Glucagon-Like Peptide 1 Induces Pancreatic β-Cell Proliferation Via Transactivation of the Epidermal Growth Factor Receptor

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We previously provided evidence that glucagon-like peptide 1 (GLP-1) induces pancreatic β-cell growth nonadditively with glucose in a phosphatidylinositol (PI) 3-kinase- and protein kinase C ζ-dependent manner. However, the exact mechanism by which the GLP-1 receptor (GLP-1R), a member of the G protein–coupled receptor (GPCR) superfamily, activates the PI 3-kinase signaling pathway to promote β-cell growth remains unknown. We hypothesized that the GLP-1R could activate PI 3-kinase and promote β-cell proliferation through transactivation of the epidermal growth factor (EGF) receptor (EGFR), an event possibly linked to GPCRs via activation of c-Src and the production of putative endogenous EGF-like ligands. Both the c-Src inhibitor PP1 and the EGFR-specific inhibitor AG1478 blocked GLP-1–induced proliferation of mouse islet cells as well as in isolated rat islets, while only AG1478 inhibited the proliferative action of beta-cellulin (BTC), an EGFR agonist. Both compounds also suppressed GLP-1–induced PI 3-kinase activation. A time-dependent increase in tyrosine phosphorylation of the EGFR in response to GLP-1 was observed in INS(832/13) cells. Not only was this transactivation of the EGFR sensitive to both the pharmacological agents PP1 and AG1478, the action of GLP-1 and BTC on INS(832/13) cells proliferation was found to be not additive. Overexpression of a dominant-negative EGFR in INS(832/13) cells caused a decrease in cell surface–associated BTC, as shown by FACS analysis. Also, the metalloproteinase inhibitor GM6001 and an anti-BTC neutralizing antibody suppressed the GLP-1 proliferative effect. Finally, coculturing the prostatic cancer cell line LNCaP that lacks GLP-1 responsiveness with INS cells increased LNCaP cell proliferation in the presence of GLP-1, thus revealing that INS cells secrete a growth factor in response to GLP-1. GM6001 and an anti-BTC neutralizing antibody suppressed increased LNCaP cell proliferation in the presence of GLP-1 in the coculture experiments. The results are consistent with a model in which GLP-1 increases PI 3-kinase activity and enhances β-cell proliferation via transactivation of the EGFR that would require the proteolytic processing of membrane-anchored BTC or other EGF-like ligands. Diabetes 52: 124–132, 2003

Glucagon-like peptide 1 (GLP-1) (7–36) amide, a potent glucoincretin hormone (1,2), is secreted by the intestinal L-cells in response to fat meals and carbohydrates (3,4). It is a potentially important drug in the treatment of type 2 diabetes in light of its ability to improve insulin secretion in both subjects with impaired glucose tolerance and type 2 diabetes (5,6). GLP-1 is also an insulinotropic agent through its ability to stimulate insulin gene expression and proinsulin biosynthesis (7,8). GLP-1 increases the expression level of the β-cell-specific transcription factor pancreatic and duodenal homeobox gene-1 (PDX-1) (9), which is implicated in the regulation of the expression of insulin, GLUT2, and glucokinase genes and in β-cell differentiation (10–12). In addition, the glucocorticoids increases in vitro β-cell proliferation nonadditively with glucose via a phosphatidylinositol (PI) 3-kinase/protein kinase C ζ signaling pathway in β(INS-1) cells (9,13), as well as the islet mass in mouse pancreas in vivo (14). Finally, GLP-1 induces several immediate early response genes and proto-oncogenes in INS cells that are implicated in cell growth/apoptosis control, such as c-fos, c-jun, junD, and nur77 (15,16).

GLP-1 signal transduction in the β-cell is now being extensively studied. GLP-1 interaction with its specific high-affinity receptor (GLP-1R), a member of the G protein–coupled receptor (GPCR) superfamily, increases cAMP levels in several β-cell models to activate the protein kinase A signal transduction system (4,17,18). There is also evidence that this rise in cAMP levels leads to an increase in cytosolic Ca2+ (19,20), possibly via cAMP-regulated guanine nucleotide exchange factor II (Epac2) (21). However, the precise mechanism by which GLP-1R activates the PI 3-kinase signaling pathway to mediate the proliferative action of GLP-1 has not been elucidated.

Recent studies investigating the mitogenic effects of GPCR agonists have led to the concept that some GPCRs that lack intrinsic kinase activity transactivate the epidermal growth factor (EGF) receptor (EGFR) to promote cell proliferation and mitogenic signal transduction pathways (22–24). The EGFR has been recently identified as an essential element in the GPCR-mediated activation of
mitogen-activated protein kinase and PI 3-kinase signaling pathways in cells treated with various GPCR agonists, such as lysophosphatidic acid (23,24), thrombin (24), and angiotensin II (22,25). In some instances, the EGFR transactivation by GPCRs has been documented to require the non-receptor-type tyrosine kinase c-Src (26,27). The mechanism involves the production of endogenous EGFR ligands from transmembrane precursors via GPCR-induced c-Src activation of an endoprotease (28).

Receptor molecules for EGFR-like growth factor receptors are encoded by the c-erbB gene and its relatives. Upon binding of their ligands, EGFRs undergo dimerization and auto-phosphorylation on tyrosine residues in order to recruit non-receptor-type tyrosine kinase c-Src (26,27). The activated c-Src in turn phosphorylates on tyrosine residues in order to recruit Src homology 2 containing proteins responsible for signal transduction (24). There are at least four known members for the EGFR family: c-erbB-1 (EGFR), c-erbB2, c-erbB-3, and c-erbB4. The c-erbB-1/EGFR is expressed throughout the human fetal pancreas and in adult β-cells (29). Huotari et al. (29) showed that only the c-erbB-1/EGFR gene is expressed in INS-1 cells and that betacellulin (BTC), a member of the EGFR family and activator of erbB-1/EGFR and erbB-4 (30), displayed mitogenic activity as it stimulated INS-1 cell replication at picomolar concentrations (29). BTC, a 9.5-kDa glycoprotein expressed in several mouse tissues, including kidney, liver, and pancreas (31), is synthesized from a 32-kDa membrane-anchored precursor that is thought to be proteolytically cleaved to generate an active and soluble secreted form of the molecule (32), as occurs with other members of the EGFR family. Since BTC is expressed in all pancreatic β-cell types examined thus far, such as various insulinomas (33), the β-cell line BT2C (31), and normal β-cells (34), as well as in pancreatic duct cells (34), it is reasonable to think that BTC could link the GLP-1R to the EGFR and be responsible for the mitogenic action of GLP-1 on the β-cell.

In this study, our aim was to investigate the possible implication of EGFR transactivation in GLP-1-induced β-cell proliferation and PI 3-kinase stimulation. Using both pharmacological and molecular biology approaches, we provide evidence supporting the concept that c-Src-dependent transactivation of the EGFR and proteolytic processing of membrane-anchored BTC or other EGF-like ligands link GLP-1R signaling to PI 3-kinase activation and β-cell proliferation.

RESEARCH DESIGN AND METHODS

Reagents. Pertussis toxin was purchased from Calbiochem (La Jolla, CA). AG1478 and PP1 were purchased from Bimol (Plymouth Meeting, PA). Human GLP-1 fragment 7–36 amide and human recombinant BTC were obtained from Sigma (St. Louis, MO). The anti-EGFR antibody was purchased from Cell Signaling Technology (Mississauga, ON). The PY20 and 4G10 anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. Monoclonal anti-β-trytic primary antibody was from R&D System (Minneapolis, MN). GM6001, a pan-specific pharmacological metalloproteinase inhibitor, was from Chemicon International (Temecula, CA). RPMI-1640 and the cell culture supplements, including FCS, were purchased from Gibco BRL (Burlington, Canada). Monoclonal [3H]thymidine was from ICN (Costa Mesa, CA). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Cell culture and incubation. INS832/3 (35 cells) (passages 36–70) were grown in monolayer cultures as described previously (36) in regular RPMI-1640 medium supplemented with 10 mmol/l HEPES, 10% heat-inactivated FCS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 100 μM L-arginine, and 100 μg/ml streptomycin at 37°C in a humidified (5% CO2/95% air) atmosphere. This clone (832/13) of INS-1 cell was used because it shows better differentiation characteristics in terms of glucose-stimulated insulin secretion than the original INS-1 cell line (35). When cells reached 80% confluence (after ~7 days), they were washed with PBS and incubated at 37°C for 12 h in a humidified KRBH medium containing 1 mmol/l CaCl2, 5 mmol/l NaHCO3, 25 mmol/l HEPES (pH 7.4) supplemented with 3 mmol/l glucose, and 0.1% defatted BSA (Fraction V; Sigma). The pharmacological inhibitors were added to the medium during the last 30 min of the preincubation period. Cells were then washed with PBS and incubated for the indicated times in the same supplemented KRBH medium containing the substances to be tested.

LNCaP cells were kindly given by Dr. Mes-Masson (University of Montreal) and cultured as described above for INS832/13 cells.

[3H]Thymidine incorporation assay. A previously described procedure (9,37) was used for [3H]thymidine incorporation assay. In brief, cells were seeded 2 days before use in 96-well plates [8 × 103 INS832/13 cells or 1.5 × 105 LNCaP cells per well] and cultured in regular complete RPMI medium as described above. Cells were then washed with PBS and preincubated for a period of 24 h in minimal RPMI medium (i.e., without serum but with 3 mmol/l glucose and 0.1% BSA). They were then incubated for 24 h in minimal RPMI medium with various test substances. Proliferation was determined by incorporation of [3H]thymidine (1 μCi/well) during the final 4 h of the 24-h incubation period. Cells were then harvested with a PHD cell harvester from Cambridge Technology (Watertown, MA), and the radioactivity retained on the dried glass fiber filters was measured by liquid scintillation.

β-cells labeling and detection. Cells were cultured on glass coverslips at 80% confluence, attached onto polyornithine-coated coverslips, and cultured as described for the [3H]thymidine incorporation assay. For the coculture experiments, INS832/13 and LNCaP cells were mixed and seeded together at a 5.1 ratio. BrdU labeling and detection was performed with a commercially available kit (BrdU labeling and detection kit I; Roche Diagnostics, Laval, Canada) according to manufacturer’s protocol. In brief, BrdU labeling was performed for the last hour of a 24-h incubation period. After cell fixation and permeabilization of 10 nmol/l GLP-1 or 5 ng/ml BTC with or without an anti-BTC antibody (0.25 μg/ml) or 2.5 μmol/l GM6001 (Ionomastat). Cells were then washed, fixed in ethanol, and incubated with a mouse monoclonal anti-BrdU antibody and a rabbit anti-insulin antibody (Santa Cruz Biotech, Santa Cruz, CA) working solution. After several washes with PBS, cells were stained with a sheep anti-mouse fluorescein-conjugated antibody and a donkey anti-rabbit rhodamine-conjugated antibody (Jackson Immunoresearch, West Grove, CA). The fluorescence of bound anti-BrdU and anti-insulin antibodies was visualized under a fluorescence microscope at 400× magnification. At least 200 cells were analyzed for each condition of an experiment. INS832/13 cells and LNCaP cells were identified by their different insulin staining and cell morphology.

Rat islets isolation and incubation. Pancreatic islets were obtained from male (200 g) Wistar rats as described (38). Isolated rat islets (50 islets per condition) were seeded in 12-well plates and cultured in regular RPMI containing 11 mmol/l glucose and 10% FCS for 24 h. Islets were then washed with PBS and preincubated for a period of 24 h in minimal RPMI medium. They were then incubated for 24 h in minimal RPMI medium containing 3 mmol/l glucose with or without GLP-1. [3H]Thymidine (5 μCi/well) was added during the final 6-h incubation period. Then, islets were lysed and DNA precipitated in 10% trichloroacetic acid (TCA) using a published procedure (39). After several washes of the TCA pellets, DNA was solubilized in 0.4 mol/l NaOH and radioactivity measured.

Immunoprecipitation and Western blotting. INS832/13 cells were grown and incubated as described above in 100-mm petri dishes, washed twice with PBS, and lysed in 1 ml of ice-cold lysis buffer (50 mmol/l Tris-HCl, pH 8.0, 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 5 mmol/l sodium pyrophosphate, and 1 mmol/l orthovanadate) for 30 min at 4°C. Samples were centrifuged (16,000g for 20 min), and the supernatants were used for protein determination and immediately submitted to immunoprecipitation. EGFR was immunoprecipitated for 4 h at 4°C from 2 mg of cell lysates with 5 μg sheep polyclonal anti-EGFR (Fitzgerald, Concord, MA) adsorbed to 30 μl protein A-Sepharose. After four washes in lysis buffer, proteins were denatured in SDS-PAGE sample buffer, subjected to electrophoresis on 7% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked with 1% BSA in PBS and probed with a cocktail of monoclonal anti-phosphorylated EGFR primary antibodies (800 ng/ml PY20 and 200 ng/ml 4G10) in PBS/BSA and subsequently with peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). After chemiluminescence detection with enhanced chemiluminescence (Amersham-Pharmacia, Buckingham, England), membranes were stripped for 30 min at 50°C in a buffer containing 62.5 mmol/l Tris-HCl (pH 8.0), 2% SDS, and 100 mmol/l β-mercaptoethanol and reprobed with a sheep polyclonal anti-EGFR primary antibody.
increase in proliferation observed with the \[3H\]thymidine (see also Fig. 10). Thus, the relatively modest

Reactions were stopped after 4 min at room temperature with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 100 mM EGTA. After a 10-min incubation period with 10 μCi of \( \text{[3H]P} \) ATP were added with 10 mM MgCl\(_2\). Lipids were extracted and spotted on silica gel plates for thin-layer chromatography. The plates were then dried and exposed to films for autoradiography.

Flow cytometric analysis of cell surface receptors into signaling-incompetent heterodimers (24). GPE-Isf4E1 and GPE-CDS33 cells generating empty retroviral particles and dominant-negative EGFR retroviral particles, respectively, were kindly provided by Dr. A. Ullrich (Max-Planck-Institut für Biochemie, Martinsried, Germany) and grown to 80% confluency in 100-mm petri dishes in 10 ml of regular RPMI medium. Then, culture media were changed and new media containing retroviruses were collected after 48 h and filtered through 0.45-μm filters and kept frozen at −80°C. Retroviruses containing RPMI media were diluted 1:4 before infections of INS(832/13) cells in 96-well plates. Cells were washed with PBS 24 h postinfection and serum starved to performed \([3H]\)thymidine incorporation assays as described above. The dominant-negative EGFR mutant lacks the cytoplasmic domain and disrupts EGFR downstream signaling by forcing endogenous wild-type receptors into signaling-incompetent heterodimers (24).

Flow cytometric analysis of cell surface–anchored BTC content. INS(832/13) cells were cultured as described above in 60-mm petri dishes. Cells were harvested and incubated for 30 min at 4°C with a monoclonal anti-BTC primary antibody (0.5 μg/ml) in PBS and subsequently with biotin-conjugated goat anti-mouse IgG (7 μg/ml in PBS) (Jackson ImmunoResearch, West Grove, PA) for another 30 min. After incubation with a streptavidin-conjugated FITC, cells were fixed in 1% parafomaldehyde (pH 7.4), and sample acquisition was performed on a Epics XL flowcytometer from Coulter (Miami, FL) using Septon II software.

Calculations and statistics. Data are presented as means ± SE. Statistical analyses were done with the SPSS for Windows. Differences between two conditions were assessed with Student’s t test for related samples. Differences were deemed significant at \( P < 0.05 \).

RESULTS

GLP-1–induced β-cell proliferation requires c-Src and EGFR transactivation. To assess the early signaling events implicated in GLP-1–induced β-cell proliferation, we first tested the effect of various pharmacological agents, including pertussis toxins, PP1, and AG1478 (specific inhibitors for Gi, c-Src, and the EGFR, respectively). As shown in Fig. 1, GLP-1 increased INS(832/13) cell proliferation by ~40%, as assessed by \([3H]\)thymidine incorporation. However, when proliferation was assessed using the BrdU incorporation assay, the percentage of positive INS cells nuclei increased from <1% for the control situation to ~4% when cells were cultured with 10 nmol/l GLP-1 (see also Fig. 10). Thus, the relatively modest increase in proliferation observed with the \([3H]\)thymidine incorporation assay might be due to intrinsic factors of the assay and to the low increased percentage of cells that respond to GLP-1. Indeed, the low labeling index found in response to GLP-1 with \([3H]\)thymidine might be due to self-decomposition products of the thymidine and incorporation of catabolic products of the thymidine in cellular fractions other than DNA, thus raising background incorporation in the control situation. It has also been considered that the BrdU-labeled index is determined visually by counting positive nuclei and that this method, in contrast to the thymidine incorporation measurements assay, does not account for the general background

![FIG. 1. c-Src and EGFR inhibitors suppress GLP-1–induced DNA synthesis in INS(832/13) cells.](image)

inclusion nor quantify the level of BrdU incorporation. Because of the convenience of the \([3H]\)thymidine incorporation assay, we used this method in most subsequent experiments. GLP-1–induced proliferation was suppressed by AG1478 and PP1, whereas pertussis toxin had no effect (Fig. 1). Pertussis toxin also reduced basal proliferation. However, no apparent cytotoxicity was observed, as evaluated by morphological examination of the cells under the microscope. This suggests that GLP-1 induces β-cell DNA synthesis via c-Src and provides
pharmacological evidence for the implication of EGFR transactivation in GLP-1–induced H9252-cell proliferation. Figure 2 shows that BTC increased [3H]thymidine incorporation in INS(832/13) cells and that its action was not additive to that of GLP-1. This observation is compatible with the view that GLP-1 induces H9252-cell proliferation by transactivation of the EGFR after production of endogenous ligands such as BTC.

We next investigated whether the cell proliferation results obtained with the INS(832/13) cell line are applicable to normal islet tissue. Figure 3A and B show that both PP1 and AG1478 suppressed GLP-1–induced [3H]thymidine incorporation in cultured rat islets.

GLP-1 transactivates the EGFR via c-Src. As shown in Fig. 4, GLP-1 induced a transient tyrosine phosphorylation of the EGFR in INS(832/13) cells. EGFR tyrosine phosphorylation showed a time-dependent increase, with a maximal effect observed as early as 2 min after exposure to 10 nmol/l GLP-1 and a return to basal value after ~30 min. Figure 5 shows that GLP-1 significantly increased EGFR phosphorylation by ~50% and that GLP-1–induced EGFR phosphorylation was abolished by PP1 or AG1478 treatments, thus supporting the implication of c-Src or c-Src–like proteins in GLP-1–induced EGFR transactivation. In contrast, only the EGFR inhibitor AG1478 blocked BTC-induced EGFR phosphorylation, since PP1 had no effect.

GLP-1 increases PI 3-kinase activity via a c-Src–dependent EGFR transactivation. The activation of PI 3-kinase by GLP-1 plays a central role in the proliferative action of the glucoincretin (9). We therefore tested the functional role of EGFR transactivation in GLP-1–induced PI 3-kinase activation. Figure 6 shows that GLP-1–induced PI 3-kinase activation was sensitive to both pharmacological inhibitors, PP1 and AG1478, whereas only AG1478 suppressed BTC-induced PI 3-kinase activation. This result suggests a role for a c-Src–dependent EGFR transactivation in GLP-1–induced PI 3-kinase activation.

GLP-1–induced β-cell proliferation is suppressed by overexpressing a dominant-negative EGFR. To complement the pharmacological studies by a molecular approach, a retroviral construct was used to overexpress a dominant-negative EGFR (lacking kinase activity) (24). INS(832/13) cells were infected with the empty vector as control or with the dominant-negative EGFR viral construct 24 h before serum starvation to subsequently perform [3H]thymidine incorporation experiments. The dominant-negative construct suppressed both GLP-1– and BTC–induced INS(832/13) cell proliferation (Fig. 7), thus providing direct evidence for an implication of EGFR transactivation in the proliferative action of the glucoincretin. The basal [3H]thymidine incorporation of noninfected cells was not significantly different from that of cells infected with the empty virus or with the dominant-negative EGFR viral construct (not shown).
GLP-1 reduces the amount of membrane-associated BTC in INS(832/13) cells. The possibility that BTC might be implicated in the cell growth–promoting action of GLP-1 was evaluated. We investigated whether cell membrane–associated BTC is processed after GLP-1 treatment in an attempt to identify the putative EGF-like ligand generated during GLP-1–induced EGFR transactivation. Because no radioimmunoassays are available for the detection of BTC in either its membrane-bound or secreted form, BTC cleavage was indirectly measured by FACS analysis. Figure 8 shows that GLP-1 caused a decrease in the content of cell surface–anchored BTC in INS(832/13) cells and that this reduction in cell surface BTC was abolished by the c-Src inhibitor PP1, suggesting that c-Src would act upstream of the metalloproteinase and therefore would not directly activate the EGFR.

GLP-1–induced proliferation is inhibited by a pan-specific metalloproteinase pharmacological inhibitor and by an anti-BTC neutralizing antibody. To directly test the possible implication of a BTC processing event in the glucoincretin proliferative effect, an anti-BTC antibody was used in a proliferation assay to determine whether BTC neutralization can suppress GLP-1–induced proliferation. Because a juxtacrine effect of BTC on EGFR without requirement of BTC processing by metalloproteinase(s) is an alternative possibility to consider, the action of GM6001 (also known as llimostat), a pan-specific metalloproteinase pharmacological inhibitor, was also tested. As shown in Fig. 9, both the anti-BTC neutralizing antibody and GM6001 completely suppressed GLP-1–induced INS(832/13) cell proliferation, suggesting that the action of GLP-1 on β-cell proliferation requires proteolytic cleavage of membrane-anchored BTC. A similar observation was made using the BrdU incorporation assay (Fig. 10).

FIG. 5. GLP-1 induces EGFR transactivation via a c-Src–dependent mechanism. INS cells were incubated for 5 min at 3 mmol/l glucose in the presence or absence of 10 nmol/l GLP-1 (A) or 5 ng/ml BTC (B) with or without pharmacological inhibitor (10 μmol/l PP1 or 250 nmol/l AG1478 added 30 min before incubations). Cells were then lysed and EGFRs were immunoprecipitated from total protein extracts to perform Western blot analysis. Representative immunoblots (IB) for phosphotyrosine (PY) and EGFR are shown. A: PY/EGFR ratios of six different experiments were quantitated and the data expressed as percent of control. *P < 0.05.

FIG. 6. Effects of c-Src and EGFR inhibitors on GLP-1– and BTC–induced PI 3-kinase activation. Cells were incubated for 5 min in 3 mmol/l glucose KRBH in the absence or presence of 10 nmol/l GLP-1 or 5 ng/ml BTC, with or without pharmacological inhibitors (10 μmol/l PP1 or 250 nmol/l AG1478). PI 3-kinase was assayed as described in RESEARCH DESIGN AND METHODS. vehicle; □, PP1; ■, AG1478. Data represent means ± SE of three experiments. *P < 0.05.

FIG. 7. Effects of retroviral overexpression of a dominant-negative EGFR on GLP-1–induced proliferation in INS(832/13) cells. Cells were infected as detailed in RESEARCH DESIGN AND METHODS for overexpression of a dominant-negative (DN) EGFR construct or with the empty virus as control. At 24 h postinfection, cells were washed and cultured at 3 mmol/l glucose for 24 h in serum-free RPMI medium in the presence or absence of 10 nmol/l GLP-1 to perform [3H]thymidine incorporation assays as described in Fig. 1. control; □, GLP-1; ■, BTC. Data represent means ± SE of three different experiments, each comprising three to four wells. *P < 0.02.
cells release a growth factor when treated with GLP-1. As shown in Fig. 10C, GLP-1–induced LNCaP cell proliferation was abolished by the metalloproteinase inhibitor GM6001 and an anti-BTC neutralizing antibody, strengthening the evidence that GLP-1 induces proteolytic cleavage of membrane-anchored BTC. It can also be observed that the anti-BTC neutralizing antibody reduced the basal level of proliferation of LNCaP cells when cocultured with INS(832/13) cells. Although not significant \( (P = 0.07) \), this result suggests that INS(832/13) cells might secrete some BTC under nonstimulatory conditions. This finding is consistent with a previous report demonstrating that INS-1 cells produce and secrete GLP-1 \( (41) \), revealing the possibility of an autocrine signaling mechanism.

**DISCUSSION**

Because diabetes results in part from an impaired balance between β-cell proliferation and apoptosis, it becomes highly relevant to study the molecular mechanisms whereby insulinotropic hormones promote β-cell growth. This is particularly relevant for GLP-1, which is considered for diabetes treatment \( (5,6) \). Using complementary pharmacological, biochemical, and molecular biology approaches, we provide evidence that GLP-1 transactivates the EGFR via a BTC-dependent mechanism and that GLP-1–induced EGFR transactivation is involved in PI 3-kinase stimulation and β-cell proliferation induced by the glucoincretin.

Recently, several GPCR agonists have been shown to induce EGFR transactivation \( (22–25) \) by a mechanism that was shown in some instances to implicate c-Src \( (26,27) \). It has been hypothesized that c-Src activates some metalloproteinases that would in turn process proactive transmembrane growth factor precursors to release mature EGFR ligands \( (28) \). Upon ligand binding, the activated/dimerized EGFR can initiate a variety of signal transduction cascades comprising the PI 3-kinase signaling pathways \( (23) \).

The experimental evidence supporting the model depicted in Fig. 11, whereby GLP-1 activation of PI 3-kinase signaling and β-cell proliferation involves EGFR transactivation, can be summarized as follows. 1) The functional role of EGFR was studied using the pharmacological agents pertussis toxin, PP1, and AG1478 to inhibit Gi, EGFR, and c-Src, respectively. AG1478 and PP1 suppressed GLP-1–induced β-cell proliferation, providing evidence of the implication of the EGFR and c-Src in GLP-1–induced β-cell growth. 2) GLP-1 induced a transient phosphorylation of the EGFR that was sensitive to PP1 and AG1478, suggesting that GLP-1 is able to transactivate the EGFR via c-Src. 3) Both PP1 and AG1478 blocked the action of GLP-1 on PI 3-kinase, suggesting that PI 3-kinase activation by the glucoincretin occurs via c-Src and EGFR transactivation. 4) A dominant-negative EGFR construct lacking the tyrosine kinase activity suppressed the action of GLP-1 on INS(832/13) cell proliferation. 5) GLP-1 decreased cell surface–associated BTC, and this action of the glucoincretin was suppressed by PP1. 6) GLP-1–induced proliferation was completely suppressed by an anti-BTC neutralizing antibody and GM6001, a pan-specific metalloproteinase inhibitor. 7) The actions of GLP-1 and BTC on β-cell proliferation were found to be

**FIG. 9.** Flow cytometric analysis of cell surface–associated BTC. Cells were incubated for 5 min in 3 mmol/l glucose KRBH in the absence or presence of 10 nmol/l GLP-1, without \((A)\) or with 10 μmol/l PP1 \((B)\). Cells were then labeled for BTC content evaluation as described in METHODS and FACS analyses were conducted. A representative experiment that was repeated three times is shown.

**FIG. 10.** Porcine insulinoma INS(832/13) cells were incubated for 5 min in 3 mmol/l glucose KRBH in the absence or presence of 10 nmol/l GLP-1 with or without an anti-BTC antibody (BTC-Ab) \((0.25\) μg/ml) or 2.5 μmol/l GM6001 (Ilostatat), a pan-specific metalloproteinase inhibitor. Data represent means ± SE of three experiments, each comprising four wells. \(*P < 0.05\) vs. control; \(**P < 0.001\) vs. GLP-1 treatment condition in the absence of inhibitor.
Finally, GLP-1-treatment induced LNCaP cell proliferation when cocultured with INS(832/13) cells, while LNCaP cells did not respond to GLP-1 when cultured alone. The enhanced LNCaP proliferation caused by GLP-1 in the coculture system was abolished by GM6001 and an anti-BTC neutralizing antibody. These results are consistent with the view that GLP-1 induces EGFR transactivation via the production of BTC, although we cannot exclude at this stage the possibility that other EGF-like ligands participate in this process. Additional work is required to conclusively identify the EGFR ligand(s) and define the metalloproteinase(s) that mediates GLP-1 action on LNCaP cell proliferation, as well as to directly prove that c-Src or a c-Src-like protein is involved in this process.

In accordance with the present study, EGFR signaling is thought to play a role in β-cell growth and islet development. Thus, mice lacking EGFR show disturbed formation of pancreatic islets (42), and EGF increases the proliferation of undifferentiated pancreatic embryonic cells in vitro (43). There are at least four members known in the EGFR family: c-erbB-1/EGFR, c-erbB2, c-erbB-3, and c-erbB4. A study by Huotari et al. (29) showed that only the c-erbB-1/EGFR gene is expressed in the INS-1 cell line and that, paradoxically, while BTC showed mitogenic activity, EGF itself did not affect INS-1 proliferation (29). We also confirmed this observation in INS(832/13) cells (not shown). A proposed explanation for the different effects of EGF and BTC on INS cell proliferation is perhaps, as discussed before (29), that EGF and BTC do not interact with the same erbB dimers. For example, EGF signaling might require an erbB1/erbB2 or erbB3 dimer but since erbB2 and erbB3 are not expressed in INS cells, EGF would not be active. By contrast, an erbB1 homodimer might be the target of BTC but not of EGF. Our observations that GLP-1 and BTC do not have additive effects on

FIG. 10. GLP-1 increases the proliferation of the prostatic cancer cell-line LNCaP when cocultured with INS(832/13) cells. A: LNCaP cells were cultured in the absence or presence of GLP-1 (10 nmol/l) or BTC (5 ng/ml) as described for INS(832/13) cells, and [3H]thymidine incorporation measurements were carried out as described in Fig. 1. Data represent means ± SE of three experiments, each comprising four wells. *P < 0.001. B: LNCaP cells were incubated with or without 10 nmol/l GLP-1 or BTC (5 ng/ml) and labeled with BrdU for the last hour of a 24-h incubation period. Cells were visualized under a fluorescence microscope for their immunofluorescence staining. Data represent means ± SE of three experiments, each totaling ~100 cells per condition. *P < 0.002. C: INS(832/13) and LNCaP cells were cocultured at a 5:1 ratio, and BrdU labeling and incorporation assay was conducted as described in B, in the absence or presence of 10 nmol/l GLP-1 or 5 ng/ml BTC with or without an anti-BTC neutralizing antibody (BTC-Ab) (0.25 μg/ml) or 2.5 μmol/l GM6001, a metalloproteinase inhibitor. Data represent means ± SE of three experiments, each totaling >200 cells per condition. *P < 0.01 vs. INS control condition; **P < 0.05 vs. LNCaP control condition; §P < 0.05 vs. corresponding conditions without inhibitor.

FIG. 11. Model illustrating the mode of action of GLP-1 on β-cell proliferation. EGF-like, EGF-like ligand. See text for details.
[3H]thymidine incorporation, that an anti-BTC neutralizing antibody can suppress the GLP-1 proliferative effect, and that GLP-1 reduces membrane-bound BTC are compatible with the possibility that BTC is a natural ligand generated by β-cells following binding of GLP-1 to its receptor.

In conclusion, the data allow us to propose the model depicted in Fig. 11, whereby GLP-1–induced EGFR transactivation, involving a c-Src-mediated event, metalloprotease activation, and BTC, plays a role in the pleiotropic effects (DNA synthesis, reduced apoptosis, gene expression, and insulin biosynthesis) of the glucocincretin.

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