for β -cells, stimulates the expression of cyclin D2 in INS-1 in a partially STAT5-dependent manner (Friedrichsen *et al.* 2001). Furthermore, we have recently shown that this up-regulation is mediated by a direct action of STAT5 on the cyclin D2 promoter (Friedrichsen *et al.* 2003). In the present study, we have investigated the effect of GLP-1 on cyclin D mRNA and protein expression in INS-1E cells, and found that this treatment is associated with a specific elevation of cyclin

D1 in a time-dependent manner with maximal induction at 6 and 12 h, respectively. The reason for the lack of sensitivity in islets compared with the INS-1 cells may be the heterogeneous expression of cyclin D1 and D2s in the β -cell monolayer cultures (data not shown) which is probably due to 1) lack of synchronization with cell cycle position and/or 2) only a subpopulation of β -cells responds to growth stimuli by replication within a given time period. In contrast, INS-1 cells can be experimentally synchronized in the cell cycle in low serum and all cells are responsive to growth stimuli. Thus, a low basal mRNA level and high induction rate may be achieved in the cell line compared with the primary cells. No effects were observed on the cyclin D2 and cdk 4 levels. However, a minor effect was detected on the cyclin D3 mRNA levels but no effect was observed at the protein level. Using inhibitors of protein kinases that have been suggested to play a role in β -cell replication, we found that GLP-1 and GIP-induced β -cell replication as well as cyclin D1 promoter transcriptional induction were dependent on both cAMP/PKA, PI3K and p 42 MAPK activities.

Both the mouse cyclin D1 and D3 promoter have been found to contain a cAMP regulatory element (CRE) (Wang *et al.* 1996, Eto 2000). The relatively weak effect we detected at the cyclin D3 mRNA level after 6 and 12 h may be due to more rapid kinetics and transient induction compared with that of cyclin D1. Moreover, recent data from knockout mice presented by Kushner *et al.* (2005), indicates that cyclin D3 plays a minor role in promoting islet function compared with cyclin D1 and D2. Whereas, cyclin D1 can partially compensate for the absence of cyclin D2, this does not seem to be the case for cyclin D3. However, additional experiments are required to clarify the exact extent and time course of the cyclin D3 regulation we observed in the β -cells. Forskolin has been shown to augment heregulin-induced cyclin D3 in Schwann cells via the CCAAT/enhancer binding protein-beta (C/EBPbeta) element indicating a cAMP-dependent regulation of cyclin D3 expression (Fuentelba *et al.* 2004). This is furthermore in accordance with our findings showing that forskolin was capable of significantly stimulating cyclin D3 expression after 6 and 12 h, respectively. In most cell types the response to CRE is associated with anti-mitogenic effects (Sabbah *et al.* 1999, Eto 2000, Richards 2001). However, in certain endocrine cell types, cAMP is stimulatory to S-phase progression (Richards 2001). The transcription factors that may potentially interact with the CRE element belong to the cAMP regulatory element binder (CREB)/activating transcription factor (ATF) superfamily of transcription factors. The MAPK signalling cascade may be activated by cAMP via guanine nucleotide exchange factors (Leech *et al.* 2000). MAPK may influence cell proliferation by activation of the transcription factor Elk-1, which binds as a heterodimer to other transcription factors like serum response factor (SRF) involved in the expression of the

immediate early genes, Fos, Jun and ATF subgroups of transcription factors, that in turn bind as heterodimers to the AP-1 elements of the cyclin D1 or D3 promoter (Dinarelo 1996, Lavoie *et al.* 1996). Activation of the PI3K/protein kinase B (PKB)/AKT pathway is linked to cell survival in many systems possibly involving the PKB/serum and glucocorticoid-inducible kinase substrates ribosomal kinase p70S6K, glycogen synthase kinase-3 (GSK-3), Bcl-2 family member, BAD, and the forkhead transcription factors FKHL1 and AFX1 (Brunet *et al.* 1999, Nakamura *et al.* 2000, Lawlor & Alessi 2001, Suhara *et al.* 2002). The role of this pathway in relation to cell cycle progression is not well understood. However, FKHL1 has in its un-phosphorylated form been shown to activate genes involved in cell cycle arrest, e.g. p27 kip (Nakamura *et al.* 2000), and death, e.g. Fas ligand (Suhara *et al.* 2002). Un-phosphorylated GSK-3 is involved in mediating proteasomal cyclin D1 degradation and has been shown to inhibit cyclin D1 transcription (Diehl *et al.* 1998). Furthermore, has the PI3K-pathway been linked to SRF-dependent activation of the c-fos promoter, independently of the MAPK pathway (Poser *et al.* 2000). The identification of the multiple elements and transcription factors potentially involved in incretin-induced cyclin D1 and eventually cyclin D3 promoter activation in β -cells will require thorough analysis of the promoters.

In conclusion, we have identified cyclin D1 as a target gene in β -cells for incretins. Multiple signalling pathways activated by incretins may contribute to the up-regulation of this gene. Cyclin D3 may be a target gene in β -cells for incretins as well but this has to be clarified. The finding that GH/PRL and incretins in rodents stimulate distinct cyclin D genes may in part explain the additive/synergistic effect of these hormones. Combination of the growth factors may be relevant for *in vitro* amplification of β -cells and as potential co-treatment in islet transplantations to maintain graft survival. Furthermore, based on the experienced stimulation of pancreatic cells with GLP-1, GIP and liraglutide we conclude that these hormones have the potential to increase β -cell regeneration/replication that, if possible to transfer to humans, would have significant impact on long term diabetes treatment.

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