Pancreatic Glucagon-Like Peptide-1 Receptor Couples to Multiple G Proteins and Activates Mitogen-Activated Protein Kinase Pathways in Chinese Hamster Ovary Cells*

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ABSTRACT

Chinese hamster ovary (CHO) cells stably expressing the human insulin receptor and the rat glucagon-like peptide-1 (GLP-1) receptor (CHO/GLPR) were used to study the functional coupling of the GLP-1 receptor with G proteins and to examine the regulation of the mitogen-activated protein (MAP) kinase signaling pathway by GLP-1. We showed that ligand activation of GLP-1 receptor led to increased incorporation of GTP-azidoanilide into Gaα, Gqα11α, and G11β2α, but not Gsα. GLP-1 increased p38 MAP kinase activity 2.5- and 2.0-fold over the basal level in both CHO/GLPR cells and rat insulinoma cells (RIN 1046–38), respectively. Moreover, GLP-1 induced phosphorylation of the immediate upstream kinases of p38, MKK3/MKK6, in CHO/GLPR and RIN 1046–38 cells. Ligand-stimulated GLP-1 receptor produced 1.45- and 2.7-fold increases in tyrosine phosphorylation of 42-kDa extracellular signal-regulated kinase (ERK) in CHO/GLPR and RIN 1046–38 cells, respectively. In CHO/GLPR cells, these effects were insensitive to either toxin. Our study indicates a direct coupling between the GLP-1 receptor and several G proteins, and that CTX-sensitive proteins are required for GLP-1-mediated activation of MAP kinases. (Endocrinology 140: 1132–1140, 1999)

GluCAGON-LIKE peptide-1 (GLP-1) is an incretin hormone that is released from epithelial cells of the gastrointestinal mucosa after food intake. GLP-1 produces multiple physiological effects, including enhancement of glucose-mediated insulin secretion in pancreatic β-cells, increased glucose metabolism in cultured adipocytes stimulated with insulin, inhibition of food intake, and modulation of gastric acid secretion (1–6). In addition, GLP-1 stimulates the expression of genes encoding insulin, GLUT-1, GLUT-2, hexokinase I, and glucokinase in pancreatic cells (7–9). The initial step in GLP-1 action requires binding of the hormone to the GLP-1 receptor, which belongs to the superfamily of G protein-coupled receptors (GPCRs) (10). GPCRs interact with and promote the dissociation of heterotrimeric G proteins into Ga-GTP and Gβγ subunits, each of which may then associate with effector molecules (11, 12). Activation of the GLP-1 receptor results in stimulation of adenyl cyclase, leading to an increase in intracellular cAMP and PKA activation in the pancreas (13–16). Treatment with an anti-Gα antibody inhibits the ability of GLP-1 to increase cAMP levels in parietal cell membranes (6). Moreover, GLP-1 stimulation of COS cells overexpressing the GLP-1 receptor results in increased intracellular Ca2+ via release from intracellular stores, concomitant with phospholipase C activation (17). Similarly, GLP-1 treatment of Chinese hamster ovary (CHO) cells that express both the cloned rat GLP-1 receptor and the human insulin receptor (CHO/GLPR) results in increased cAMP levels and intracellular Ca2+ (18, 19). Taken together, these findings support the idea that the GLP-1 receptor might associate with multiple G proteins. However, no information is available about the identity of G proteins coupled to the GLP-1 receptor.

Mitogen-activated protein (MAP) kinases are cytoplasmic serine/threonine kinases that transmit extracellular signals to the nucleus via phosphorylation of transcription factors and other signaling molecules (20, 21). MAP kinase pathways are activated by a number of extracellular stimuli, including growth factors that interact with cell surface receptor tyrosine kinases (20). A number of receptors that couple to heterotrimeric G proteins have recently been shown to stimulate the activity of two members of the MAP kinase superfamily, which includes the extracellular signal-regulated kinase (ERK) (22–24) and p38 MAP kinase (25). Whether the two different classes of receptor (GPCRs and receptor tyrosine kinases) require distinct

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signaling pathways in MAP kinase activation remains unclear.

The objective of this study was 3-fold: 1) to characterize the Gq proteins that interact with the cloned GLP-1 receptor in CHO/GLPR cells, 2) to examine whether ligand-stimulated GLP-1 receptor activates ERK and p38 MAP kinase, and 3) to determine the possible cross-talk between the GLP-1 receptor and the insulin receptor toward MAP kinase activation in these cells.

Materials and Methods

Cell culture

CHO/GLPR cells were generated as described previously (18, 19). In brief, CHO cells overexpressing the human insulin receptor (CHO/HIR) were cotransfected with plasmids containing the gene for hygromycin resistance and the full-length rat GLP-1 receptor complementary DNA (a gift from Dr. B. Thorens, University of Lausanne, Lausanne, Switzerland) under the control of the simian virus 40 promoter. Cells were selected by culture in the presence of hygromycin (700 μg/ml). As controls, CHO/HIR cells were transfected with the corresponding hygromycin plasmid alone. Cells were grown in Ham’s F-12 medium containing 10% FCS and maintained in a humidified air incubator with 5% CO2.

Binding of GLP-1 and insulin

Cells were seeded on 12-well dishes and grown to confluence. Four hours before the start of the experiment, culture medium was changed to serum-free medium. For insulin binding, cells were washed twice with 0.5 ml ice-cold buffer A (10 mM HEPEs (pH 7.6), 120 mM NaCl, 1.2 mM MgSO4, 1 mM EDTA, 15 mM sodium acetate, and 10 mM glucose) and then incubated overnight at 4 C with 0.5 ml buffer A containing 1% BSA plus various concentrations of insulin (0.06–30 nm) and 30,000 cpm [125I]insulin (Amersham, Arlington Heights, IL). The next day, the supernatant was discarded, and cells were washed three times with 0.5 ml ice-cold Dulbecco’s PBS and lysed with 0.5 ml 0.5% NaOH-0.1% SDS for 10 min at room temperature. For GLP-1 binding, serum-starved cells were washed twice with 0.5 ml binding buffer B (10 mM Tris (pH 7.6), 120 mM NaCl, 1.2 mM MgSO4, 15 mM sodium acetate, and 5 mM KCl) and then incubated with 0.5 ml buffer B containing 2% BSA, 500 U/ml aprotinin, 10 μg/ml aprotinin, 10 mM glucose, various concentrations of GLP-1 (0.03–30 nm), and 30,000 cpm [32P]GLP-1 (Amersham). After an overnight incubation at 4 C, the supernatant was discarded, and the cells were washed three times and lysed as indicated above. The radioactivity was measured using a ICN Apec Series γ-counter (Costa Mesa, CA).

Membrane preparation

Confluent cells that had been cultured on 150-cm2 dishes were placed in serum-free Ham’s F-12 for 2 h at 37 C. Cells were washed once with 10 ml ice-cold buffer C (10 mM HEPEs (pH 7.5), 0.25 mM sucrose, and 5 mM EDTA) and scraped into 5 ml ice-cold buffer C containing 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and the cell suspension was centrifuged at 600 g for 10 min at 4 C. The pellets obtained from 10 dishes were homogenized on ice in 10 ml chilled buffer C containing protease inhibitors using a Polytron (Brinkmann Instruments, Inc., Westbury, NY) at setting 7 and twice for 15 sec each time. The homogenate was centrifuged at 12,000 g for 20 min at 4 C, and the clarified supernatant was centrifuged at 100,000 g for 1 h at 4 C. The membrane pellet was reconstituted in buffer D (50 mM HEPEs (pH 7.5), 0.25 mM sucrose, 1 mM MgCl2, 1 mM dithiothreitol (DTT), 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.2 mM PMSE), homogenized using a Dounce homogenizer, and stored in aliquots at −80 C.

Preparation of [32P]GTP-azidoanilide

[32P]GTP-azidoanilide was synthesized as previously described (26). After purification, [32P]GTP-azidoanilide was divided into aliquots, dried on a speed vacuum centrifuge, and stored at −20 C until use.

Labeling and immunoprecipitation of membrane-associated G proteins

Aliquots of purified membranes (70–100 μg protein) were centrifuged at 8000 g for 15 min, and after which pellets were resuspended in buffer E (50 mM HEPEs (pH 7.5), 5 mM MgCl2, 30 mM KCl, 1 mM benzamidine, and 0.1 mM EDTA) and incubated for 10 min at 25 C with 5–10 μCi [32P]GTP-azidoanilide in the absence or presence of different concentrations of GLP-1. The reaction was stopped by incubation on ice for 1 min, and the samples were centrifuged at 8000 g for 10 min at 4 C. Pellets were resuspended in buffer E containing 20 mM glutathione and irradiated for 10 min at 4 C using UV light at 254 nm (100-watt lamp at a distance of 10 cm).

The immunoprecipitation of G protein α-subunits was performed as described previously (27). The photolabeled membranes were precipitated at 8000 g for 15 min at 4 C, and the pellets were solubilized in 40 μl 2% SDS at room temperature, followed by the addition of 130 μl buffer F (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5% SDS, 1% deoxycholate, 1% Triton X-100, 0.2 mM PMSE, and 10 μg/ml aprotinin). The samples were centrifuged at 8000 g for 10 min at 4 C to remove insoluble material. The clarified supernatents (160 g) were transferred to tubes containing 5-10 μl polyclonal rabbit antisera raised against Gs, Gq, Gi1,2, and Gi3 (1 mg/ml protein; New England Nuclear Corp., Boston, MA). After an overnight incubation at 4 C with constant rotation, 40 μl reconstituted protein A-Sepharose (10% suspension in buffer F) were added to each sample, and the incubation was continued for another 2 h with rotation. Thereafter, the immune complexes were recovered by centrifugation and subjected to a series of washes as followed: two washes of 1 ml each containing 50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.5% SDS, and 600 mM NaCl; one wash of 1 ml containing 100 mM Tris (pH 7.4), 300 mM NaCl, and 10 mM EDTA; and one wash of 1 ml containing 20 mM Tris (pH 7.4) and 1 mM EDTA. The immune pellets were resuspended in Laemml sample buffer and boiled for 10 min. An aliquot (20 μl) of the samples was subjected to SDS-PAGE under reducing conditions (28). To separate the Gi1,2 protein isoforms, eluted materials were electrophoresed in gels containing 9% acrylamide and 6 μm urea. Gels were dried, and band intensity was quantified by electronic autoradiography using a Packard Instant Imager (Packard Instruments, Meriden, CT). Antibodies against Gsα and Gqα are specific and do not cross-react with other Gα proteins (29, 30). Although some weak cross-reactivity has been shown previously (29) between antibodies for Gi1,2 and Gqα, we did not observe any significant cross-reactivity, as evident by the absence of contaminating bands (Figs. 1, B and C). The protein concentration was determined by the method of Bradford (31).

Western blot analysis

Cells were lysed directly in Laemml sample buffer containing 5% 2-mercaptoethanol, and 1 mM orthovanadate, and equal amounts of proteins from each sample were electrophoresed on 4–12% polyacrylamide gels (Novex, San Diego, CA) under reducing conditions and then transferred by electrotransfer onto polyvinylidene difluoride membranes. The membranes were incubated in blocking buffer (20 mM Tris (pH 7.5), 157 mM NaCl, and 0.1% Tween-20 (TBS-T) with 5% (wt/vol) nonfat dry milk) overnight at 4 C. The membranes were then incubated with primary antibody (1:1,000 to 1:10,000 dilution) in TBS-T containing 5% nonfat dry milk for 1 h at room temperature or overnight at 4 C. After a series of washes in TBS-T, the membranes were incubated with horse-radish peroxidase-conjugated secondary antibody (Amersham) for 1 h at room temperature. The immunoblots were visualized using the ECL chemiluminescence detection system (Amersham), and the signals were quantified by laser densitometry (ImageQuant, Molecular Dynamics, Inc., Sunnyvale, CA). The polyclonal anti-MKK3 and phospho-specific anti-MKK3/MKK6 antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). The polyclonal anti-p38 MAP kinase antibody (1:1000) was obtained from Promega Corp. (Madison, WI).
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Determination of MAP kinase activity

MAP kinase activity was determined in anti-ERK1/ERK2 immunoprecipitates as described by Kole et al. (33). p38 MAP kinase activity was determined as recently reported (34) with some modifications. Confluent cells grown in 35-mm dishes were treated with various factors, as described in the figure legends, and lysed at 4°C for 15 min in 0.5 ml lysis buffer containing 30 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 1 mM orthovanadate, 0.15 mM Pefabloc SC, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μM okadaic acid, 10 μM tautomycin, and 10 μM cypermethrin. Equal amounts of protein from clarified lysates were immunoprecipitated with anti-p38 MAP kinase antibody (gift from Dr. Roger J. Davis, University of Massachusetts, Worcester, MA), 25 μg/ml cAMP-dependent protein kinase inhibitor peptide (Upstate Biotechnology, Lake Placid, NY), 20 μM [γ-32P]ATP (10 cpm/nmol), and 1 mM DTT for 15 min at 22°C. The reaction was stopped by the addition of Laemmli sample buffer, and the samples were electrophoresed using 4–12% SDS-polyacrylamide gels under reducing conditions. Gels were dried and subjected to autoradiography at −70°C.

Statistical methods

Data are expressed as the mean ± SEM, and differences between groups were analyzed by two-tailed Student’s t test or ANOVA. Significant differences were determined when P < 0.05.

Results

Stable expression of rat GLP-1 receptor in CHO/GLPR cells

We obtained seven independent CHO cell lines in which [125I]GLP-1 binding was significantly above the basal levels. Of these clones, the cell line with the highest binding activity, designated CHO/GLPR, was used for this study. CHO/GLPR cells specifically bound [125I]GLP-1 in a saturable manner. Scatchard analysis revealed that the dissociation constant (Kd) and the maximal binding were 4.9 ± 1.6 nM (mean ± SEM; n = 4) and 112,000 ± 18,000 GLP-1 receptor sites/cell, respectively. Similar to parental CHO/HIR cells, the Kd and binding capacity for [125I]insulin were 1.2 ± 0.7 nM and 166,000 ± 36,000 receptor sites/cell, respectively, in CHO/GLPR cells.

Functional coupling of GLP-1 receptor with G proteins

The interaction of GLP-1 receptor with specific G proteins was determined by incorporation of a radiolabeled nonglycylizable GTP analog into various Gα proteins present in CHO/GLPR cell membranes. [32P]GTP incorporation into Gα, Gα11, and Gα13 (Fig. 1A) as well as Gα12, Gα13, and Gα11 (Fig. 1B) increased after GLP-1 stimulation. In contrast, GLP-1 did not affect GTP incorporation into Gα, Gα11, and Gα13 (Fig. 1C). This experiment was repeated several times using various concentrations of GLP-1, and the compilation of the results is presented in Fig. 2. GLP-1 caused a concentration-dependent increase in Gα protein labeling, with maximal responses at 10 nM for Gα and 100 nM for Gα11 and Gα13. Concentrations of GLP-1 as high as 1 μM failed to stimulate Gα labeling over the levels seen in untreated controls. To assess whether the effect of GLP-1 on Gα protein activation was via the transfected GLP-1 receptor and not due to other endogenous receptors expressed in CHO cells, we analyzed the effect of GLP-1 on Gα protein coupling in membranes from CHO/HIR cells transfected with empty vector (CHO/HPH cells). As shown in Fig. 1D, GLP-1 increased labeling of Gα, Gα11, and Gα13. The degree of GTP cross-linking in Gα, Gα11, and Gα13 is shown.

Additional experiments were performed to determine the relative expression of Gα, Gα11, Gα12, and Gα proteins in total cell extracts from CHO/GLPR cells.
HIR cells, and RIN1046–38 cells, an insulinoma cell line containing endogenous pancreatic GLP-1 receptors (35). Figure 3 shows a representative Western blot analysis of Ga protein levels in these three different cell lines. Although RIN1046–38 cells showed levels of Go, Goq1,1, Go1,2, and Go3, the insulinoma cell line contained higher levels of Goq1,1 than Go1,2. The pattern of Ga protein expression in CHO/GLPR cells was similar to that of CHO/HIR cells, which indicates that overexpression of the GLP-1 receptor did not change levels of Ga protein in CHO/GLPR cells.

**Activation of p38 MAP kinase**

p38 MAP kinase was recently shown to play a key role in glucose-mediated induction of insulin gene expression (36). As p38 MAP kinase can be activated by G protein-coupled receptors (25, 37), we were interested in determining whether ligand-stimulated GLP-1 receptors could stimulate p38 MAP kinase in CHO/GLPR cells. p38 MAP kinase activity was measured in an immunoprecipitation-based kinase assay that uses recombinant GST-ATF-2 fusion protein as the substrate for phosphorylation (37).

A representative experiment is shown in Fig. 4, upper panel. The autoradiograms of the dried gels from several experiments were quantified by scanning densitometry, and values are expressed as the fold increase over that in untreated cells (Fig. 4, lower panel). Treatment of CHO/GLPR cells with 10 nM GLP-1 for 30 min increased activity 2.5-fold over the basal value (P < 0.01). Similarly, 1-h treatment with arsenite (0.4 mM) led to activation of p38 MAP kinase, in agreement with earlier findings by Liu and collaborators (38). In contrast to the effect of GLP-1, 10 μM forskolin or 1 mM 8-bromo-cAMP had no effect on ATF-2 phosphorylation (1.07 ± 0.08-fold; n = 4). We next measured the degree of stimulation of p38 MAP kinase in RIN1046–38 insulinoma cells and found a significant 2-fold increase in activity produced by GLP-1 (P < 0.05; Fig. 5A). Similarly, increasing glucose concentrations in the medium from 5 to 11 mM increased p38 activity by 1.3-fold (n = 2), which is in accord with previously published data (36). These results suggest that activation of GLP-1 receptors generates signals necessary for the stimulation of p38 MAP kinase in GLP-1-responsive cells.

Next, we studied the effect of ligand-stimulated insulin receptor activation on p38 MAP kinase activity in CHO/GLPR cells. A 15-min incubation with 100 nM insulin resulted in an increase in p38 MAP kinase activity of 1.84 ± 0.3-fold over the basal value (mean ± SEM; n = 7; P < 0.05; Fig. 6A). As GLP-1 can also stimulate the p38 MAP kinase pathway, the possibility exists that GLP-1 and insulin may have additive effects. To test this hypothesis, CHO/GLPR cells were

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**Fig. 2.** Concentration dependency of G protein coupling to the GLP-1 receptor. The counts per min of [32P]GTP-azidoanilide incorporation into Go subunits was quantified by electronic autoradiography using a Packard Instant Imager. Values represent the fold increase over values in the untreated control group (mean ± SEM; n = 3–6). *, **, and ***, P < 0.05, 0.01, and 0.001, respectively, compared with the untreated group.

**Fig. 3.** Immunodetection of Go subunits. CHO/GLPR, CHO/HIR, and RIN1046–38 cells were lysed, and equal amounts of total cell proteins were fractionated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The Go subunits (indicated to the right) were detected by immunoblotting using specific polyclonal antibodies. Molecular mass markers are shown on the left.

**Fig. 4.** Effect of GLP-1 on p38 MAP kinase activity. CHO/GLPR cells were treated without (−) or with (+) 10 nM GLP-1 for 30 min or with 400 μM arsenite for 1 h. The cells were lysed, and p38 MAP kinase activity was determined in an immunoprecipitation-based kinase assay using recombinant GST-ATF-2 fusion protein as the substrate. A representative autoradiogram is shown (upper panel). The results of densitometric analyses of ATF-2 phosphorylation are represented as fold induction over untreated cells (lower panel). Values are the mean ± SEM (n = 7). ***, P < 0.01 compared with untreated cells.
pretreated without or with 10 nM GLP-1 for 2 h followed by the addition of 100 nM insulin for 15 min. Under these experimental conditions, GLP-1 increased insulin-stimulated values by 1.96 ± 0.4-fold (P < 0.05; n = 10) over the effect of insulin alone. These results suggest a possible convergence of signaling events between the insulin receptor tyrosine kinase and the G protein-coupled GLP-1 receptor.

Treatment of CHO/GLPR cells with GLP-1 for 2 h did not alter the affinity and binding capacity for [125I]insulin (data not shown). Furthermore, neither autophosphorylation nor the tyrosine kinase activity of the insulin receptor was affected by GLP-1 (data not shown).

To characterize the G proteins that transduce the stimulatory action of GLP-1 on p38 MAP kinase activity, the effects of pertussis toxin (PTX) and cholera toxin (CTX) were tested (Fig. 6). Pretreatment of CHO/GLPR cells with 10 nM GLP-1 for 2 h completely blocked the GLP-1-stimulated increase in p38 activity, whereas PTX pretreatment at 0.5 µg/ml had no effect. Similarly, the additive effects of GLP-1 and insulin on p38 activation were not affected by PTX, but were blocked by CTX pretreatment. These effects were not due to uneven p38 protein recovery in the immune complexes (data not shown). Control experiments confirmed that CTX treatment was able to reduce the increase in intracellular cAMP mediated by GLP-1, and that PTX treatment blocked the signal induced by lysophosphatidic acid, a Gαi-coupled receptor ligand (data not shown).

**Activation of MKK3/MKK6**

MKK3 and MKK6, the upstream regulators of p38 MAP kinase, are dual specificity kinases whose activities depend on serine and threonine phosphorylation (39, 40). As no information is currently available regarding MKK3/MKK6 activation by GLP-1, we performed an immunoblot analysis of their phosphorylation status using a phosphospecific antibody against MKK3/MKK6. GLP-1 incubation increased MKK3/MKK6 phosphorylation in CHO/GLPR cells (Fig. 7A, upper panel) and in RIN 1046–38 insulinoma cells (Fig. 5B, upper panel).
added at 100 nM (B) for 15 min where indicated. Immunoblot analysis was performed on whole cell lysates using antiphosphospecific MKK3/ MKK6 antibody (upper panels) or an antibody against MKK3 protein (lower panels). This experiment was repeated twice with comparable results.

third panel) by 3.4 ± 0.6 (mean ± SEM; n = 4; P < 0.05) and 3.4 ± 0.1 (n = 3; P < 0.05) fold over the basal value, respectively. Pretreatment of CHO/GLPR cells with CTX had a modest effect on basal MKK3/MKK6 phosphorylation levels, but fully blocked the stimulatory effect of GLP-1. In contrast, PTX treatment did not affect GLP-1-stimulated MKK3/MKK6 activation. Reprobing the blot with anti-MKK3 antibody revealed similar quantities of MKK3 protein loaded on the gel (Fig. 7A, lower panel).

Phosphorylation of MKK3/MKK6 was increased 3-fold over the basal value by a 15-min incubation with 100 nm insulin in CHO/GLPR cells (Fig. 7B, upper panel). The activation of MKK3/MKK6 by insulin was not affected after preincubation with PTX (lanes 5 and 6 vs. lanes 1 and 2) or CTX (lanes 9 and 10 vs. lanes 1 and 2). GLP-1 increased insulin-stimulated values by 1.6-fold over the effect of insulin alone (Fig. 7B, lane 4 vs. 2). Determination of the effect of bacterial toxins on the activation of the MKK3/MKK6 pathway mediated by insulin plus GLP-1 indicated that CTX inhibited the enhancing role of GLP-1. By comparison, MKK3/MKK6 phosphorylation levels stimulated by insulin and GLP-1 were not affected by pretreatment with PTX.

Activation of ERK

ERK activity was first measured in an immunoprecipitation-based assay in which the addition of GLP-1 to CHO/GLPR cells led to a 1.7 ± 0.1-fold increase in 3H incorporation in myelin basic protein compared with that in unstimulated cells (mean ± SEM; n = 5; P < 0.01). Similar results were obtained when the levels of the active form of ERK1 and ERK2 were determined by Western immunoblotting with phospho-specific ERK antibody (32). Treatment of CHO/ GLPR cells (Fig. 8A, upper panel) and RIN 1046–38 insulina (Fig. 5B, first panel) with 10 nm GLP-1 resulted in 1.5- and 2.7-fold increases in ERK dual phosphorylation, respectively. This enhancing effect of GLP-1 in CHO/GLPR cells was abolished in the presence of CTX, but not in the presence of PTX. Reprobing the blot with anti-ERK antibody revealed similar quantities of ERK protein loaded on the gel (Fig. 8A, lower panel). Control experiments showed that similar to the effect on p38 MAP kinase activation, simply raising cAMP levels by incubation of cells with 1 nm 8-bromo-cAMP did not enhance ERK tyrosine phosphorylation (n = 2).

Figure 8B (upper panel) shows that ERK phosphorylation was increased 14-fold over the basal level by a 15-min incubation with 100 nm insulin in CHO/GLPR cells. Activation of the ERK pathway by insulin was not affected after preincubation with PTX (lanes 5 and 6 vs. lanes 1 and 2) or CTX (lanes 9 and 10 vs. lanes 1 and 2). GLP-1 increased insulin-stimulated values by 2-fold over insulin alone (Fig. 8B, lane 4 vs. 2). Determination of the effect of bacterial toxins on the activation of ERK by insulin plus GLP-1 indicated that CTX inhibited the enhancing role of GLP-1. By comparison, ERK phosphorylation levels stimulated by insulin and GLP-1 were not affected by pretreatment with PTX.

Discussion

In previous work, it has been reported that the rat GLP-1 receptor possesses the characteristic structure of the GPCR superfamily (10). The established signal transduction linked to GLP-1 receptor is an increase in intracellular cAMP in β-cells (13). However, GLP-1 is also able to elicit phospholipase C activation (17) and increases in intracellular free calcium levels (17, 19) when the GLP-1 receptor is overexpressed in heterologous systems. A similar observation was made with other members of this glucagon-secretin receptor family, including receptors for glucagon, secretin, PTH, and calcitonin (41). This indicates that GLP-1 action might be mediated either via a direct coupling of the GLP-1 receptor with different G proteins or via the activation of a single G
protein that modulates various functions through different subunits (42). In that regard, G protein βγ-subunits from Gα or Gβ have been shown to activate pathways such as phospholipase C (43, 44), phosphatidylinositol 3′-kinase (45, 46), and MAP kinase (22). In this study we showed that the cloned pancreatic GLP-1 receptor is able to couple directly to different G protein α-subunits, including Gα11, a11/Gα11, and, to a certain extent, Gα11/Gα11α, but not Gα5, in CHO/GLPR cells. The ability of the GLP-1 receptor to couple with several G proteins might be a function of overexpression; however, it remains to be seen whether such diversity in coupling exists in other cell types expressing GLP-1 receptors endogenously. In pancreatic β-cells, the rise in intracellular Ca²⁺ caused by GLP-1 appears to be mediated by either activation of plasma membrane calcium channels (14, 47, 48) or a mechanism involving Ca²⁺-induced calcium release (49). The fact that Gα11α and Gα contents were identical in CHO/GLPR cells and RIN1046–38 cells supports the idea that GLP-1 signal transduction is not dictated by G protein availability in different cell types. However, as we did not assess the presence of multiple G protein coupling in pancreatic β-cells, the possibility still exists that they are present only in CHO cells overexpressing GLP-1 receptor.

It has been reported that p38 MAP kinase might be involved in glucose-induced insulin gene expression in MIN6 insulinoma cells (36). In addition, the p38 MAP kinase has been shown to have an important role in various cellular functions, including the phosphorylation of transcription factors (50), the control of apoptosis (51–53), and cell cycle-mediated events (54). In mammalian cells, p38 MAP kinase is activated by endotoxins, cytokines, and environmental stresses. Very recently, Yamauchi and collaborators (25) identified novel activators of p38 MAP kinase, and they proposed that GPCRs, such as those for Gα11α-coupled m1 muscarinic acetylcholine receptors, Gα-coupled m2 muscarinic acetylcholine receptors, and Gα-coupled β-adrenergic receptors, activate p38 MAP kinase via Gβγ subunits. In this study, we show that the cloned GLP-1 receptor triggers the activation of p38 MAP kinase. Neither forskolin nor 8-bromo-cAMP could mimic the enhancing effect of GLP-1 on p38 MAP kinase activity, which suggests that increased cAMP levels are not responsible for triggering this GLP-1-mediated response. Of interest, GLP-1 also induces the phosphorylation/activation of MKK3/MKK6, two direct upstream activators of p38 MAP kinase (37, 39, 40). p38 MAP kinase and MKK3/MKK6 followed a similar activation pattern, consistent with the sequential activation of MKK3/MKK6 and p38 MAP kinase in GLP-1-stimulated cells.

Other studies have found that GLP-1 induces activation and tyrosine phosphorylation of 42- and 44-kDa ERKs in INS-1 cells (23). Our results show that in CHO/GLPR cells, insulin and GLP-1 both increase ERK tyrosine phosphorylation. However, insulin was much more potent than GLP-1. This is in sharp contrast to the relative effects of insulin and GLP-1 on p38 activation, where similar levels of activation were observed. This result argues that the rise in intracellular cAMP levels mediated by GLP-1 might attenuate Raf1 kinase activity (55), thereby inhibiting ERK activity. Alternatively, cAMP might enhance the expression and activity of dual specificity Thr/Tyr phosphatases, a class of enzymes that directly and specifically regulate MAP kinase family members (56) through enhanced tyrosine and threonine dephosphorylation. In support of this view, Kusari and collaborators (57) observed a significant increase in MKP-1 expression with a concomitant decrease in ERK activity by cAMP derivatives.

To elucidate the role of heterotrimeric G proteins in GLP-1-induced MAP kinase cascade activation, we compared the effects of bacterial toxins on the signals mediated by GLP-1. CTX and PTX exerted different inhibitory effects on the GLP-1 signals. Induction of ERK and p38 MAP kinase activities by GLP-1 was abolished totally by CTX, but was not affected by PTX. As a simple rise of intracellular cAMP after incubation with 8-bromo-cAMP was unable to activate ERK or p38 MAP kinase, these observations suggest the possibility that Gβγ associated with Gα protein could be involved in GLP-1-induced ERK and p38 MAP kinase activation.

To analyze further the pathway that links the GLP-1 receptor to the MAP kinase activation, we explored the role of receptor tyrosine kinase activation. It is well established that the insulin receptor tyrosine kinase mediates 42- and 44-kDa ERK activation in a Ras-dependent manner (58). Furthermore, insulin induces a second class of MAP kinases, the JNK/SAPK, in Rat1/HIRc cells (59). However, it is not yet clear whether insulin can enhance p38 MAP kinase activity. On the one hand, insulin was found to have no effect on p38 MAP kinase activity in muscle (60) while causing a marked inhibition in PC12 cells and Rat1 fibroblasts (52). On the other hand, insulin has recently been reported to induce p38 MAP kinase activity in hepatoma cells (61). These controversial observations raise the possibility that insulin’s ability to modulate p38 MAP kinase may be related to the cell type studied. Thus, we assessed the ability of insulin to induce MKK3/MKK6 and p38 MAP kinase activation in CHO/GLPR cells that express human insulin receptors. Insulin elevated both activities severalfold. Further stimulation was observed with the combination of insulin and GLP-1, which might indicate that the GLP-1 receptor uses many of the same intermediates involved in insulin receptor signaling. The potential of bacterial toxins to regulate the insulin signal differed from that of GLP-1; insulin signaling was insensitive to CTX or PTX, whereas GLP-1 action was fully blocked by CTX. These observations suggest that toxin-sensitive G proteins play a minor role in the insulin-mediated increase in MAP kinase activity in CHO cells.

In conclusion, we have directly demonstrated the coupling of activated GLP-1 receptor to multiple G proteins by GTP-azidoanilide photoaffinity labeling. Although coupling to Gα11α or Gα may be important for some of the actions of GLP-1, stimulation of the MAP kinase pathways seems to require functional coupling between the activated GLP-1 receptor and the Gα subunit. Lastly, the ability of insulin to enhance MKK3/MKK6, p38 MAP kinase, and ERK activities is augmented by GLP-1 pretreatment. Our findings provide new insight into the mechanisms of GLP-1 action and indicate that modulation of GLP-1 receptor function may have important effects on cellular responsiveness to insulin.
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References


