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Glucose-Dependent Insulinotropic Polypeptide Is a Growth Factor for β (INS-1) Cells by Pleiotropic Signaling

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Activation of the G-protein-coupled receptor for glucose-dependent insulinotropic polypeptide facilitates insulin-release from pancreatic β-cells. In the present study, we examined whether glucose-dependent insulinotropic polypeptide also acts as a growth factor for the β-cell line INS-1. Here, we show that glucose-dependent insulinotropic polypeptide induced cellular proliferation synergistically with glucose between 2.5 mM and 15 mM by pleiotropic activation of signaling pathways. Glucose-dependent insulinotropic polypeptide stimulated the signaling modules of PKA/cAMP regulatory element binder, MAPK, and PI3K/protein kinase B in a glucose- and dose-dependent manner. Janus kinase 2 and signal transducer and activators of transcription 5/6 pathways were not stimulated by glucose-dependent insulinotropic polypeptide. Activation of PI3K by glucose-dependent insulinotropic polypeptide and glucose was associated with insulin receptor substrate isoforms insulin receptor substrate-2 and growth factor bound-2 associated binder-1 and PI3K isoforms p85α, p110α, p110β, and p110γ. Downstream of PI3K, glucose-dependent insulinotropic polypeptide-stimulated protein kinase Bα and protein kinase Bβ isoforms and phosphorylated glycogen synthase kinase-3, forkhead transcription factor FKHR, and p70^{S6K}. These data indicate that glucose-dependent insulinotropic polypeptide functions synergistically with glucose as a pleiotropic growth factor for insulin-producing β-cells, which may play a role for metabolic adaptations of insulin-producing cells during type II diabetes. (*Molecular Endocrinology* 15: 1559–1570, 2001)

THE GLUCO-INCRETIN EFFECT results in higher insulin response and consecutive smaller increase in blood sugar after an oral glucose load compared with intravenous administration. Two major insulinotropic incretin hormones have been characterized: glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (1, 2).

GLP-1 is synthesized in entero-endocrine L-cells of the ileum and colon and released into circulation as bioactive truncated GLP-1 (7–36 amide). GLP-1 receptors are expressed on pancreatic β-cells, and activation of GLP-1 receptors at high glucose levels results in potentiation of glucose-dependent insulin secretion and activation of insulin gene transcription (1, 2). The incretin effect of GLP-1 is preserved in type II diabetes mellitus, which is currently exploited in clinical studies for the therapy of type II diabetes mellitus (2). In addition to its insulinotropic characteristics, GLP-1 also functions as a growth and differentiation factor for pancreatic β-cells by pleiotropic activation of mitogenic signaling modules (3–8).

GIP is synthesized in duodenal K cells as a 42-amino acid peptide and secreted as a hormone by the stimulation of the duodenum with nutrients, mainly by fat and glucose. The GIP receptor belongs the family of G protein-linked seven-transmembrane receptors and is highly expressed on β-cells. Stimulation of GIP receptors induces a rise in cAMP and intracellular calcium, which facilitates glucose-dependent insulin release from pancreatic β-cells physiologically (1, 9–11). However, the incretin effect of GIP is not preserved in type II diabetes or in relatives of type II diabetic subjects (1). Thus, defective insulinotropic signaling by the GIP receptor has been implicated in the pathogenesis of type II diabetes (1, 12). In addition to its role as an insulinotropin, GIP is involved in metabolic regulation of insulin-sensitive tissues by sensitizing adipose cells to insulin (12). Furthermore, several lines of evidence indicate a function of GIP as a growth and metabolic factor for β-cells. In Chinese hamster ovary (CHO) cells stably expressing the GIP receptor, GIP activates MAPK, which could be partially blocked by wortmannin, an inhibitor of PI3K (13). Wortmannin also partially inhibits GIP-mediated insulin secretion in β-cells providing indirect evidence that GIP signaling in β cells may involve activation of mitogenic lipid

Abbreviations: BrdU, 5-bromo-deoxyuridine; CREB, cAMP regulatory element binder; ERK, extracellular signal-regulated kinase; Gab-1, growth factor bound-2 associated binder-1; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; GSK, glycogen-synthase kinase; IRS, insulin receptor substrate; JAK, janus kinase; PKB, protein kinase B; pY, phosphorylated tyrosine; STAT, signal transducer and activator of transcription.

kinase PI3K (14). A knockout of the GIP receptor demonstrated not only a defect in entero-insular axis but also the failure of β cells to adapt metabolically to insulin resistance (15). Although it was not reported whether islet size is decreased in GIP receptor knockout mice, a recent study showed that dominant negative overexpression of the GIP receptor in pancreatic β-cells leads to diminished islet size (Göke, B., and A. Volz, personal communication).

In the light of these studies, we examined whether GIP acts as a β-cell growth factor using the differentiated β-cell line INS-1 (16). In addition, we elucidated patterns of signaling by GIP on major mitogenic signaling modules in β- cells, namely the pathways of PKA/cAMP regulatory element binder (CREB), MAPK, PI3K/protein kinase B (PKB), and janus kinase 2 (JAK2)/signal transducers and activators of transcription 5/6 (STATs5/6).

RESULTS

Mitogenic Effects of GIP and Glucose

To determine whether GIP and glucose act as growth factors for β-cells, proliferation of INS-1 cells was determined by rate of DNA synthesis by enzyme-linked detection of 5-bromo-deoxyuridine (BrdU) incorporation (17). BrdU incorporation at 2.5 mM glucose without GIP served as control and was set at 1. The addition of glucose at rising concentrations in the absence of GIP induced a dose-dependent increase in proliferation, which was maximal at 15 mM glucose (2.8 ± 0.14 mean ± SD of control; n = 12; Fig. 1A) and stagnated at higher concentrations of glucose up to 25 mM (data not shown). Addition of 10^{-7} M GIP instigated a further 1.5- to 1.9-fold rise in INS-1 cell proliferation at glucose concentrations between 2.5 and 15 mM (Fig. 1A). GIP-induced INS-1 cell proliferation was absent at 0 mM glucose and stagnated at glucose concentrations higher than 15 mM (data not shown). Statistical analysis by ANOVA revealed that the increase of proliferation at basal levels and after stimulation with GIP were always highly significant when compared with control levels at 2.5 mM glucose. Furthermore, the GIP-induced rise in proliferation was highly significant when compared with respective basal levels (Fig. 1A).

To elucidate whether mitogenic effects by GIP were dose-dependent, INS-1 cells were stimulated by a rising concentration of GIP between 10^{-10} M and 10^{-6} M at 15 mM glucose. At low concentrations of 10^{-10} M and 10^{-9} M, GIP induced only a minor rise in BrdU-incorporation, which was not statistically significant in the ANOVA analysis. However at higher concentrations of GIP between 10^{-8} M and 10^{-6} M, INS-1 cell proliferation increased 1.4- to 1.5 fold, which was highly statistically significant (Fig. 1B).

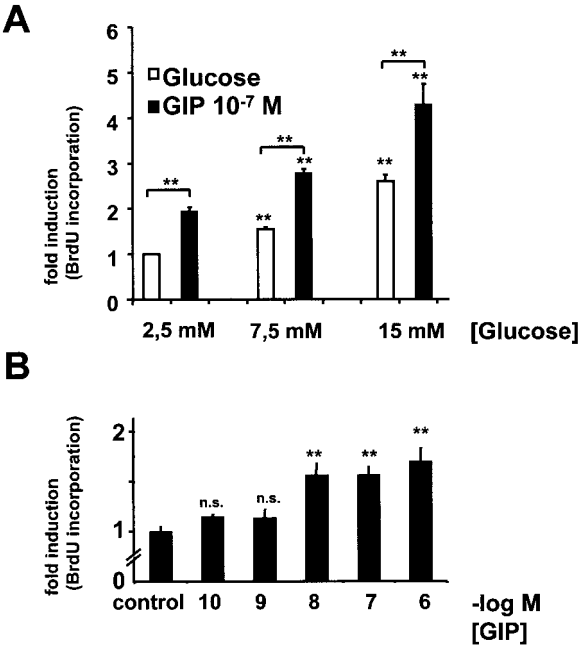

Fig. 1. Mitogenic Effects of GIP and Glucose

A, Proliferation of INS-1 cells stimulated with a glucose gradient and GIP. INS-1 cells were starved overnight and stimulated for 24 h with 10^{-7} M GIP at 2.5 mM, 7.5 mM, and 15 mM glucose. DNA synthesis was measured by adding BrdU for the last 6 h of the stimulation period and subsequent detection by ELISA. Each *bar* represents the mean ± SD of 12 independent experiments. They are expressed as relative to control assigning a value of 1 to cells stimulated with 2.5 mM glucose in the absence of GIP. B, Dose-response of INS-1 cell proliferation by GIP at 15 mM glucose. INS-1 cells were stimulated with 15 mM glucose and a GIP gradient between 10^{-10} and 10^{-6} M at 15 mM glucose for 24 h. Cellular proliferation was measured by BrdU incorporation. Each *bar* represents the mean ± SD of six independent experiments. They are expressed as relative to control assigning a value of 1 to cells stimulated with 15 mM glucose. Statistical analysis was performed by ANOVA. n.s., Nonsignificant, $P = 0.08$ for 10^{-10} and 10^{-9} M GIP; *, $P < 0.05$; **, $P < 0.005$.

Activation of PKA/CREB by GIP and Glucose

Activation of the PKA/CREB signaling module was measured by phosphorylation of CREB in a transactivation assay using luciferase as a reporter gene (Fig. 2A) and by immunoblotting with an antibody specific for phosphorylated CREB at serine[133] (Fig. 2, B–D). Dose-response of GIP-induced CREB phosphorylation was examined at 2.5 mM and 15 mM glucose (Fig. 2A). CREB phosphorylation at 2.5 mM glucose served as control and was set at 1. Elevation of glucose to 15 mM initiated a 4.1-fold rise in CREB phosphorylation (Fig. 2A). At 2.5 mM glucose, GIP instigated a dose-dependent increase in CREB phosphorylation, which was maximal at 10^{-7} M by 21-fold with an EC_{50} of approximately 10^{-8} M. At 15 mM glucose, GIP stimulated CREB phosphorylation in a dose-dependent manner in a similar pattern as at 2.5 mM, although at a

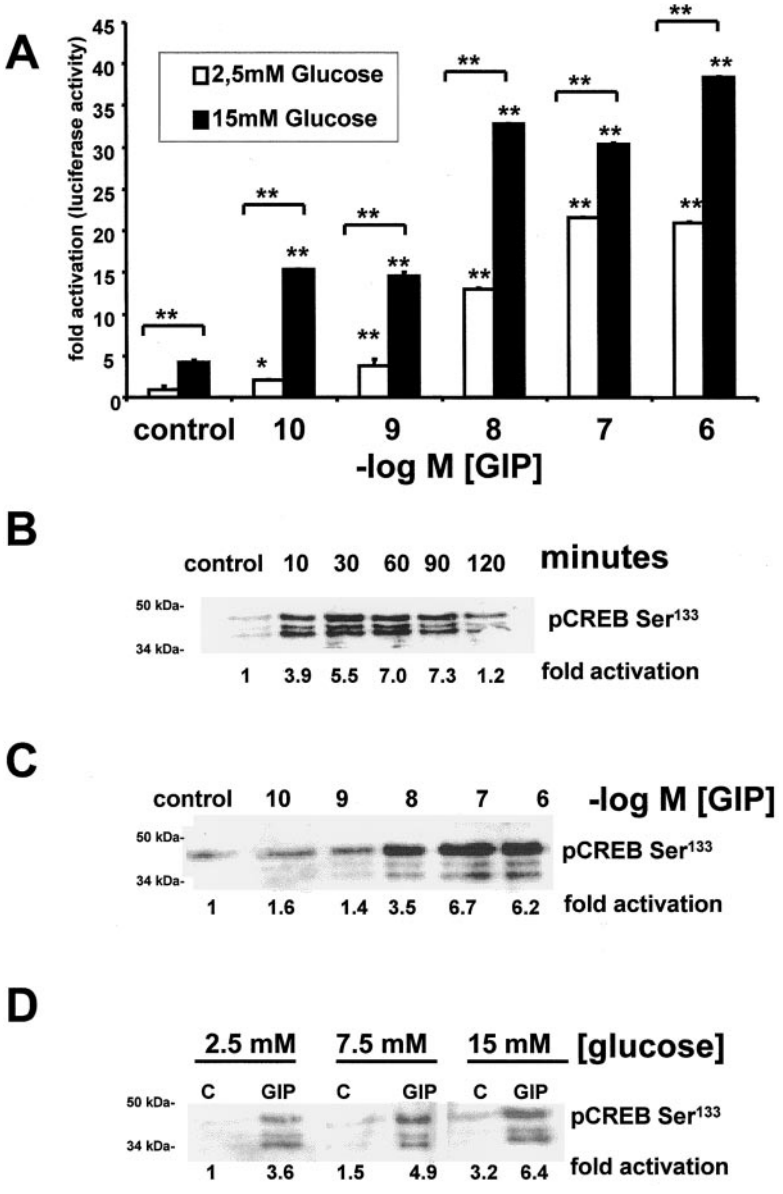

Fig. 2. Activation of the PKA/CREB Signaling Module by GIP and Glucose

A, Dose response of GIP-induced CREB phosphorylation at low (2.5 mM) and high (15 mM) glucose concentrations. INS-1 cells were transfected with CREB transactivator plasmid as described in *Materials and Methods*. INS-1 cells were stimulated for 16 h with different concentrations of GIP and glucose. CREB phosphorylation was determined by the luciferase activity of a cotransfected reporter plasmid. The luciferase activity of INS-1 cells stimulated with 2.5 mM glucose without GIP served as control and was set at 1. Each *bar* represents the mean ± SD of four to five independent experiments. Statistical analysis was performed by ANOVA. *, $P < 0.05$; **, $P < 0.005$. B, Time course of CREB phosphorylation by GIP. INS-1 cells were starved overnight and stimulated with 10^{-7} M GIP at indicated times. Cells were lysed and 100 μg of proteins were separated by SDS-PAGE and immunoblotted by Western analysis. The degree of CREB phosphorylation was determined using activation-specific antibody for pCREB Ser[133]. Proteins were detected using enhanced chemiluminescence, and band densities were quantified by densiometry. Data represent a typical blot of n = 3. They are expressed as relative to control assigning a value of 1 to nonstimulated cells. C, Dose response of CREB phosphorylation by GIP. Experiment was performed as in panel B except that cells were stimulated with indicated concentrations of GIP for 60 min. D, Glucose dependency of CREB phosphorylation by GIP. INS-1 cells were stimulated with 10^{-7} M GIP at 2.5 mM, 7.5 mM, and 15 mM glucose for 60 min. CREB phosphorylation of INS-1 cells stimulated with 2.5 mM glucose only was set at 1.

much higher level of activation (Fig. 2A). Here, maximal activation was 38-fold at 10^{-6} M with an EC_{50} between 10^{-9} M and 10^{-8} M GIP. At both glucose concentrations, a sharp rise in CREB phosphorylation was observed between 10^{-9} M and 10^{-8} M and a slower elevation at higher concentrations of GIP (Fig. 2A). Additional elevation of glucose concentrations above 15 mM did not further increase basal and GIP-stimu-

lated CREB phosphorylation (data not shown). ANOVA analysis revealed that glucose-induced rise of basal CREB phosphorylation was highly statistically significant. Furthermore, GIP-induced elevation of CREB phosphorylation was significant ($P < 0.05$) or highly significant ($P < 0.005$) at all GIP and glucose concentrations compared with basal levels and also when GIP-induced CREB phosphorylation was compared between low (2.5 mM) and high (15 mM) glucose concentrations (Fig. 2A).

The kinetics of GIP-induced CREB phosphorylation was examined by immunoblotting. CREB phosphorylation was rapid with an almost 4-fold rise in phosphorylation at 10 min. Maximal phosphorylation was increased 7-fold at 60 min. After 60 min, CREB phosphorylation decreased down to almost control levels at 120 min (Fig. 2B). The dose response of CREB phosphorylation demonstrated by immunoblotting (Fig. 2C) was similar to that shown by transactivating luciferase assays (Fig. 2A). In the immunoblot, maximal phosphorylation of CREB was observed at 10^{-7} M GIP with an EC_{50} at about 10^{-8} M GIP. Again, there was a sharp elevation in CREB phosphorylation between 10^{-9} M and 10^{-8} M GIP (Fig. 2C) as in the transactivating luciferase assay (Fig. 2A). Glucose dependency of GIP-stimulated CREB phosphorylation was examined by immunoblotting using a glucose gradient of 2.5 mM, 7.5 mM, and 15 mM glucose and by stimulation with 10^{-7} M GIP (Fig. 2D). Elevation of glucose from 2.5 mM to 15 mM instigated a rise in CREB phosphorylation to 3.2-fold compared with the control, which was further stimulated 2- to 3-fold above basal levels by the addition of GIP (Fig. 2D). Since elevation of glucose without GIP also stimulated CREB phosphorylation, we examined the kinetic glucose-stimulated CREB phosphorylation. When 7.5 or 15 mM glucose was added after glucose starvation of INS-1 cells of 24 h, increased phosphorylation was only noted after 6 h and was maximal by 12 h (data not shown), implicating a much slower kinetics of glucose-induced CREB phosphorylation compared with the CREB activation by GIP. Therefore, to determine the glucose dependency of CREB phosphorylation by GIP in the immunoblot analysis, we equilibrated INS-1 cells in 7.5 and 15 mM glucose concentration overnight. Equal loading of protein lysates was verified by immunoblotting for nonphosphorylated CREB (data not shown).

Activation of MAPK by GIP and Glucose

Activation of the MAPK pathway signaling module was examined by phosphorylation of transcription factor Elk-1 in a transactivation assay using luciferase as a reporter gene (Fig. 3A) and by immunoblotting with an antibody specific for phosphorylated MAPK [extracellular signal-regulated kinases 1 and 2 (ERK 1/2)] (Fig. 3, B–D). The experimental design was as described for CREB phosphorylation. Glucose at 15 mM stimulated Elk-1 phosphorylation 4.6-fold. At low and high glucose, GIP induced a maximal Elk-1 phosphorylation at 10^{-6} M (10-fold at 2.5 mM and 23-fold at 15 mM). However, the EC_{50} was slightly shifted to the left at 15 mM glucose (2.5 mM glucose: EC_{50} at 10^{-8} M GIP; 15 mM glucose: EC_{50} between 10^{-9} M and 10^{-8} M GIP; Fig. 3A). As in the case of CREB phosphorylation, there was a sudden approximately 2-fold rise in Elk-1 phosphorylation between 10^{-9} M and 10^{-8} M GIP (compare Figs. 2 and 3). ANOVA analysis revealed that glucose-induced Elk-1 phosphorylation was highly statistically significant. Elk-1 phosphorylation by GIP became significant at 10^{-10} at 15 mM glucose and at 10^{-9} at 2.5 mM glucose and was highly statistically significant at higher concentrations of GIP. The difference between GIP-induced CREB activation at low (2.5 mM) and high (15 mM) glucose concentrations was always highly significant (Fig. 3A).

The kinetics, dose response, and glucose response of MAPK activation were examined by immunoblotting for activated MAPK kinases ERK 1/2 (Fig. 3, B–D). GIP at 10^{-7} M instigated a fast rise in ERK phosphorylation at 10 min, which was maximal between 30 and 90 min and decreased at 120 min (Fig. 3B). A dose response similar to that for Elk-1 phosphorylation was demonstrated for ERK with a maximal phosphorylation at 10^{-7} M and 10^{-6} M GIP with an EC_{50} between 10^{-9} M and 10^{-8} M (Fig. 3C). Elevation of glucose to 7.5 mM and 15 mM stimulated basal ERK phosphorylation 2.4-fold, which was further increased by the addition of GIP (Fig. 3D). Kinetics and dose responses of glucose-induced Elk-1 and ERK phosphorylation were similar to that described for CREB phosphorylation. Equal loading of protein lysates was verified by immunoblotting for nonphosphorylated ERK (data not shown).

Activation of PI3K/PKB by GIP and Glucose

Stimulation of PI 3K by GIP and glucose was examined at the level of signaling molecules known to activate PI3K using antibodies for phosphorylated tyrosine (pY), insulin receptor substrate (IRS) isoforms IRS-1, IRS-2, and growth factor bound-2 associated binder-1 (Gab-1) (18, 19). In addition, PI3K activation was elucidated at the level of PI3K applying antibodies for PI3K regulatory subunit p85α and catalytic subunits p110α, p110β, and p110γ (18–21). INS-1 cells were stimulated at 2.5 mM, 7.5 mM, and 15 mM glucose overnight and subsequently with 10^{-7} M GIP for 60 min, since we could show that GIP-induced PI3K activation was maximal at this time point (data not shown). Cell lysates were subjected to immunoprecipitation, and PI3K activity was determined as described in *Materials and Methods*. PI3K activation by GIP was difficult to reproduce at 2.5 mM glucose. Thus, only stimulation of PI3K by GIP at 7.5 mM and 15 mM glucose was included in the analysis. Basal levels of PI3K were elevated by increasing glucose concentration to 15 mM, which was most pronounced for Gab-1- and p110β-associated PI3K activity (Fig. 4). The addition of GIP instigated modest amplifications in PI3K activity at 7.5 mM glucose except for p110γ-

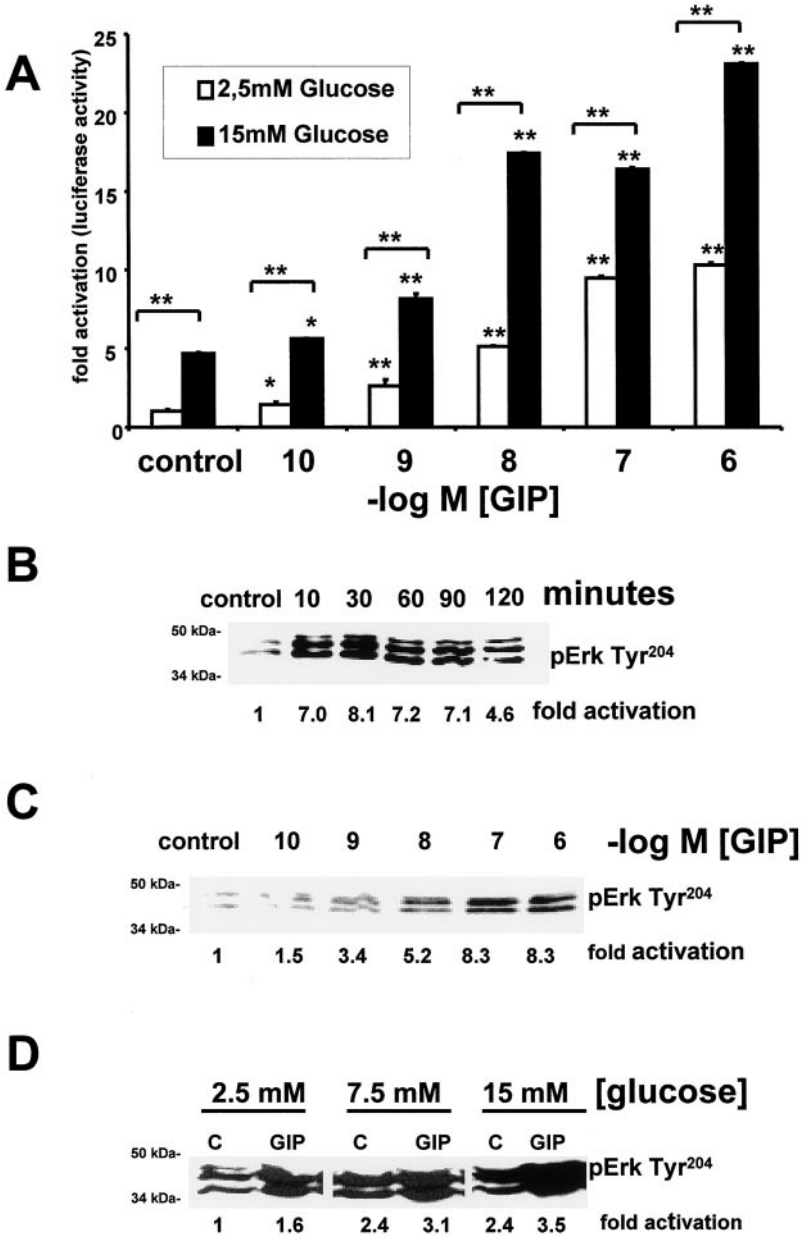

Fig. 3. Activation of the MAPK Signaling Module by GIP and Glucose at the Level of Transcription Factor Elk-1 (A) and the MAPK ERK-1 and ERK-2 (B–D)

A, Dose response of GIP-induced Elk-1 phosphorylation at low (2.5 mM) and high (15 mM) glucose concentrations. INS-1 cells were transfected with Elk-1 transactivator plasmid as described in *Materials and Methods*. INS-1 cells were stimulated for 16 h with different concentrations of GIP and glucose. Elk-1 phosphorylation was determined by the luciferase activity of a cotransfected reporter plasmid. The luciferase activity of INS-1 cells stimulated with 2.5 mM glucose without GIP was set at 1. Each *bar* represents the mean ± SD of four to five independent experiments. Statistical analysis was performed by ANOVA. *, $P < 0.05$; **, $P < 0.005$. B, Time course of ERK phosphorylation by GIP. INS-1 cells were starved overnight and stimulated with 10^{-7} M GIP at indicated time points. Cells were lysed and 100 μg of proteins were separated by SDS-PAGE and immunoblotted by Western analysis. The degree of ERK phosphorylation was determined using activation-specific antibody for pTyr (204) of ERK-1 and ERK-2. Proteins were detected using enhanced chemiluminescence, and band densities were quantified by densiometry. Data represent a typical blot of n = 3. They are expressed as relative to control, assigning a value of 1 to nonstimulated cells. C, Dose-response of ERK phosphorylation by GIP. Experiment was performed as in panel B except that cells were stimulated with indicated concentrations of GIP for 60 min. D, Glucose dependency of ERK phosphorylation by GIP. INS-1 cells were stimulated with 10^{-7} M GIP at 2.5 mM, 7.5 mM, and 15 mM glucose for 60 min. ERK phosphorylation of INS-1 cells stimulated with 2.5 mM glucose only was set at 1.

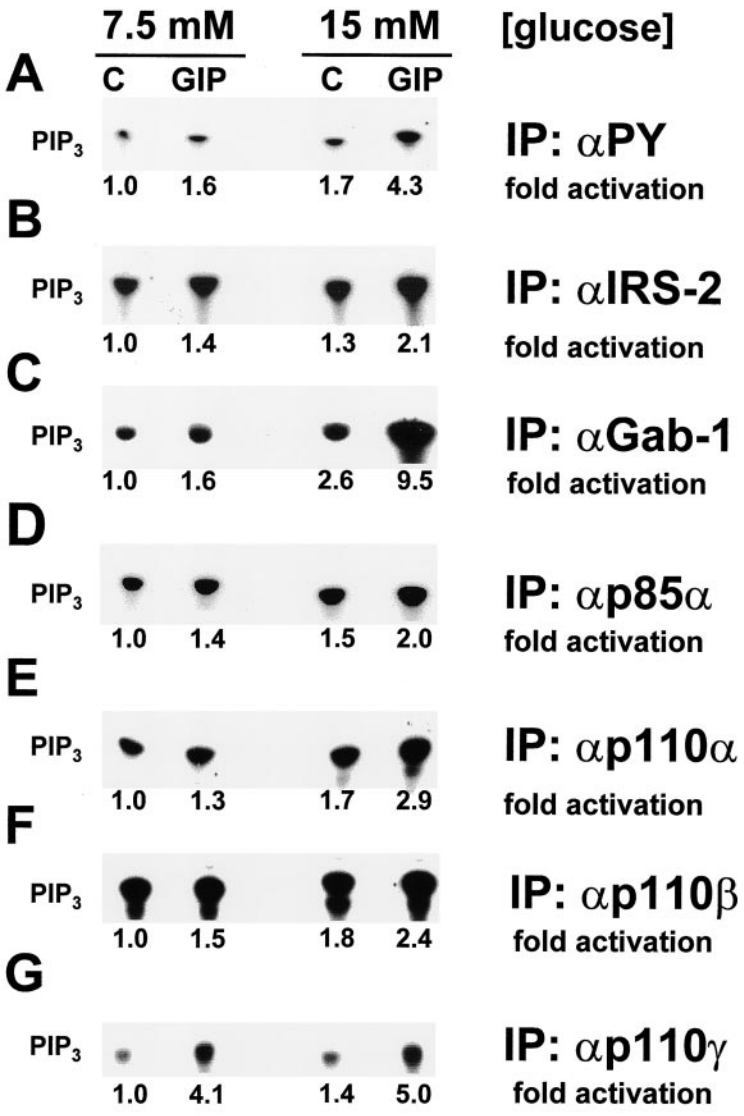

Fig. 4. PI3K Activation by GIP and Glucose

Cells were starved overnight and stimulated with 10^{-7} M GIP for 60 min at 7.5 and 15 mM glucose. Equal amounts of cell lysates were subjected to immunoprecipitation with antibodies to pY (A), IRS-2 (B), Gab-1 (C), p85α (D), p110α (E), p110β (F), and p110γ (G). PI3K assays were performed as described in *Materials and Methods*. ^{32}P-incorporation into phosphatidylinositol 3-P was quantified after separation by TLC using densiometry. The band shown represents phosphatidylinositol 3-P. Shown are representative autoradiographs of n = 3. They are expressed as relative to control assigning a value of 1 to nonstimulated cells at 7.5 mM glucose.

associated PI3K activity, which was stimulated 4.1-fold (Fig. 4G). At 15 mM glucose, a more robust amplification of PI3K activity in all immunoprecipitates was observed, most notably in anti-Gab-1 and anti-p110γ associated PI3K activity (Fig. 4). No stimulation of GIP-induced PI3K activity was associated with anti-IRS-1, anti-insulin receptor β-chain, anti-JAK2, and p85β immunoprecipitates (data not shown).

The serine-threonine kinase PKB is activated by PI3K. To elucidate whether PKB is stimulated by GIP in β-cells, PKB activation was detected by immunoblotting INS-1 cell lysates with an activation-specific antibody for pPKBSer^{473}. This method was used to examine the kinetic (Fig. 5A), dose response (Fig. 5B), and glucose dependency (Fig. 5C) of PKB stimulation by GIP. Compared with PKA/CREB and MAPK, PKB activation by GIP exhibited slower kinetics with a maximal 2.6- to 3.5-fold phosphorylation between 30 and 90 min and a decline to almost control levels at 360 min (Fig. 5A). ANOVA revealed a highly significant elevation in PKB phosphorylation between 30 and 120 min (Fig. 5A). The dose-response curve of PKB phosphorylation by GIP demonstrated maximal 2.6-fold phosphorylation between 10^{-8} M and 10^{-6} M with a sudden rise of activation between 10^{-9} M and 10^{-8} M, which also corresponded to the EC_{50} (Fig. 9B). At 10^{-9} M, GIP-induced PKB phosphorylation became highly statistically significant. Using a glucose gradient

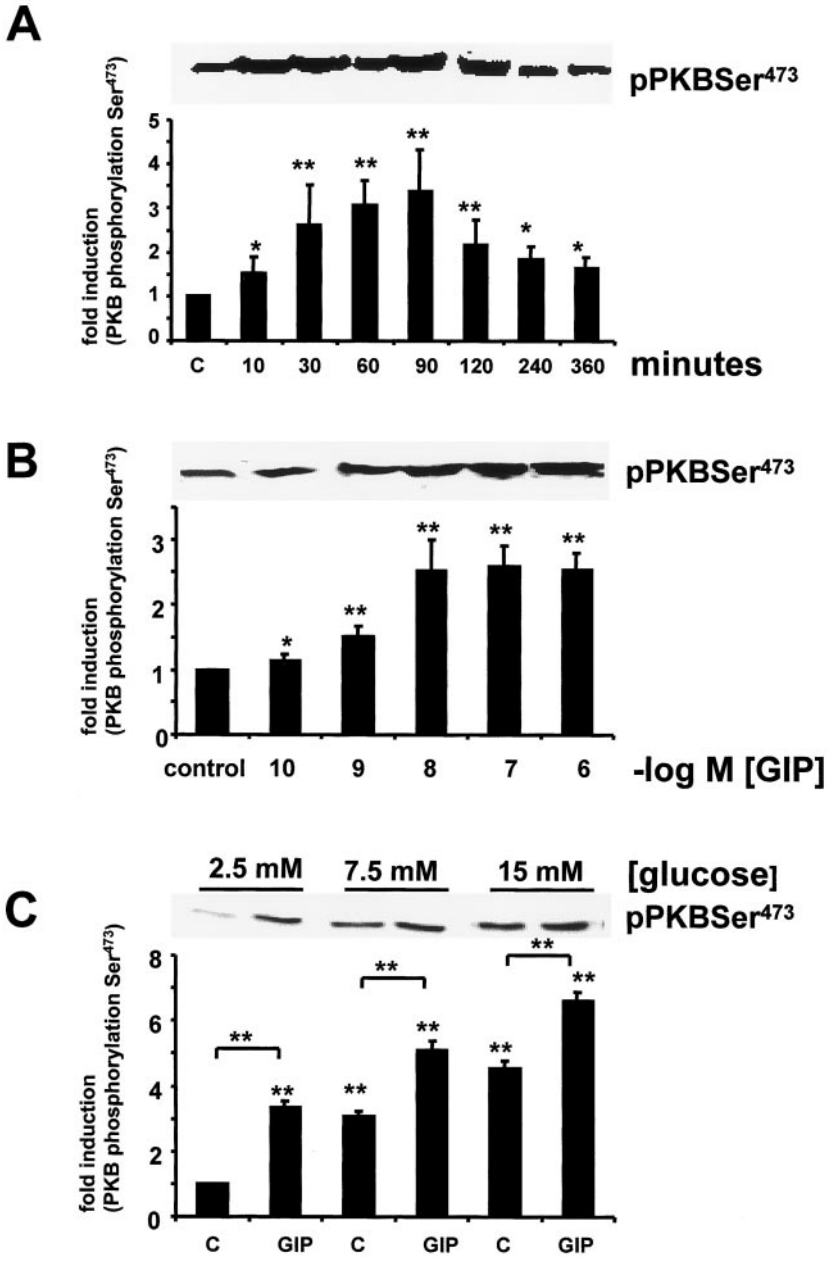

Fig. 5. Activation of PKB by GIP and Glucose

A, Time course of PKB phosphorylation by GIP. INS-1 cells were starved overnight and were stimulated with 10^{-7} M GIP at 11 mM glucose. Cells were lysed and 100 μg of proteins were separated by SDS-PAGE and immunoblotted by Western analysis. The degree of PKB phosphorylation was determined using activation-specific antibody for pSer^{473} PKBα as described in *Materials and Methods*. Immunoblots using an antiserum recognizing total PKBα served as a control for equal loading. Proteins were detected using enhanced chemiluminescence, and band densities were quantified by densiometry. Data are the mean ± SEM of 6–10 independent experiments. They are expressed as relative to control, assigning a value of 1 to nonstimulated cells at 360 min. B, Dose-response of GIP-induced PKB phosphorylation. Experiment was performed as in panel A except that cells were stimulated with indicated concentrations of GIP for 60 min. C, Glucose dependency of PKB phosphorylation by GIP. INS-1 cells were stimulated with 10^{-7} M GIP at 2.5 mM, 7.5 mM, and 15 mM glucose for 60 min. PKB phosphorylation of INS-1 cells stimulated with 2.5 mM glucose only was set at 1. Statistical analysis was performed by ANOVA. *, $P < 0.05$; **, $P < 0.005$.

of 2.5 mM, 7.5 mM, and 15 mM, we could show that basal and GIP-stimulated PKB phosphorylation increased with rising glucose concentrations (Fig. 5C) similar to the glucose response of CREB and ERK phosphorylation (Figs. 2D, 3D, and 5C). Statistical analysis by ANOVA revealed that the increase in basal levels and after stimulation with GIP by elevation of glucose was statistically significant as well as the difference between basal levels and GIP-induced PKB phosphorylation at 2.5 mM, 7.5 mM, and 15 mM glu-

cose (Fig. 5C). Equal loading of protein lysates was verified by immunoblotting for nonphosphorylated PKBα (data not shown).

Multiple isoforms of PKB are expressed in pancreatic β-cells (21, 22) and may be differentially activated by glucose and GIP. We therefore examined the isoform-specific stimulation of PKB using specific antisera for PKBα, PKBβ, and PKBγ to perform isoform-specific PKB assays in immunoprecipitates of glucose and GIP-stimulated INS-1 cell lysates as described in *Materials and Methods*. GIP induced activation of PKB serine kinase activity glucose dependently between 7.5 mM and 15 mM glucose in PKBα- and PKBβ- (Fig. 6A and B) but not in PKBγ-immunoprecipitates (not shown). The transcription factor FKHR and cytoplasmatic kinases, glycogen-synthase 3α/β (GSK-3) and $p70^{S6K}$ (21) are downstream targets of PKB. To find out whether FKHR, GSK-3, and $p70^{S6K}$ are stimulated by glucose and GIP, we used phosphorylation-specific antibodies for activated FKHR, GSK, and $p70^{S6K}$. All targets of PKB were phosphorylated by stimulation by glucose and GIP at 7.5 and 15 mM glucose (Fig. 6, C–F). We noticed in the PKB assays that native GSK-3 was coimmunoprecipitated by PKBα and PKBβ antibodies (data not shown) in a similar time course as the phosphorylation in straight cell lysates (Fig. 6D), indicating that GSK is associated with stimulated PKB and that PKB is a GSK kinase. Equal loading of protein lysates was verified by immunoblotting for nonphosphorylated GSK-3, FKHR, and $p70^{S6K}$ (data not shown).

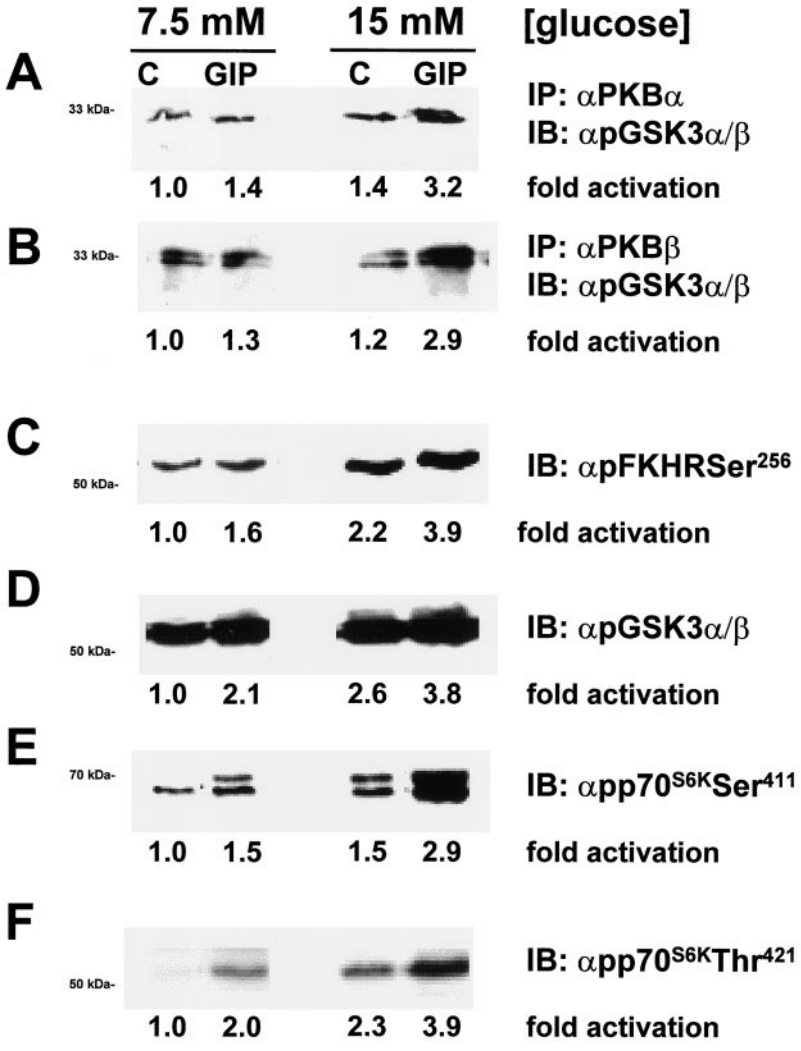

Fig. 6. Activation of PKB Isoforms PKBα and PKBβ, the Transcription Factor FKHR, and Cytoplasmatic Kinases GSK-3 and $p70^{S6K}$ by GIP and Glucose

Cells were starved overnight and stimulated with 10^{-7} M GIP for 60 min at 7.5 and 15 mM glucose. A and B, Equal amounts of cell lysates were subjected to immunoprecipitation with antibodies to PKBα (A) and PKBβ (B). PKB assays were performed as described in *Materials and Methods*. Shown are representative autoradiographs of n = 3. They are expressed as relative to control assigning a value of 1 to nonstimulated cells at 7.5 mM glucose. Phosphorylation of FKHR (C), GSK-3 (D), and $p70^{S6K}$ (E and F) was determined by immunoblotting using phosphorylation-specific antibodies. Shown are representative autoradiographs of n = 3.

JAK/STAT Pathways

GH is a major mitogen of β-cells by the activation of JAK/STAT signaling module, especially JAK2 and STAT5 (23, 24). To determine whether GIP shares this mitogenic pathway with GH, we stimulated INS-1 cells with 10^{-8} M human recombinant GH and 10^{-7} M GIP and immunoblotted cell lysates with phosphorylation-specific antibodies for tyrosine^{701} of STAT1, serine^{727} of STAT3, tyrosine^{705} of STAT3, tyrosine^{694} of STAT5, and tyrosine^{641} of STAT6. Neither human GH nor GIP induced phosphorylation of STAT1 and STAT3. In contrast, GIP failed to phosphorylate STAT5 and STAT6, which were phosphorylated by stimulation with GH in INS-1 cells, indicating that GIP does not activate the mitogenic JAK2/STATs5/6 pathway in INS-1 cells (data not shown).

DISCUSSION

Insulin-secreting β-cells of the pancreas are highly specialized cells with a low mitogenic index. The β-cell pool may be replenished by neogenesis of β-cells from precursor cells in pancreatic ducts or by islet cell replication (25–27). IGF-I, GH, and PRL have been characterized as growth factors for β-cells. Elevated glucose levels also induce cellular proliferation in β-cells (23, 24, 28–32). Recently, it could be shown that the gluco-incretin hormone GLP-1 acts as a growth, antiapoptotic, and differentiation factor for β-cells in animal models, in isolated islets, and clonal β-cells (4–8). These results prompted us to examine whether and how the other gluco-incretin hormone GIP acts as growth factor for β-cells. Here, we show that GIP induced β-cell proliferation in the β-cell line INS-1 by pleiotropic signaling. Our results indicate that GIP may be added to the growing list of β-cell growth factors. Recent animals studies corroborate the hypothesis of GIP as a β-cell growth factor. Selective expression of a dominant negative mutant of the human GIP receptor in β-cells as a transgene yielded diminished islet size and overt diabetes in mice (Göke, B., and A. Volz, personal communication). In addition, the failure of GIP receptor knockouts to respond to high fat diet with hyperinsulinemia like wild-type mice points to a role of GIP signaling in the regulation of appropriate β-cell mass in insulin resistance (15).

The GIP receptor belongs to the family of G protein-coupled seven-transmembrane receptors and is expressed not only in pancreatic β-cells but also in brain, adipose tissue, intestine, and heart (9, 10). Stimulation of the GIP receptor leads to rise of cytosolic cAMP by the activation of membrane-bound adenylate cyclase and a rise in intracellular Ca^{++} (9–11). We examined whether GIP activates major mitogenic signaling modules and demonstrated pleiotropic stimulation of PKA/CREB, p44/p42 MAPK, and PI3K/PKB signaling pathways by the GIP receptor in a similar dose response. These effects were synergistic with glucose between 2.5 mM and 15 mM. In contrast, there was no evidence of JAK2/STATs5/6 pathway activation by GIP in INS-1 cells (Fig. 7). These results indicate that signaling of β-cell growth factors is transduced by pleiotropic, yet specific, pathways. Receptor tyrosine kinases such as insulin and IGF-I receptors activate PI3K/PKB and MAPK (32–34). Cytokine-like receptors such as the GH receptor stimulate JAK2/STAT5 and PI3K (23, 24, 30, 35). Finally, G protein-coupled receptors such as GIP- and GLP-1 receptors induce activation of PKA/CREB, MAPK, and PI3K/PKB (3, 31, 36), but not JAK/STAT pathways (Fig. 7). In this context, it is interesting to note that mitogenic signaling pathways activated by both receptors for GIP and GLP-1 are similar in β-cells (3, 31, 35, 36), indicating a mitogenic signaling redundancy of both incretin hormones (37).

Glucose concentration in the stimulation medium was essential for the activation of all signaling modules examined. Synergistic effects of glucose and GIP were additive in the immunoblotting analysis of CREB, ERK1/2, and PKB phosphorylation with a relative short stimulation of 60 min whereas transactivating luciferase assays for CREB and Elk-1 demonstrated superadditive effects at a stimulation period of 16 h. Thus, the superadditive effects seen in the transactivating luciferase assays may be caused by the accumulation of reporter luciferase. On the other hand, detailed analysis of PI3K signal transmission by glucose and GIP revealed additive and superadditive effects, implying differential substrate specificity of glucose and GIP induced signal transduction by PI3K. Blockage of glucokinase by alloxan (38) and prevention of glucose phosphorylation by 2-deoxy-D-glucose (39) indicated signal transduction by derivatives of glucose metabolism in β-cells (Ref. 40 and Trümper, A., and D. Hörsch; unpublished results). Clearly, ATP generated from pyruvate in mitochondria and glutamate (40) are favorite candidates as mitogenic and antiapoptotic second messengers of glucose-induced signaling as well as for glucose-induced synergism in GIP signaling. However, it has to kept in mind that activation of signal transduction pathways by glucose may not be the result of a second messenger-like ATP but reflect the altered ADP:ATP ratio in β-cells at high glucose levels (40). ATP is a necessary cofactor for kinase reactions (41). The increased availability of ATP for kinase reactions at high glucose levels may facilitate kinase reactions and thus mimic a second messenger effect.

Growth factors signal to the nucleus by cytoplasmatic signaling cascades inducing the transcription of cell-specific sets of genes by the activation of transcription factors (23–25, 28, 29, 42, 43). Here, we showed that GIP and glucose phosphorylate transcription factors CREB, Elk-1, and FKHR. In addition, glucose and GIP phosphorylated p70^{S6K}, a crucial regulator of translation (44). These data indicate that mitogenic effects of glucose and GIP in β-cells may not only be caused by activation of transcription but also by initiation of protein translation. Recently, it has been shown that a major part of the mitogenic re-

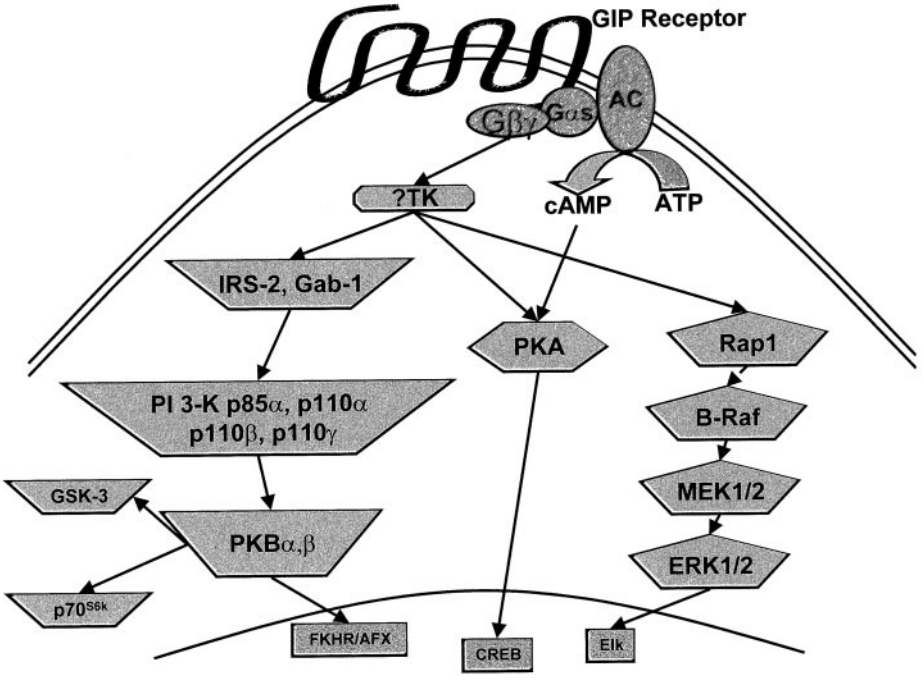

Fig. 7. Schematic Presentations of Signaling Cascades Stimulated by GIP in Pancreatic β-Cells

AC, Membrane-coupled adenylate cyclase; AFX, forkhead transcription factor AFX; Elk, transcription factor Elk; FKHR, Forkhead in rhabdomyosarcoma; Gαs: α-subunit of stimulatory G protein; Gβγ: βγ-subunits of G proteins; MEK, MAPK/ERK kinase; B-Raf, serine/threonine kinase B-Raf; RAS, small GTPase, cellular protein homolog to v-ras; ?TK: unidentified tyrosine kinase.

sponse of β-cells to glucose may be attributed to the activation of $p70^{S6K}$ (45). Furthermore, a knockout of $p70^{S6K}1$ leads to diminished β-cell size and glucose resistance in mice (44), indicating that stimulation of $p70^{S6K}$ is essential for regulation of β-cell growth.

Altered β-cell function is pivotal for the development of type 2 diabetes. As the disease develops, the ability of β-cells to secrete compensatory amounts of insulin decreases, thereby increasing hyperglycemia and insulin resistance (27, 46–48). Thus, mitogenic and antiapoptotic activation of G protein-coupled receptors, strongly expressed on β-cells, may prove to be a therapeutic approach for late stages of type II diabetes. Although, short-term antidiabetic effects of GIP are not preserved in type II diabetes and glucose intolerance due to lack of insulinotropic action (1), long-term application of GIP may prove to be an therapeutic approach for type II diabetes. We have shown that GIP is a growth and antiapoptotic factor for β-cells by pleiotropic activation of PKA/CREB, MAPK, and PI3K/PKB pathways. Thus, long-term application of GIP may be beneficial in the treatment of type II diabetes as a β-cell growth factor by improving β-cell function.

MATERIALS AND METHODS

Materials

Silica-gel TLC plates were obtained from Merck & Co., Inc. (Darmstadt, Germany), protein G-agarose was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nitrocellulose paper (Optitran BA-S85) was from Schleicher & Schuell, Inc. (Keene, NH) and [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (Arlington Heights, IL). GIP (1–42) and recombinant human GH were obtained from Bachem (Bubendorf, Switzerland). 5-Bromo-deoxyuridine (BrdU) incorporation and cell death detection ELISAs were from Roche (Mannheim, Germany). New England Biolabs, Inc. (Beverly, MA) supplied the PKBα antiserum, phospho-specific antibodies for serine^{473} and threonine^{308} of PKBα, serine^{133} of CREB, serine^{411} of $p70^{S6K}$, threonine^{421}/serine^{424} of $p70^{S6K}$, tyrosine^{701} of STAT1, serine^{727} of STAT3, tyrosine^{705} of STAT3, tyrosine^{694} of STAT5, tyrosine^{641} of STAT6, and serine$^{21/9}$ of glycogen-synthase kinase 3α/β (GSK-3), and GSK-3α/β cross-tide protein, along with respective control antibodies for nonphosphorylated kinases as well as enhanced chemiluminescence reagents. Antibodies for Gab-1, insulin receptor substrate (IRS) IRS-1, IRS-2, p110α, p110β, p85α, pY, and PKB-isoforms α, β, and γ were from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies for phosphorylated ERK-1 and ERK-2 (pERK Tyr^{204}), control ERK-antibodies, and p110γ antibodies were from Santa Cruz Biotechnology, Inc.. Reagents for SDS-PAGE were from Bio-Rad Laboratories, Inc. (Hercules, CA), cell culture reagents were from Life Technologies, Inc.(Karlsruhe, Germany), and all other chemicals were from Sigma (St. Louis, MO).

Cell Culture

INS-1 cells (passage 80–120) were grown as previously described (16) in regular RPMI-1640 medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 C in a humidified (5% CO_2, 95% air) atmosphere. Before stimulation, INS-1 cells were starved in medium without serum, glucose, and sodium pyruvate.

BrdU Incorporation

Cells were seeded at a density of 3×10^3 in 96-well plates, grown for 24 h in regular medium, washed once with 10 mM PBS (pH 7.4), and subsequently starved for 24 h. They were then incubated for 24 h in RPMI medium with different glucose concentrations and test substances. During the last 6 h of stimulation, 20 μl of a BrdU solution was added and ELISA (17) was performed according to guidelines provided by the manufacturer.

Transreporting System for Elk1 and CREB Phosphorylation

INS-1 cells were grown for 48 h in normal medium in six-well plates until they reached 60–80% confluency. Cells were then washed twice with PBS, transfected with luciferase reporter gene (pFR-Luc) and either Elk1 (pFA-2-Elk1) or CERB (pFA2-CREB) transactivator domains (all from Stratagene, La Jolla, CA) by lipid-based transfection (Pfx-6; Invitrogen, Groningen, The Netherlands) for 8 h in INS-1 medium without serum. Subsequently, cells were grown in INS-1 medium with 5 mM glucose and 5% FBS and then stimulated for 16 h in INS-1 medium containing 1% FBS with GIP at different glucose concentrations.

Immunoprecipitation and Immunoblotting

INS-1 cells were starved for 12 h and were then equilibrated for another 12 h in the indicated glucose concentrations. One hour before stimulation, the stimulation medium was changed. Cells were lysed after stimulation in ice-cold lysis buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium vanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1.5 mg/ml benzamidine, and 34 μg/ml phenylmethylsulfonyl fluoride) and sonicated for 15 sec, and insoluble material was removed by centrifugation at 15,000 rpm in a microfuge for 10 min. For immunoblotting, 100 μg of protein per lane were separated by 10% SDS-PAGE, Western-transferred on nitrocellulose membranes, and immunoblotted as previously described (18). For immunoprecipitation experiments, 500 μg of protein lysate were immunoprecipitated with indicated antibodies and 60 μl of protein G- or protein A-agarose, respectively, for 2–4 h at 4 C. Beads were washed twice with protein lysis buffer and either used immediately for SDS-PAGE or frozen at −80 C. Protein bands were visualized with enhanced chemiluminescence. Autoradiographs were scanned, and band density was determined using Gelscan 3D software (BioSciTec, Marburg, Germany). Statistical analysis was performed by ANOVA.

PI3K Assays

Immune-complexed PI3K activity was determined as previously described (18). Immune complexes were incubated in a 55 μl reaction mixture containing 200 μM ATP, 5 μCi [γ-^{32}P]ATP, 200 mM $MgCl_2$, and 5 μg phosphatidylinositol for 20 min at room temperature. Reactions were stopped by the addition of 150 μl of $CHCl_3/CH_3OH$/11.6 N HCl (33:66:0.6) and subsequently of 120 μl of $CHCL_3$. The organic phase was washed once with 150 μl of CH_3OH/1 N HCl (1:1), 20 μl 8 N HCl, and 160 μl $CHCl_3$/methanol 1:1. The organic phase was removed by centrifugation and applied to silica gel TLC plates, developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.3:2), dried, and visualized by autoradiography. The band representing phosphatidylinositol 3-P was quantified as described.

PKB Assays

Immunocomplexed PKB activity was determined by incubating washed antibodies with 200 μmol/liter ATP and 1 μg GSK3α/β cross-tide fusion protein. Phosphorylation of the substrate was determined by immunoblotting with a phosphorylation-specific antibody for serine$^{21/9}$ of GSK3α/β at 30 kDa (New England Biolabs, Inc.).

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REFERENCES

1. Fehmann HC, Göke R, Göke B 1995 Cell and molecular biology of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulin releasing polypeptide. Endocr Rev 16:390–410
2. Kieffer TJ, Habener JF 1999 The glucagon-like peptides. Endocr Rev 20:876–913
3. Buteau J, Roduit R, Susini S, Prentki M 1999 Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in β (INS-1)-cells. Diabetologia 42: 856–864
4. Gang X, Stoffers A, Habener JF, Bonner-Weir S 1999 Exendin-4 stimulates both β-cell replication and neogenesis resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276
5. Zhou J, Wang X, Pineyro MA, Egan JM 1999 Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon and insulin secreting cells. Diabetes 48:2358–2366
6. Perfetti R, Zhou J, Doyle ME, Egan JM 2000 GLP-1 induces cell proliferation, PDX-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. Endocrinology 141:4600–4605
7. Stoffers DA, Kieffer TJ, Hussain MA 2000 Insulinotropic glucagon-like peptide-1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes 49:741–748
8. Hui H, Wright C, Perfetti R 2001 Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. Diabetes 50:785–796
9. Usdin TB, Mezey E, Button DC, Brownstein MJ, Bonner TI 1993 Gastric inhibitory polypeptide receptor, a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and in the brain. Endocrinology 133:2861–2870
10. Wheeler MB, Gelling RW, McIntosh CH, Georgiou J, Pederson RA 1995 Functional expression of the rat islet glucose-dependent insulinotropic polypeptide receptor: ligand binding and intracellular signaling properties. Endocrinology 136:4629–4639
11. Volz A, Göke R, Lankat-Buttgereit B, Fehmann HC, Bode HP, Göke B 1996 Molecular cloning, functional expres-

sion, and signal transduction of the GIP-receptor cloned from a human insulinoma. FEBS Lett 373:23–29
12. Yip RGC, Wolfe MM 2000 GIP biology and fat metabolism. Life Sci 66: 91–103
13. Kubota A, Yamada Y, Yasuda K, et al. 1997 Gastric inhibitory polypeptide activates MAP kinase through the Wortmannin-sensitive and -insensitive pathways. Biochem Biophys Res Commun 235:171–175
14. Straub S, Sharp GWG 1996 Glucose-dependent insulinotropic polypeptide stimulates insulin secretion via increased cyclic AMP and (Ca 2+) and a Wortmannin-sensitive signaling pathway. Biochem Biophys Res Commun 254: 369–374
15. Miyawaki K, Yamada Y, Yano H, et al. 1999 Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. Proc Natl Acad USA 96:14843–14847
16. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB 1992 Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology 130:167–178
17. Huong PL, Kolk AH, Eggelte TA, Verstijnen CP, Gilis H, Hendriks JT 1991 Measurement of antigen specific lymphocyte proliferation using 5-bromo-deoxyuridine incorporation. An easy and low cost alternative to radioactive thymidine incorporation. J Immunol Methods 5:243–245
18. Kerouz N, Hörsch D, Pons S, Kahn CR 1997 Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol-3 kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. J Clin Invest 100:3164–3172
19. Holgado-Madruga M, Emlet DR, Moscatello DK, Godwin AK, Wong AJ 1996 A Grb2-associated docking protein in EGF- and insulin-receptor signalling. Nature 379:560–563
20. Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann MP 1998 Bifurcation of lipid and protein kinase signals of PI3Kγ to the protein kinases PKB and MAPK. Science 282:293–296
21. Coffer PJ, Jin J, Woodgett JR 1998 Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J 335:1–13
22. Holst LS, Mulder H, Manganiello V, et al. 1998 Protein kinase B is expressed in pancreatic β-cells and activated upon stimulation with insulin-like growth factor 1. Biochem Biophys Res Commun 250:181–186
23. Cousin SP, Hügl SR, Myers MG, White MF, Reifel-Miller A, Rhodes CJ 1999 Stimulation of pancreatic β-cell proliferation by growth hormone is glucose-dependent: signal transduction via Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) with no crosstalk to insulin receptor substrate-mediated mitogenic signalling. Biochem J 344:649–658
24. Friedrichsen BN, Galsgaard ED, Nielsen JH, Moldrup A 2001 Growth hormone- and prolactin-induced proliferation of insulinoma cells, INS-1, depends on activation of STAT5 (Signal transducer and activator of transcription 5). Mol Endocrinol 15:136–148
25. Rane SG, Reddy EP 2000 Cell cycle control of pancreatic β cell proliferation. Front Biosci 5:1–26
26. Scharfmann R, Czernichow P 1996 Differentiation and growth of pancreatic β-cells. Diabetes Metab 22:223–228
27. Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S, Sharma A 2001 β-cell adaption and decompensation during the progression of diabetes. Diabetes 50 (Suppl 1):S154–159
28. Josefsen K, Sorensen LR, Buschard K, Birkenbach M 1999 Glucose induces early growth response gene (Egr-1) expression in pancreatic β cells. Diabetologia 42:195–203
29. Susini S, Roche E, Prentki M, Schlegel W 1998 Glucose and glucoincretin peptides synergize to induce c-fos, c-jun, junB, zif-268, and nur-77 gene expression in pancreatic beta (INS-1) cells. FASEB J 12:1173–1182
30. Nielsen JH, Møldrup A, Billestrup N, Petersen ED, Allevato G, Stahl M 1992 The role of growth hormone and prolactin in β cell growth and regeneration. Adv Exp Med Biol 321:9–17
31. Frödin M, Sekine N, Roche E, et al. 1995 Glucose, other secretagogues, and nerve growth-factor stimulate mitogen- activated protein-kinase in the insulin-secreting β-cell line, INS-1. J Biol Chem 270:7882–7889
32. Hügl SR, White MF, Rhodes CJ 1998 Insulin-like-growth-factor-1 (IGF-1)-stimulated pancreatic β-cell growth is glucose-dependent—synergistic activation of insulin-receptor substrate-mediated signal-transduction pathways by glucose and IGF-1 in INS-1 cells. J Biol Chem 273:17771–17779
33. Leibiger IB, Leibiger B, Moede T, Berggren PO 1998 Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 s6 kinase and CaM kinase pathways. Mol Cell 1:933–938
34. Xu GX, Rothenberg PL 1998 Insulin receptor signaling in the β cell influences insulin gene expression and insulin content. Diabetes 47:1243–1252
35. Trümper A, Trümper K, Trusheim H, Arnold R, Hörsch D 2000 Protein kinase B activation by glucose-dependent insulinotropic polypeptide and growth hormone in beta (INS-1) cells. Diabetologia 43 (Suppl 1):136 (Abstract)
36. Trusheim H, Trümper A, Trümper K, Wilmen A, Göke B 2000 Glucagon-like peptide-1 induced signal transduction and immediate early gene expression in beta (INS-1) cells. Diabetologia 43 (Suppl 1):121 (Abstract)
37. Pederson RA, Satkunarajah M, McIntosh CH, et al. 1998 Enhanced glucose-dependent insulinotropic polypeptide secretion and insulinotropic action in glucagon-like peptide 1 receptor −/− mice. Diabetes 47:46–52
38. Lenzen S, Brand FH, Panten U 1988 Structural requirements of alloxan and ninhydrin for glucokinase inhibition and of glucose for protection against inhibition. Br J Pharmacol 95:851–859
39. Schuit FC, Huypens P, Heimberg H, Pipeleers DG 2001 Glucose-sensing in pancreatic β-cells: a model for the study of other glucose-regulated cells in gut, pancreas and hypothalamus. Diabetes 50:1–11
40. Isihara H, Wollheim CB 2000 What couples glycolysis to mitochondrial signal generation in glucose-stimulated insulin secretion. IUBMB Life 49:391–395
41. Davies SP, Reddy H, Caivano M, Cohen P 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105
42. Pawson T, Saxton TM 1999 Signaling networks—do all roads lead to the same genes? Cell 97:675–678
43. Simon MA 2000 Receptor tyrosine kinase: specific outcomes from general signals. Cell 103:13–15
44. Pende M, Kozma SC, Jaquet M, et al. 2000 Hypoinsulinaemia, glucose intolerance and diminished β-cell size in S6K1-deficient mice. Nature 408:994–997
45. Dickson LM, Lingohr MK, McCuaig J, et al. 2001 Differential activation of PKB and $p70^{S6K}$ by glucose and IGF-1 in pancreatic β-cells (INS-1). J Biol Chem 276:21110–21120
46. Virkamäki A, Ueki K, Kahn CR 1999 Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest 103:931–941
47. DeFronzo RA 1997 Insulin resistance: a multifaceted syndrome responsible for NIDDM, hypertension, dyslipidemia and atherosclerosis. Neth J Med 50:191–197
48. Taylor S 1999 Deconstructing type 2 diabetes. Cell 97:9–12