

Activation of IRS-2–Mediated Signal Transduction by IGF-1, but not TGF- α or EGF, Augments Pancreatic β -Cell Proliferation

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Transforming growth factor (TGF)- α - and epidermal growth factor (EGF)-induced signal transduction was directly compared with that of glucose and insulin-like growth factor-1 (IGF-1) in INS-1 cells. TGF- α /EGF transiently (<20 min) induced phosphorylation of extracellular-regulated kinase (Erk)-1/2 (>20-fold), glycogen synthase kinase (GSK)-3 (>10-fold), and protein kinase B (PKB) (Ser⁴⁷³ and Thr³⁰⁸), but did not increase [³H]thymidine incorporation. In contrast, phosphorylation of Erk1/2, GSK-3, and PKB in response to glucose and IGF-1 was more prolonged (>24 h) and, though not as robust as TGF- α /EGF, did increase β -cell proliferation. Phosphorylation of p70^{S6K} was also increased by IGF-1/glucose, but not by TGF- α /EGF, despite upstream PKB activation. It was found that IGF-1 induced phosphatidylinositol 3-kinase (PI3K) association with insulin receptor substrate (IRS)-1 and -2 in a glucose-dependent manner, whereas TGF- α /EGF did not. The importance of specific IRS-2–mediated signaling events was emphasized in that adenoviral-mediated overexpression of IRS-2 further increased glucose/IGF-1–induced β -cell proliferation (more than twofold; $P < 0.05$) compared with control or adenoviral-mediated IRS-1 overexpressing INS-1 cells. Neither IRS-1 nor IRS-2 overexpression induced a β -cell proliferative response to TGF- α /EGF. Thus, a prolonged activation of Erk1/2 and PI3K signaling pathways is important in committing a β -cell to a mitogenic event, and it is likely that this sustained activation is instigated by signal transduction occurring specifically through IRS-2. *Diabetes* 51: 966–976, 2002

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AdV-GFP, GFP-expressing adenovirus; EGF, epidermal growth factor; EGFR, EGF receptor; Erk, extracellular-regulated kinase; GSK, glycogen synthase kinase; IGF-1, insulin-like growth factor-1; IRS, insulin receptor substrate; MEK, Erk kinase; mTOR, mammalian target of rapamycin; p70^{S6K}, 70-kDa S6 protein kinase; PDK, 3-phosphoinositide-dependent kinase; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; SH2, *src* homology 2; Shc, *Src* homologous and collagen-like protein; TGF, transforming growth factor.

Pancreatic β -cells are the only cells of the body that produce and secrete insulin in a manner finely controlled by blood glucose concentrations. Diabetes is characterized by uncontrolled hyperglycemia and is linked to β -cell loss and dysfunction, resulting in an insulin-deficient state. Type 1 diabetes manifests from the autoimmune destruction of β -cells, and in type 2 diabetes, β -cell mass fails to expand to compensate for the accompanying peripheral insulin resistance (1,2). Therefore, β -cell survival and proliferation play a pivotal role in preventing the pathogenesis of diabetes. Thus, it is of interest to identify those growth factors and the key elements of their mitogenic and/or survival signal transduction pathways so that they may be used for in vitro propagation of β -cells (for use in β -cell replacement therapy) or pharmacologically maintaining compensatory increases of β -cell mass in vivo (3).

Growth of β -cells can take place by at least two pathways: replication of differentiated β -cells or neogenesis (4). Either process occurs during neonatal development of the pancreas. However, in adulthood β -cells expanding via mitogenesis make up only 0.5–6% of the total β -cell population, which is primarily the result of replication of differentiated pre-existing β -cells (5–7). Nutrients (such as glucose) and certain growth factors (including IGF-1 and somatotrophs [8–11]) will induce β -cell proliferation and provide significant increases in β -cell mass, as observed during pregnancy (12) and obesity without diabetes (3,13–15).

IGF-1, prolactin, and growth hormone stimulate primary β -cell growth in a glucose-dependent manner (3,5,6,10,11,16). Early events in the signal transduction pathways leading to mitogenesis have recently been characterized in INS-1 β -cells (3,10,11,17). In the pancreatic β -cell, IGF-1 action is glucose dependent (10,11). Stimulatory glucose concentrations can slightly increase endogenous insulin receptor substrate (IRS)-1 and -2 tyrosine phosphorylation independently in the β -cell, but there is a marked potentiation of glucose on IGF-1–induced IRS-1 and -2 tyrosine phosphorylation (10,11). IRS proteins provide cytosolic docking sites for *src* homology 2 (SH2) domain-containing signaling proteins, such as the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (10,11). IGF-1 and glucose increase IRS-mediated PI3K activity (17), and this is required for glucose-, glucose/IGF-1–

dependent-, and growth hormone-stimulated- β -cell proliferation (11). However, despite this pattern of PI3K activation in β -cells, downstream IGF-1 activation of protein kinase B (PKB) occurs independently of glucose, and glucose does not activate PKB (18). Downstream of PKB, unlike IGF-1, glucose does not appreciatively phosphorylate glycogen synthase kinase (GSK)-3. Nonetheless, glucose does cause phosphorylation of p70^{S6K} (70-kDa S6 protein kinase), which is augmented by IGF-1 and occurs via direct upregulation of mammalian target of rapamycin (mTOR) (18,19). IRSs also engage Grb2 (growth factor receptor-bound-2) and/or Shc (Src homologous and collagen-like) proteins, leading to the activation of the Ras/Raf/Erk pathway, which contributes to glucose-dependent IGF-1-stimulated β -cell proliferation (11). Additionally, glucose has been shown to induce activation of extracellular-regulated kinase (Erk)-1/2 independently of tyrosine-phosphorylated IRS, which, together with glucose-induced activation of mTOR/p70^{S6K} (18), also significantly contributes to the glucose-dependent aspect of IGF-1/growth hormone-induced β -cell proliferation (19–21).

Transforming growth factor (TGF)- α and epidermal growth factor (EGF) primarily bind to Erb1, a member of the EGF receptor (EGFR) family, which also includes Erb2, Erb3, and Erb4 (22). Similar to stimulation of the IGF-1R, binding of TGF- α /EGF to the EGFR causes homodimerization and transphosphorylation of the receptor with the appearance of newly created phosphotyrosine binding domains, with which SH2-containing proteins can interact (23,24). Stimulation of the EGFR leads to the activation of various downstream targets including Erk1/2 (25) and PI3K (26) in some pancreatic tumor cell lines. Erk1/2 activation occurs via protein-complex formation of the EGFR with the Grb2/mSOS (mammalian Son of Sevenless) complex and subsequent activation of the Ras/Raf-1/MEK signaling pathway (27,28). TGF- α /EGF-induced stimulation of PI3K can occur via direct complex formation of tyrosine-phosphorylated sites on the activated EGFR and the SH-2 domains of the p85 regulatory subunit of PI3K, which may be further enhanced by the additional presence of Gab1 or p120^{cb1} (29,30).

TGF- α and EGF have been associated with pancreatic endocrine cell development and pancreatic exocrine tumor cell growth (31–33), but it is unclear whether they can induce mitogenesis in differentiated β -cells. It is possible that differences of pancreatic cell populations in responding to these stimuli may be related to the differential expression patterns of Erb receptor types (34–36). In the present study, the signal transduction and proliferative action of TGF- α and EGF were compared in parallel with those of glucose and IGF-1 in the glucose-responsive β -cell line, INS-1. It was found that only those growth factors that could maintain activation of signal transduction (>20 min) via recruitment of specific docking proteins, especially IRS-2, were able to induce β -cell mitogenesis.

RESEARCH DESIGN AND METHODS

Materials. The [*methyl*-³H]thymidine (25 Ci/mmol) was from Amersham (Piscataway, NJ). Antibodies to phosphorylated Erk1/2 were purchased from Promega (Madison, WI); antibodies to PKB total, phosphorylated (Ser⁴⁷³ and Thr³⁰⁸) PKB and phosphorylated GSK-3 were purchased from New England Biolabs (Beverly, MA); antibodies to GSK-3 total and EGFR were purchased from Santa Cruz (Santa Cruz, CA); and antibody to the p85 subunit of PI3K

was purchased from Upstate (Lake Placid, NY). The total Erk antiserum was a gift from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX), and the p70^{S6K} and IRS-1 and -2 antisera and cDNA were gifts from Drs. M. White and M. Myers (Joslin Diabetes Center, Boston, MA). The activity assay for PKB was purchased from New England Biolabs, human recombinant IGF-1 from Gropep (Adelaide, Australia), and murine EGF from Sigma (St. Louis, MO). Purified human recombinant TGF- α was prepared by Stem Cell Pharmaceuticals (Seattle, WA). Protein kinase/phosphatase inhibitors were purchased from Calbiochem (La Jolla, CA). All other biochemicals were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

Cell culture. The glucose-sensitive pancreatic β -cell line INS-1 (37) was used in these experiments. INS-1 cells were maintained in RPMI 1640 medium containing 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 μ mol/l β -mercaptoethanol, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10% FCS, and 11.2 mmol/l glucose and incubated at 37°C, 5% CO₂ as described (37). Under starvation conditions (before beginning experimental treatment), cells were incubated in RPMI 1640 medium containing 0.1% fatty acid-free BSA, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.5 mmol/l glucose and incubated as before. In all experiments, INS-1 cells utilized for controls were incubated with starvation media during treatment periods. Cells were subcultured at 80% confluence.

Adenoviral vector construction and infection. IRS-1 and -2 recombinant adenoviruses were prepared as previously described (38). Essentially, the cDNA encoding human IRS-1 or mouse IRS-2 was introduced into the shuttle vector pACCMVS, and homologous recombination was carried out in HEK293 cells that contain the transcription factor, E1A, which is required for viral replication. The generated recombinant adenovirus was amplified and purified from lysed 293 cells and utilized for infection of β -cells (38). Purified virus (5×10^9 pfu/10⁶ cells) was added to INS-1 cells and incubated 2 h. Cells were washed and fresh media was added. After a 24-h incubation, cells were trypsinized and plated onto 96-well plates for [³H]thymidine incorporation assay.

[³H]thymidine incorporation. Incorporation of [³H]thymidine was used as an indicator of DNA synthesis and INS-1 cell proliferation and was carried out as described previously (10,11). Briefly, INS-1 cells were cultured on 96-well plates (1×10^4 cells/well), starved for 24 h, and treated with various growth factors with or without glucose (0.5–15 mmol/l) for 24 h. For the last 4 h of treatment, 1 μ Ci [³H]thymidine/well was added. The [³H]thymidine specifically incorporated into the INS-1 cell DNA was trapped on glass fiber filters and counted by liquid scintillation counting.

Immunoblot analysis. INS-1 cells were cultured on 6-well plates to 80% confluence. Cells were starved (RPMI 1640 without serum and only 0.5 mmol/l glucose) for 24 h before treatment. Quiescent cells were treated with growth factors in the absence or presence of increasing glucose concentrations for the indicated time periods. Cells were washed once with ice-cold PBS and lysed in buffer containing 50 mmol/l HEPES (pH 7.5), 1% Triton X-100, 2 mmol/l sodium vanadate, 50 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 4 mmol/l EDTA, 10 μ mol/l leupeptin, 10 μ g/ml aprotinin, and 100 μ mol/l phenylmethylsulfonyl fluoride.

Immunoblot analysis of mitogenic signal transduction protein expression and protein tyrosine phosphorylation was performed as described previously, using horseradish peroxidase-based chemiluminescence reaction as a secondary detection method (39,40).

Coimmunoprecipitations. Cells were treated as above but grown on 15-cm plates. Lysate (1.5 ml) containing ~2.0 mg total protein was immunoprecipitated with 5 μ l antibody against either p85 or EGFR for 16 h at 4°C. The immune complexes were collected with 50 μ l protein A-Sepharose for 1 h at 4°C, washed three times with lysis buffer, and resuspended in 40 μ l of 2 \times SDS gel loading buffer. Samples were loaded onto a 7.5% SDS-PAGE gel, and coimmunoprecipitated proteins were detected via immunoblot analysis.

PKB activity. Cells were treated as above. Harvested protein (30 μ g) was added to 100 μ l PKB lysis buffer containing 20 μ l anti-PKB conjugated to agarose beads (New England Biolabs) and incubated on a rocker at 4°C for 2 h. PKB activity was measured as described in the manufacturer's directions for the PKB/Akt Kinase Assay Kit (New England Biolabs).

Other procedures. Protein assay was performed using the bicinchoninic acid method (Pierce, Rockford, IL). Data are presented as means \pm SE. Statistically significant differences between groups were analyzed using Student's *t* test, where *P* < 0.05 was considered statistically significant.

RESULTS

Glucose, IGF-1, TGF- α , and EGF increased Erk1/2 phosphorylation in β -cells. Experiments were conducted to define the signaling kinase cascades activated by

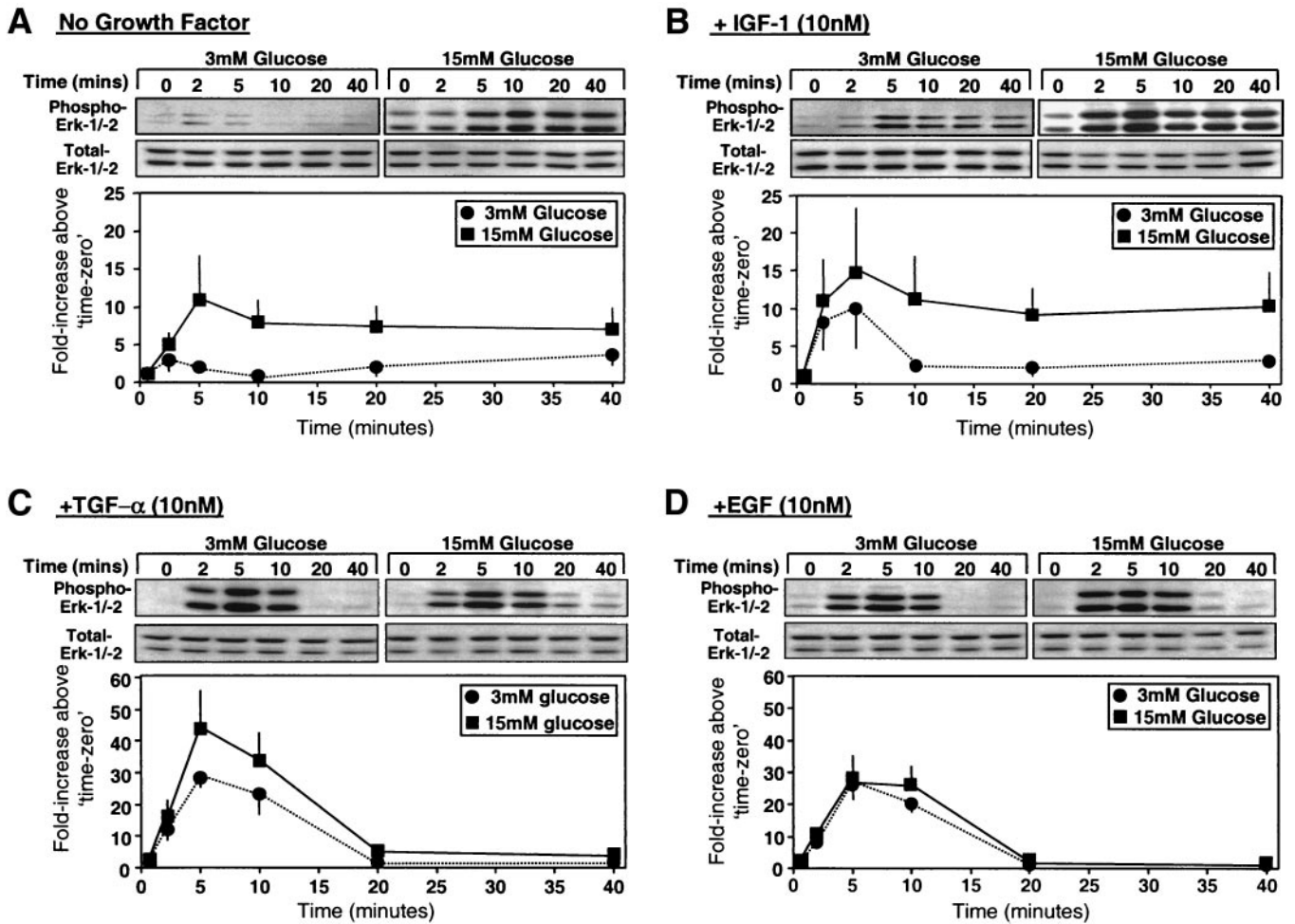


FIG. 1. Glucose- and glucose/IGF-1-dependent Erk1/2 phosphorylation is maintained, but not that induced by TGF- α /EGF. Erk1/2 phosphorylation was examined in INS-1 cells (80% confluent 3.5-cm dish) after 0, 2, 5, 10, 20, and 40 min treatment with basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone (A) or in the additional presence of 10 nmol/l IGF-1 (B), 10 nmol/l TGF- α (C), or 10 nmol/l EGF (D). Lysates were prepared as described in RESEARCH DESIGN AND METHODS and subjected to immunoblot analyses with antibodies directed against phosphorylated Erk1 or total Erk1/2. Example immunoblots for phospho-Erk1/2 and total Erk1/2 are shown. Quantification of the phosphorylation of both Erk1 (44 kDa) and Erk2 (42 kDa) was performed by densitometric scanning with Optiquant software analysis. The data are expressed as a means \pm SE ($n \geq 3$) of the fold-increase above time zero.

TGF- α or EGF in INS-1 cells. In these experiments, the kinetics of activation were examined and compared with those signaling responses initiated by glucose and IGF-1. In preliminary experiments, it was found that over a concentration range between 0.1 and 100 nmol/l TGF- α , EGF, or IGF-1, maximum Erk1/2 phosphorylation was attained between 1 and 10 nmol/l for any of these growth factors in INS-1 cells incubated for 10 min at 15 mmol/l glucose (data not shown). This is comparable to that previously found for IGF-1-induced [3 H]thymidine incorporation in INS-1 cells (11). In all of the following experiments, a 10-nmol/l concentration of growth factor was used in order to elicit a marked response. The time course of Erk1/2 phosphorylation was examined after 0, 2, 5, 10, 20 and 40 min in INS-1 cells treated with either basal (3 mmol/l) or stimulatory (15 mmol/l) glucose concentrations (Fig. 1A) alone or in the additional presence of 10 nmol/l IGF-1 (Fig. 1B), 10 nmol/l TGF- α (Fig. 1C), or 10 nmol/l EGF (Fig. 1D). Quantitative analyses of Western blots are shown in Fig. 1A–D, and data are expressed as the fold-increase above “time zero.” Glucose (15 mmol/l), in the absence of growth factors, significantly stimulated

Erk1/2 phosphorylation ≥ 10 -fold by 5 min ($P \leq 0.01$) (Fig. 1A). The effect of glucose was greatest at 5 min, and a ≥ 5 -fold effect was still readily apparent at 40 min ($P \leq 0.05$, Fig. 1A). IGF-1 potentiated Erk1/2 phosphorylation at 15 mmol/l glucose, which also peaked after 5 min (15-fold; $P \leq 0.01$) and was maintained as long as 40 min (≥ 10 -fold; $P \leq 0.01$, Fig. 1B). In contrast to the magnitude of response observed with glucose and IGF-1, TGF- α and EGF induced a much more marked increase in Erk1/2 phosphorylation/activation in INS-1 cells. Erk1/2 activation reached a maximum at 5 min, but was >40 -fold for TGF- α and >25 -fold for EGF ($P \leq 0.01$, Fig. 1C and D). Also, unlike that induced by glucose or IGF-1, phosphorylation of Erk1/2 induced by TGF- α and EGF was transient and diminished after 20 min. The increase in phosphorylation, which is still present with TGF- α /EGF plus 15 mmol/l glucose at 40 min, is equivalent to that produced by 15 mmol/l glucose in the absence of growth factor. Unlike IGF-1, TGF- α /EGF-induced Erk1/2 phosphorylation was independent of glucose concentration. In these experiments, total Erk1/2 remained unchanged under any of the conditions (Fig. 1A–D).

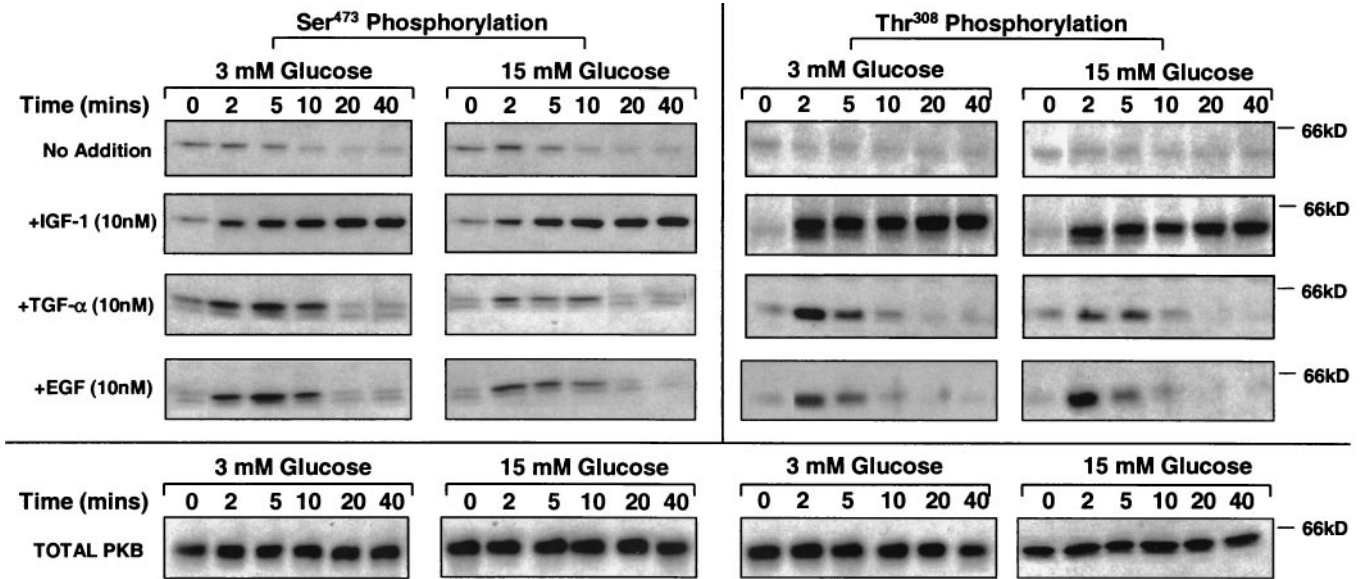


FIG. 2. IGF-1 induced a sustained glucose-independent increase in phosphorylation of PKB, whereas TGF- α /EGF induced only a transient glucose-independent response. PKB phosphorylation was examined in INS-1 cells (80% confluent 3.5-cm dish) after 0, 2, 5, 10, 20, and 40 min of treatment with basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF. Lysates were prepared as described in RESEARCH DESIGN AND METHODS and were subjected to immunoblot analyses with antibodies directed against the phosphorylated Ser⁴⁷³ of PKB in the left panel or the phosphorylated Thr³⁰⁸ of PKB in the right panel. Representative immunoblots from three different experiments are shown. In the bottom panel, an example immunoblot of total PKB is shown indicating that its levels essentially remained unchanged during the course of the experiment.

IGF-1, TGF- α , and EGF increased PKB phosphorylation/activity in β -cells. Glucose and IGF-1 have been shown to stimulate PI3K activity in INS-1 cells (17). PKB is activated upon binding to PI3K-generated lipid products in the plasma membrane via its pleckstrin homology domain and subsequent phosphorylation of Thr³⁰⁸ by 3-phosphoinositide-dependent kinase (PDK-1), followed by a second phosphorylation at Ser⁴⁷³, which may be attributed to autophosphorylation (41,42). The time course of phosphorylation of PKB was evaluated, utilizing Thr³⁰⁸ and Ser⁴⁷³ phosphospecific antibodies, in INS-1 cells incubated 0, 2, 5, 10, 20 and 40 min with 3 and 15 mmol/l glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF (Fig. 2). Glucose alone did not significantly increase PKB phosphorylation as observed previously (18), and appeared slightly lower than that at time zero at either glucose concentration (Fig. 2). Phosphorylation of PKB at both the Ser⁴⁷³ and Thr³⁰⁸ sites induced by IGF-1 occurred rapidly (≤ 2 min) and was maintained ≥ 40 min (Fig. 2). The magnitude of PKB phosphorylation induced by IGF-1 was unchanged with time and was independent of glucose concentration (Fig. 2). TGF- α and EGF also induced a marked increase in PKB phosphorylation at both the Ser⁴⁷³ and Thr³⁰⁸ sites. However, this response to TGF- α and EGF was transient, peaking between 2 and 5 min but back to basal levels by 20 min (Fig. 2). In these experiments, total PKB remained unchanged under all conditions (Fig. 2).

PKB activity was simultaneously examined in PKB immunoprecipitates of INS-1 cells and correlated with the phosphorylation response observed (Figs. 2 and 3). Glucose did not significantly affect PKB activity between 2 and 40 min of treatment (Fig. 3A). However, glucose did modestly increase PKB activity (twofold) after 2 h (Fig. 3A). IGF-1 increased PKB activity (Fig. 3B) in parallel with PKB phosphorylation state (Fig. 2). The IGF-1-induced

increase was twofold ($P \leq 0.05$) between 2 and 40 min of treatment and further increased (four- to fivefold, $P \leq 0.05$), independently of glucose, after 2 h (Fig. 3B). PKB activity was increased nearly eightfold in the presence of TGF- α and EGF irrespective of glucose concentration after 10 min of treatment ($P \leq 0.01$, Fig. 3C and D). However, as was observed with PKB phosphorylation induced by TGF- α and EGF (Fig. 2), PKB activity diminished rapidly within 20 min and was negligible after 2 h, with only the slight twofold glucose-induced increase in PKB activity still present (Fig. 3C and D).

IGF-1, TGF- α , and EGF, but not glucose, increased GSK-3 phosphorylation in β -cells in parallel to PKB activation. GSK-3 is a constitutively active kinase that is inactivated in response to insulin and IGF-1 by phosphorylation, as a downstream target of PKB (43). The time course of GSK-3 phosphorylation was examined after 0, 2, 5, 10, 20 and 40 min in INS-1 cells treated with either basal (3 mmol/l) or stimulatory (15 mmol/l) glucose concentrations (Fig. 4A) alone or in the additional presence of 10 nmol/l IGF-1 (Fig. 4B), 10 nmol/l TGF- α (Fig. 4C), or 10 nmol/l EGF (Fig. 4D). Quantitative analyses of Western blots are shown in Fig. 4A–D, and data are expressed as the fold-increase above time zero. Glucose, in the absence of growth factors, did not significantly increase GSK-3 phosphorylation between 2 and 40 min, as previously found (18) (Fig. 4A). IGF-1 rapidly increased levels of phosphorylated GSK-3 (threefold, $P \leq 0.05$), and this response was also maintained at 40 min (Fig. 4B, $P \leq 0.05$). IGF-1-induced GSK-3 phosphorylation correlated with IGF-1-induced PKB phosphorylation and was independent of glucose concentration (Figs. 2 and 4). The phosphorylation of GSK-3 was markedly increased by TGF- α and EGF, peaking at 10 min of treatment (≥ 10 -fold, $P \leq 0.05$; Fig. 4C and D). As for TGF- α - and EGF-induced PKB phosphorylation (Fig. 3C and D), TGF- α - and EGF-

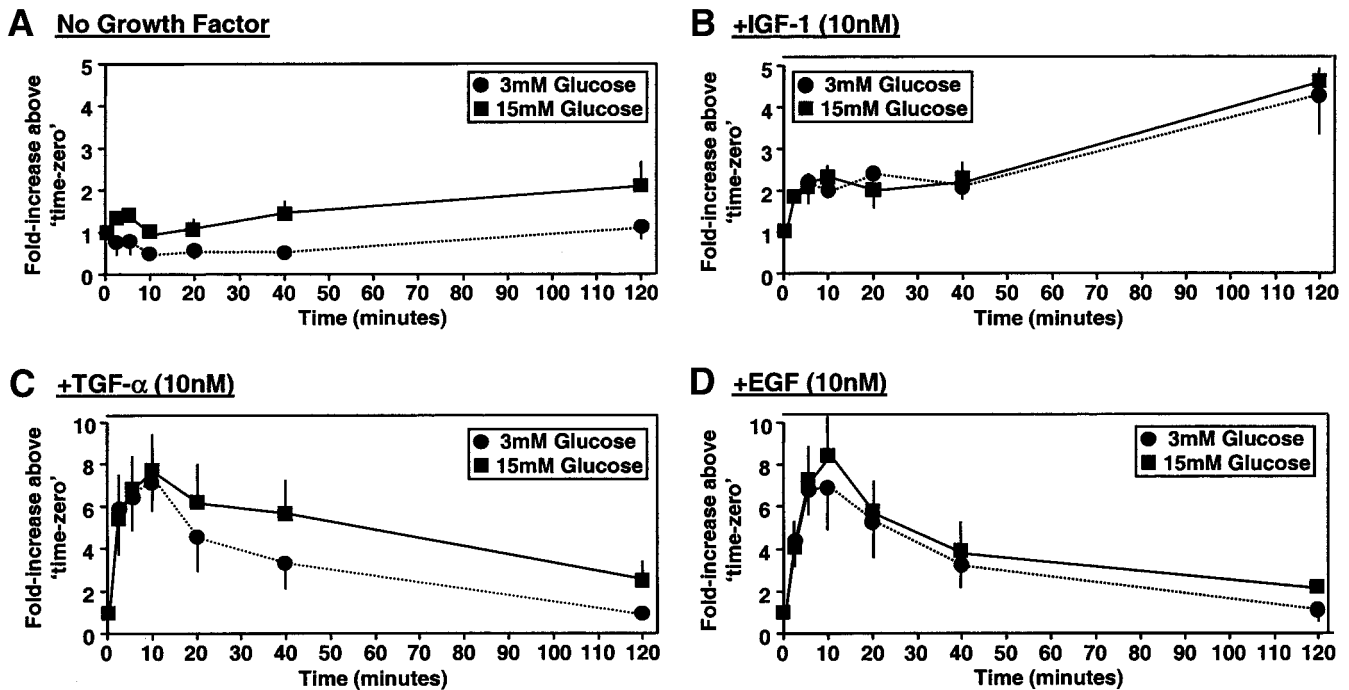


FIG. 3. IGF-1 induced a sustained glucose-independent increase in activity of PKB, whereas TGF- α /EGF induced only a transient glucose-independent response. PKB activity was examined in INS-1 cells (80% confluent 3.5-cm dish) after 0, 2, 5, 10, 20, 40, and 120 min of treatment with basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone (A) or in the additional presence of 10 nmol/l IGF-1 (B), 10 nmol/l TGF- α (C), or 10 nmol/l EGF (D). Lysates were prepared as described in RESEARCH DESIGN AND METHODS and subjected to immunoprecipitation with an antibody directed against PKB. Quantification of the PKB in vitro assay substrate, phosphorylated GSK-3, was performed by densitometric scanning with Optiquant software analysis. The data are expressed as a means \pm SE ($n \geq 3$) of the fold-increase above time zero.

stimulated GSK-3 phosphorylation quickly fell to control levels by 20 min of treatment (Fig. 4C and D).

TGF- α and EGF did not increase phosphorylation of p70^{S6K} in β -cells. p70^{S6K} activation has been proposed to lie downstream of PKB and mTOR (44). However, glucose- and amino acid-induced p70^{S6K} activation has been shown to be independent of PKB stimulation, involving an alternative route mediated directly via mTOR activation (19,45). p70^{S6K} is phosphorylated at multiple sites, which can be determined from analysis of its decrease in electrophoretic migration in a SDS-polyacrylamide gel (44,46). Phosphorylation of p70^{S6K} was evaluated in INS-1 cells at 5, 40, and 120 min incubated with 3, 6, and 15 mmol/l glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF (Fig. 5). Control cells were treated with starvation media containing 0.5 mmol/l glucose for the indicated time periods. As demonstrated previously, glucose increased the phosphorylation state of p70^{S6K}, which was further increased by the addition of IGF-1 (Fig. 5) (10). However, this increase was delayed in onset, so that a significant increase in phosphorylation of p70^{S6K} was not observed until after 40 min, but thereafter was maintained ≥ 2 h (Fig. 5). In contrast to IGF-1, TGF- α and EGF did not enhance glucose-dependent phosphorylation of p70^{S6K} (Fig. 5) and had no effect on the phosphorylation of p70^{S6K} in the absence of glucose.

Glucose and IGF-1-stimulated signaling responses, but not those of TGF- α and EGF, are maintained ≥ 2 h. Because stimulatory glucose and IGF-1 appeared to maintain activation of certain signaling molecules >40 min and the activation of p70^{S6K} and PKB was delayed in onset in response to glucose, experiments examining phosphorylation/activation of signaling proteins were extended to

2 h. After 2 h of treatment, Erk1/2 phosphorylation remained increased at stimulatory glucose concentrations independently of growth factor presence (Fig. 6). The effect of IGF-1 on Erk1/2 phosphorylation was glucose dependent and maintained for 2 h (Fig. 6). IGF-1-stimulated PKB phosphorylation (at both Ser⁴⁷³ and Thr³⁰⁸) was also maintained up to 2 h, and as observed previously (18), was independent of glucose concentration (Fig. 6). In contrast to short-term incubation (<1 h), 15 mmol/l glucose slightly increased phosphorylation of PKB at Ser⁴⁷³ and Thr³⁰⁸ (Fig. 6), which correlated with increased activity of PKB (Fig. 3A). Likewise, GSK-3 phosphorylation followed that of PKB phosphorylation/activity, including a slight increase after 2 h of exposure to 15 mmol/l glucose (Fig. 6). We have also found that the glucose-dependent IGF-1-induced phosphorylation of Erk1/2 and p70^{S6K}, as well as glucose-independent IGF-1-induced phosphorylation of PKB and GSK-3, was maintained up to 24 h (data not shown). TGF- α and EGF did not affect Erk1/2, PKB, or GSK-3 phosphorylation at 2 h (Fig. 6), characteristic of their transient activity observed in β -cells. Only an effect attributed to 15 mmol/l glucose could be observed after 2 h in the presence of TGF- α or EGF.

TGF- α /EGF does not increase pancreatic β -cell proliferation. [³H]thymidine incorporation was measured in quiescent INS-1 cells treated 24 h with 3 and 15 mmol/l glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF (Fig. 7). Control cells were treated with starvation media containing 0.5 mmol/l glucose and data are expressed as the fold-increase above control. TGF- α /EGF did not significantly enhance β -cell proliferation (Fig. 7). Moreover, neither TGF- α nor EGF potentiated the proliferative response occurring with

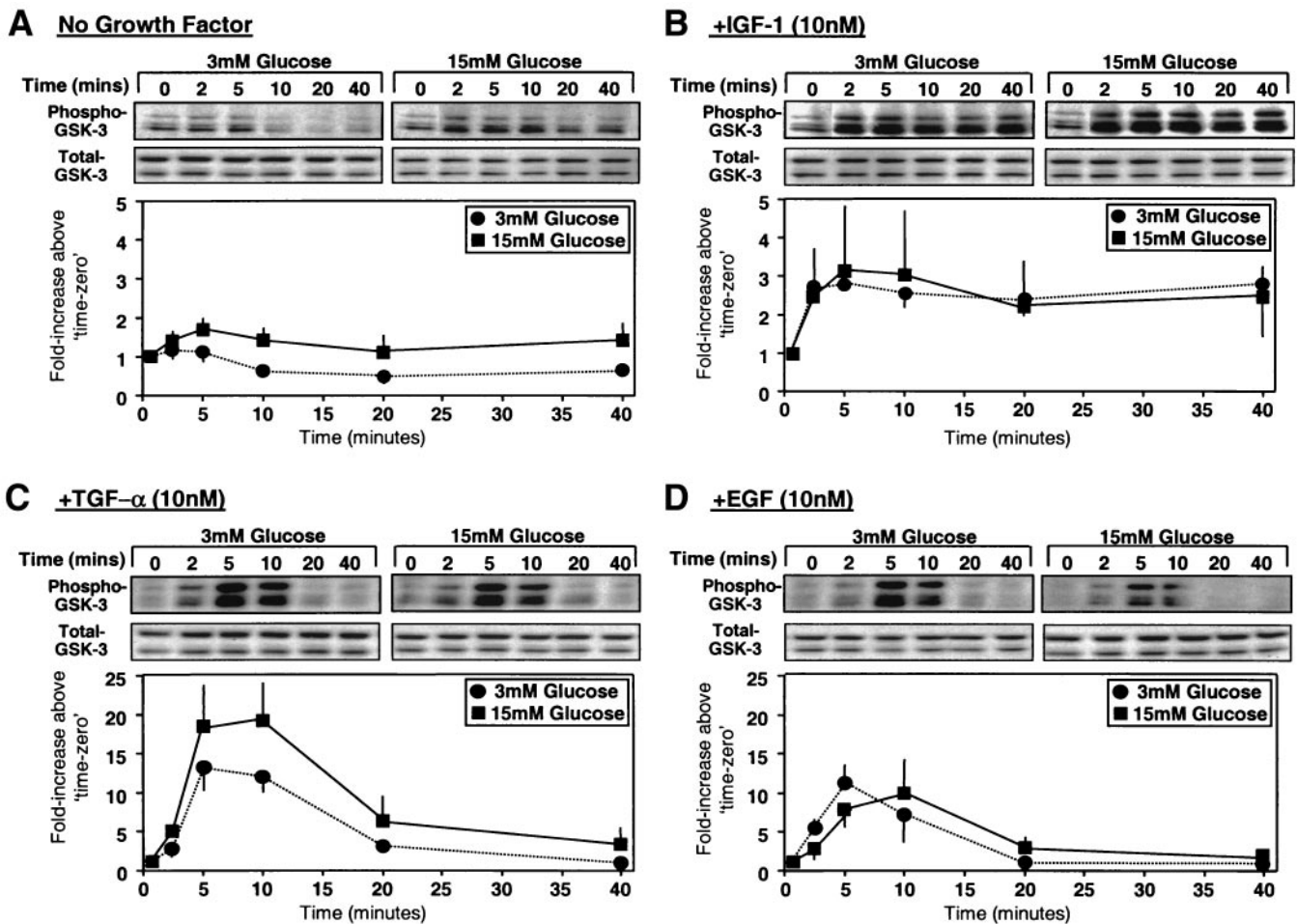


FIG. 4. IGF-1, but not glucose, induced a sustained phosphorylation of GSK-3, whereas TGF- α /EGF induced only a transient glucose-independent response. GSK-3 phosphorylation was examined in INS-1 cells (80% confluent 3.5-cm dish) after 0, 2, 5, 10, 20, and 40 min of treatment with basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone (A) or in the additional presence of 10 nmol/l IGF-1 (B), 10 nmol/l TGF- α (C), or 10 nmol/l EGF (D). Lysates were prepared as described in RESEARCH DESIGN AND METHODS and were subjected to immunoblot analyses with antibodies directed against phosphorylated GSK-3 or total GSK-3. Example immunoblots for phosphorylated and total GSK-3 are shown. Quantification of phosphorylated GSK-3 was performed by densitometric scanning with Optiquant software analysis. The data are expressed as a means \pm SE ($n \geq 3$) of the fold-increase above time zero.

15 mmol/l glucose (~ 20 -fold over basal, $P < 0.05$) (Fig. 7). However, in contrast to TGF- α /EGF, IGF-1 in the presence of high glucose concentrations (15 mmol/l) markedly increased INS-1 cell proliferation (>60 -fold, $P < 0.05$) (Fig. 7) as previously observed (10,11). The effect of IGF-1 on β -cell proliferation was glucose-dependent (10,11). In experiments in which IGF-1 was added to TGF- α and EGF, there was no synergistic effect observed on β -cell proliferation at permissive glucose concentrations, unlike that

found with the combination of IGF-1 and growth hormone (data not shown) (10).

TGF- α /EGF did not signal via IRS-1 or IRS-2. The differences in duration between TGF- α - and EGF-stimulated signaling events and those induced by IGF-1 and glucose might have been due to differential activation of early signal elements immediately downstream of the growth factor receptor. IRS-1 and -2 are cytosolic docking proteins, observed to bind to the activated, ligand-bound

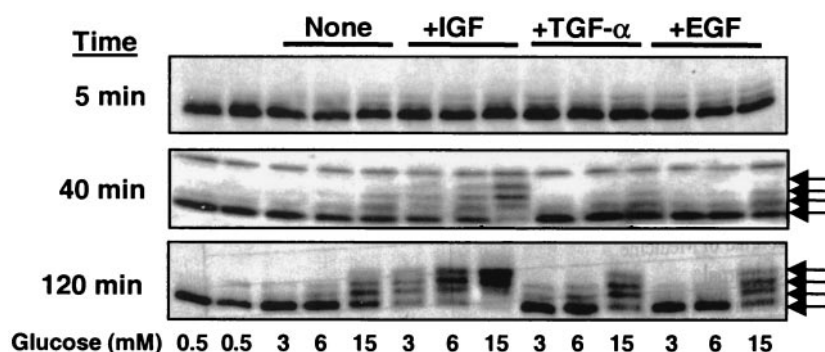


FIG. 5. Stimulated p70^{S6K} phosphorylation was dependent on glucose concentration and enhanced by the presence of IGF-1, but not TGF- α or EGF. INS-1 cells (80% confluent 3.5-cm dish) were stimulated with increasing glucose concentrations (3, 6, and 15 mmol/l) in either the absence of growth factor or in the presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF as indicated for 5, 40, or 120 min. Control cells were treated with starvation media containing 0.5 mmol/l glucose for the indicated time periods. Lysates were subjected to immunoblot analysis with an antibody directed against p70^{S6K}. Increased p70^{S6K} phosphorylation is represented by the decreased migration of the protein in a 10% SDS-PAGE gel. The arrows indicate the four detectable migrating phosphorylated forms of p70^{S6K}. Immunoblots are representative from three different experiments.

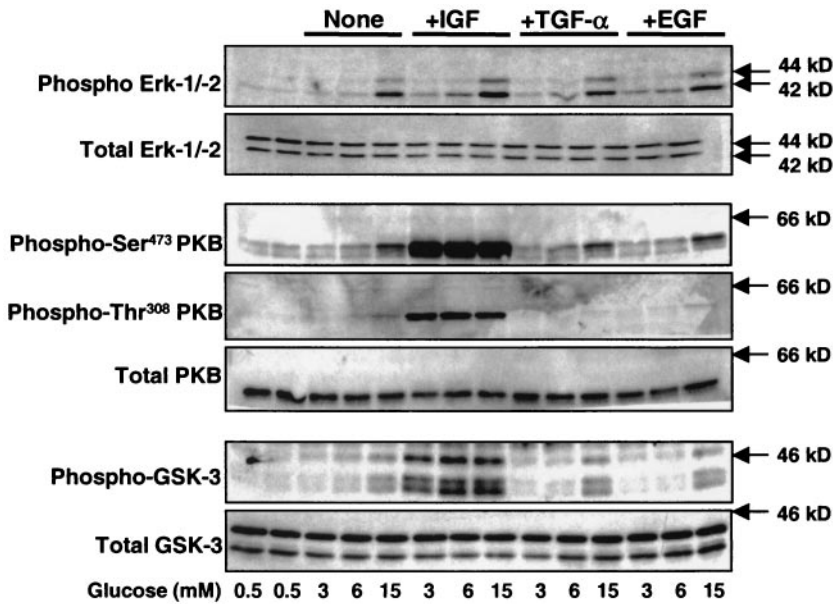


FIG. 6. Effect of glucose, IGF-1, TGF- α , and EGF on Erk1/2, PKB, and GSK-3 phosphorylation after a 2-h incubation. INS-1 cells (80% confluent 3.5-cm dish) were stimulated with increasing glucose concentrations (3, 6, and 15 mmol/l) in either the absence of growth factor or in the presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , and 10 nmol/l EGF as indicated for 2 h. Control cells were treated with starvation media containing 0.5 mmol/l glucose. Lysates were subjected to immunoblot analysis with antibodies directed against the total and phosphorylated forms of Erk1/2, PKB, and GSK-3.

IGF-1R when it is stimulated (47) and important in mitogenic responses for β -cell proliferation (3). It was examined whether TGF- α - and EGF-induced Erk1/2 and PKB signal transduction occurred via upstream activation of IRS-1 and -2 in β -cells. INS-1 cells were incubated for 5 min with 3 and 15 mmol/l glucose alone or in the additional

presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF (Fig. 8). The p85 subunit of PI3K was immunoprecipitated from the cell lysates and association with IRS-1 and -2 were measured by subsequent immunoblotting of these p85 immunoprecipitates. IGF-1 increased the association of IRS-1 and -2 with PI3K within 5 min, which was further enhanced at a stimulatory 15 mmol/l glucose concentration (Fig. 8). In parallel experiments in which the EGFR was immunoprecipitated instead of the p85 subunit of PI3K, no association of IRS-1 and -2 was detected during stimulation with TGF- α and EGF (data not shown).

Overexpression of IRS-2 specifically augmented glucose- and IGF-1-induced cell proliferation in INS-1 cells, but not that by TGF- α and EGF. The effect of adenoviral-mediated overexpression of IRS-1 and -2 on β -cell proliferation was investigated. Firstly, INS-1 cells were infected with increasing doses of adenovirus ($5\text{--}100 \times 10^8$ pfu/ 10^6 cells) containing either IRS-1 or -2, and levels of IRS-1 and -2 were evaluated by immunoblot analysis (Fig. 9A). A dose of 50×10^8 pfu, a dose in which both proteins were increased >10 -fold, was chosen for the

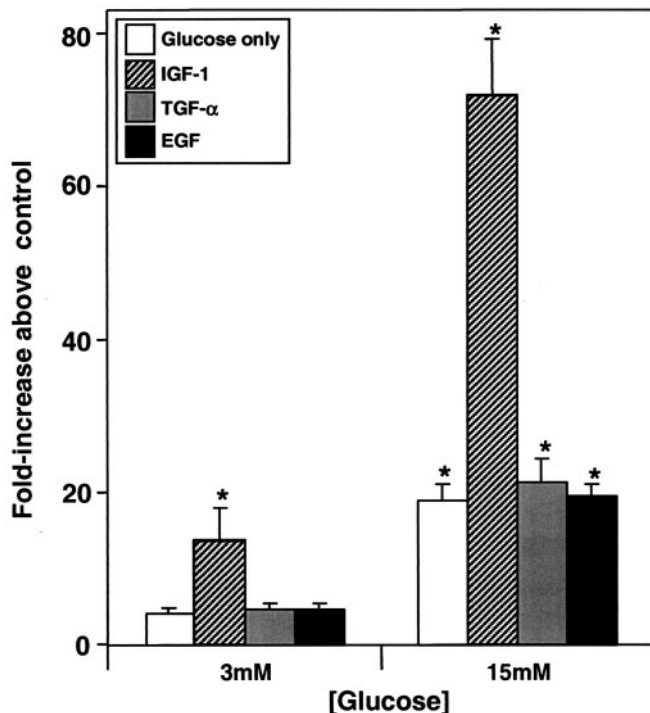


FIG. 7. TGF- α and EGF were ineffective in stimulation of [3 H]thymidine incorporation in INS-1 cells. Cells were plated at 10,000 cells/well and were incubated 24 h in RPMI 1640 medium containing 10% serum. Cells were made quiescent by incubating 24 h with starvation media and were then treated with basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF as indicated. Control cells were treated with starvation media containing 0.5 mmol/l glucose. [3 H]thymidine was added during the last 4 h of treatment, cells were harvested, and [3 H]thymidine incorporated was counted as described in RESEARCH DESIGN AND METHODS. Data are expressed as the fold-increase \pm SE above control ($n \geq 5$). * $P \leq 0.05$.

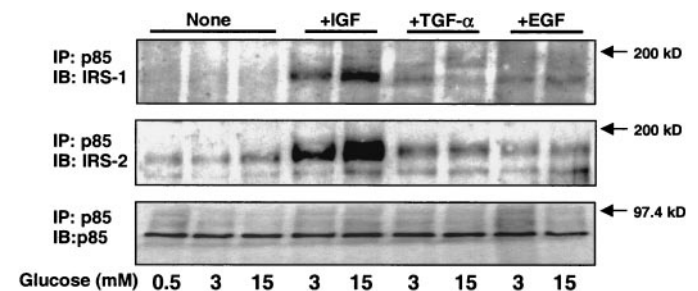


FIG. 8. IGF-1, but not TGF- α or EGF, induced glucose-dependent association of p85 with IRS-1 and -2. IRS-1 and -2 association with p85 was examined in INS-1 cells (80% confluent 10-cm dish) after 5 min of treatment with basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF as indicated. Lysates were subjected to immunoprecipitation with antibodies directed against p85. Immunoprecipitates were subjected to immunoblot analyses with antibodies directed against IRS-1, IRS-2, and p85. An example immunoblot of three experiments is shown for each coimmunoprecipitation analysis.

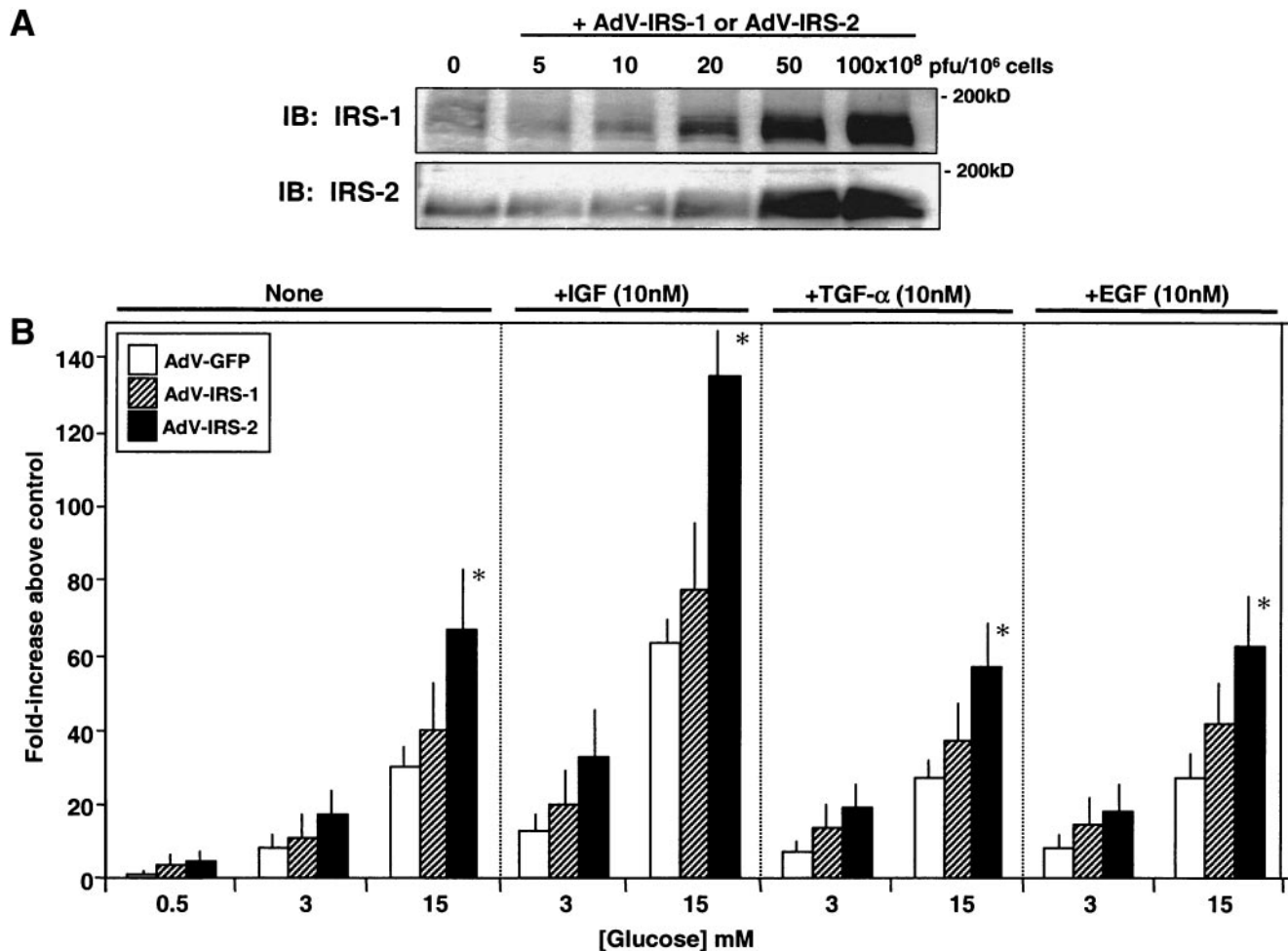


FIG. 9. Adenoviral-mediated overexpression of IRS-2-enhanced glucose- and glucose/IGF-1-dependent [^3H]thymidine incorporation in INS-1 cells. INS-1 cells were infected with increasing doses of adenovirus ($5\text{--}100 \times 10^8$ pfu/ 10^6 cells) containing either IRS-1 (AdV-IRS-1) or IRS-2 (AdV-IRS-2), and levels of IRS-1 and IRS-2 were evaluated by immunoblot analysis and displayed in A. A dose of 50×10^8 pfu was utilized for examining the effects of IRS-1 and -2 on [^3H]thymidine incorporation. AdV-GFP was used as a control for infection. Infected cells were made quiescent by incubating for 24 h with starvation media and were then treated with starvation media (0.5 mmol/l) only or basal (3 mmol/l) or stimulatory (15 mmol/l) glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF as indicated. The data are expressed as the fold-increase \pm SE above control cells (GFP-infected cells treated with starvation media) ($n \geq 5$). *Value significantly greater than the corresponding treatment in AdV-GFP-infected control cell populations; $P \leq 0.05$.

experiments examining their effects on [^3H]thymidine incorporation. An adenovirus that expressed GFP (AdV-GFP) was used as a control for these experiments and also indicated a $>90\%$ gene transfer efficiency mediated by the recombinant adenoviral vector as previously described (38). [^3H]thymidine incorporation was measured as described above in quiescent, infected INS-1 cells treated with 3 and 15 mmol/l glucose alone or in the additional presence of 10 nmol/l IGF-1, 10 nmol/l TGF- α , or 10 nmol/l EGF (Fig. 9B). Control cells were treated with starvation media containing 0.5 mmol/l glucose, and data are expressed as the fold-increase above control. In INS-1 cells overexpressing IRS-2, glucose-dependent proliferation was >60 -fold ($P < 0.05$) and glucose-IGF-1-dependent proliferation was ~ 120 -fold ($P < 0.05$) (Fig. 9B). Thus, IRS-2 overexpression enhanced glucose- and IGF-1-induced β -cell proliferation twofold above that of AdV-GFP-infected cells (Fig. 9B). Unlike IRS-2, IRS-1 did not significantly increase proliferation rates over that of control infected INS-1 cells stimulated with glucose with or without IGF-1 (Fig. 9B). In INS-1 cells overexpressing

either IRS-1 or -2 and treated with TGF- α and EGF, no change in proliferation rate was observed above that already induced by glucose alone.

DISCUSSION

IGF-1 and glucose are mitogenic to pancreatic β -cells (9–11). Glucose-dependent IGF-1 signal transduction via IRS-mediated stimulation of PI3K activity has been shown to be essential for eliciting a mitogenic response in β -cells (10,11). Additionally, Erk1/2 activation and p70^{S6K} stimulation are known to facilitate the mitogenic responses of IGF-1 and glucose (10,11,18). Similar to IGF-1, TGF- α and EGF activated similar signaling pathways, although their magnitude of activation, duration of activity, and lack of glucose-dependency differed from that of IGF-1 in β -cells. A consequence of these differences was that TGF- α and EGF did not enhance INS-1 cell proliferation. In this side by side comparative study, the importance of duration, not magnitude, of the growth factor-induced signaling response appeared to be a significant factor for inducing

β -cell mitogenesis. TGF- α and EGF induced marked signaling responses, including increased Erk1/2 phosphorylation and PKB phosphophorylation and activity, which led to increased GSK-3 phosphorylation but did not progress to p70^{S6K} activation. These responses were much greater in magnitude than that induced by IGF-1, but were transient, lasting no longer than 20 min. Although, IGF-1 produced a comparatively modest increase in Erk1/2 and PI3K/PKB/GSK-3 signaling pathways compared with TGF- α /EGF, the responses were maintained ≥ 2 h and even up to 24 h. The activation of p70^{S6K} required >30 min of stimulation by glucose and IGF-1; therefore, the lack of a mitogenic response associated with TGF- α /EGF treatment is likely the result of the transient nature of the signal.

The dissimilarities in the kinetics and amplitude of activation of signaling pathways associated with TGF- α /EGF or IGF-1 treatment may be the result of differences in protein complex formation of signaling molecules immediately downstream of their receptors, the EGFR and IGF-1R, respectively. Activation of the IGF-1R intrinsic tyrosine kinase activity leads to tyrosine phosphorylation of IRS-1 and/or IRS-2, which via docking of the p85 regulatory subunit of PI3K, causes activation of PI3K and initiation of downstream signaling events (47). We found IGF-1 induced a significant increase in the association of IRS-1 and IRS-2 with p85 in INS-1 cells. This association, though present at 3 mmol/l glucose, was substantially greater in the presence of stimulatory glucose (15 mmol/l), underlying the glucose-dependent aspect of IGF-1-induced mitogenesis in β -cells (11). In contrast, neither TGF- α nor EGF was able to promote p85 recruitment to IRS-1 or -2, indicating that the EGFR does not induce signal transduction via IRS irrespective of glucose concentration. EGFR binding directly to p85, however, is inducible by TGF- α and EGF in INS-1 cells (data not shown). Dissimilar to IGF-1R activation, TGF- α /EGF-induced activation of the EGFR intrinsic tyrosine kinase leads to EGFR autophosphorylation and the recruitment of the p85 subunit of PI3K directly to the EGFR, but might also involve alternative adapter molecules to members of the IRS family (29,30,48). In general, it has been unclear whether EGFR ligands can induce proliferation of differentiated β -cells, perhaps with the exception of betacellulin (34), although they are capable of causing mitogenesis within pancreatic tumors (25,26,31–33) and promote β -cell neogenesis (35). The differences in mitogenic responses of pancreatic cells to these growth factors have been attributed to the presence of certain ErbB receptor subtypes and receptor interactions with certain adapter molecules (22,29,30,34–36). It is possible that the lack of a prolonged signaling response by TGF- α or EGF in INS-1 cells might be due to the absence of a particular ErbB receptor subtype and/or associated adapter molecule. However, other possibilities, such as a more rapid TGF- α /EGF receptor desensitization, perhaps involving decreased TGF- α /EGF receptor recycling and/or efficient phosphotyrosine phosphatase-mediated inactivation, should also be considered; to establish such studies, however, further experimentation is required. Notwithstanding, the data revealed in this study indicate that TGF- α /EGF do not induce a mitogenic response in INS-1 cells (a model of relatively well-differen-

tiated mature β -cells [37]), despite a marked activation of Erk1/2 and PI3K signaling pathways.

The inability of TGF- α /EGF to signal via IRS, unlike IGF-1, could also contribute to the failure to maintain activation of signaling pathways leading to mitogenesis in the β -cell. Indeed, the importance of IRS-2 in particular for initiating signaling events leading to increased β -cell mitogenesis was emphasized in this study in adenoviral-mediated IRS-2 overexpressing INS-1 cells in which glucose- and IGF-1-induced β -cell proliferation was more than doubled, whereas TGF- α /EGF had no effect. Intriguingly, adenoviral-mediated IRS-1 overexpression did not significantly increase glucose or IGF-1-induced β -cell mitogenesis. These data provided further evidence suggesting a specific key role of IRS-2 in the promotion of β -cell growth (3) and are consistent with increased expression of IRS-2 within proliferating β -cells (49). Moreover, these observations are also inversely correlated with the marked reduction in β -cell mass found in IRS-2-deficient mice, but not in IRS-1-deficient mice (14,15,50,51). Therefore, in this study the data suggest that maintained activation of PI3K and Erk1/2 mitogenic signal transduction pathways required for β -cell proliferation is at least in part maintained in β -cells because of growth factor signaling that occurs specifically via IRS-2. However, other factors that associate with IRS-2 (rev. in ref. 52) could also contribute to a stable signaling complex and may also play a role in maintaining a continuous IRS-2-mediated signaling response that leads to increased β -cell proliferation.

In summary, this study further demonstrated the importance of glucose-dependent activation of Erk1/2 and p70^{S6K} signaling elements, but not necessarily PKB, to commit a β -cell to a mitogenic event. Moreover, sustained activation of these mitogenic signal transduction pathways appears to be required to drive β -cell proliferation. Such a prolonged activation could be a consequence of specific signaling via IRS-2. Growth factors, such as TGF- α and EGF, which only induce a transient activation (<20 min) of PI3K and Erk1/2 signal transduction pathways, do not signal via IRS-2 or initiate a stable complex formation capable of signaling to Erk1/2 or PI3K in INS-1 cells, thus failing to activate p70^{S6K} and to induce β -cell proliferation. Therefore, if human β -cells are to be propagated in vitro to increase the number of cells for eventual β -cell replacement therapy of type 1 diabetes, it will be important to use growth factors that maintain activation of signal transduction pathways (particularly those involving IRS-2, Erk1/2, and p70^{S6K}) in cell culturing strategies. Likewise, if the therapeutic goal of maintaining a compensatory increase in β -cell mass to adapt to peripheral insulin resistance is to be considered a viable pharmaceutical strategy to treat type 2 diabetes, such an approach must consider inducing a sustained activation of β -cell mitogenic signal transduction pathway, particularly those mediated via IRS-2.

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