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Insulin-like Growth Factor I (IGF-I)-stimulated Pancreatic β-Cell Growth Is Glucose-dependent

SYNERGISTIC ACTIVATION OF INSULIN RECEPTOR SUBSTRATE-MEDIATED SIGNAL TRANSDUCTION PATHWAYS BY GLUCOSE AND IGF-I IN INS-1 CELLS*

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Nutrients and certain growth factors stimulate pancreatic β-cell mitogenesis, however, the appropriate mitogenic signal transduction pathways have not been defined. In the glucose-sensitive pancreatic β-cell line, INS-1, it was found that glucose (6–18 mM) independently increased INS-1 cell proliferation (>20-fold at 15 mM glucose). Insulin-like growth factor I (IGF-I)-induced INS-1 cell proliferation was glucose-dependent only in the physiologically relevant concentration range (6–18 mM glucose). The combination of IGF-I and glucose was synergistic, increasing INS-1 cell proliferation >50-fold at 15 mM glucose + 10 nM IGF-I. Glucose metabolism and phosphatidylinositol 3′-kinase (PI 3′-kinase) activation were necessary for both glucose and IGF-I-stimulated INS-1 cell proliferation. IGF-I and 15 mM glucose increased tyrosine phosphorylation mediated recruitment of Grb2/mSOS and PI 3′-kinase to IRS-2 and pp60. Glucose and IGF-I also induced Shc association with Grb2/mSOS. Glucose (3–18 mM) and IGF-I, independently of glucose, activated mitogen-activated protein kinase but this did not correlate with IGF-I-induced β-cell proliferation. In contrast, $p70^{S6K}$ was activated with increasing glucose concentration (between 6 and 18 mM), and potentiated by IGF-I in the same glucose concentration range which correlated with INS-1 cell proliferation rate. Thus, glucose and IGF-I-induced β-cell proliferation were mediated via a signaling mechanism that was facilitated by mitogen-activated protein kinase but dependent on IRS-mediated induction of PI 3′-kinase activity and downstream activation of $p70^{S6K}$. The glucose dependence of IGF-I mediated INS-1 cell proliferation emphasizes β-cell signaling mechanisms are rather unique in being tightly linked to glycolytic metabolic flux.

Adult pancreatic β-cells are relatively well differentiated and consequently have a low mitotic index (1, 2). Under normal circumstances the proportion of β-cells undergoing mitosis is about 0.5% of the population of β-cells in a pancreatic islet (1). Nonetheless, β-cell proliferation can be increased by several nutrient factors, such as glucose and amino acids (2). Glucose increases mitogenesis in islet β-cells so that around 5% of the β-cell population are undergoing DNA synthesis (2–4), for which glucose metabolism is required (3). However, the intracellular signaling pathways responsible for glucose-mediated β-cell proliferation, beyond a requirement for glucose metabolism, are not particularly well understood. Nonetheless, it has been postulated that this may be via elevation of intracellular cAMP (2, 5), glucose-mediated activated protein kinase C (6), and/or a Ca^{2+}-dependent activation of the mitogen-activated protein kinase (MAPK)[1] (7, 8).

Pancreatic β-cell proliferation can also be stimulated by several growth factors (2, 9). In particular, somatolactogenic hormones (prolactin and growth hormone (GH)) and insulin-like growth factor I (IGF-I) have been shown to increase the number of replicating β-cells in rodent islets by up to 6% of the islet cell population (2, 10, 11). A prolactin-induced increase in β-cell proliferation is likely associated with an increase in β-cell mass observed during pregnancy (12). GH is perhaps the most potent of peptide growth factors to induce proliferation of differentiated β-cells (10, 13). It has been postulated that GH mediates β-cell growth via local IGF-I production (14), however, it is more likely that GH mediates a direct effect on β-cell proliferation (15) probably via a JAK2/STAT5 signal transduction pathway (16). Likewise, IGF-I stimulates β-cell proliferation independently of GH or prolactin (17), most probably via the IGF-I receptor and subsequent protein tyrosine phosphorylation signal transduction pathway found in other mammalian cell types (17–20). IGF-I binds to the IGF-I-receptor resulting in activation of its intrinsic tyrosine kinase activity that in turn tyrosine phosphorylates members of the insulin receptor substrate (IRS) family (18–20). Tyrosine-phosphorylated IRS is then able to recruit the 85-kDa regulatory subunit of phosphatidylinositol 3′-kinase (PI 3′-kinase) via its SH2 domain, leading to activation of the enzyme (17–19). This then leads to PI 3′-kinase-dependent downstream activation of the 70-kDa S6 kinase ($p70^{S6K}$) (21). IGF-I-mediated tyrosine phosphorylation of IRS also engages the bridging molecule growth factor receptor-bound protein-2 (Grb-2) via its SH2 domain to phosphotyrosine site on IRS (17–19). This results in an increased binding of IRS-docked Grb-2 to the murine Son-of-Sevenless 1

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; Grb-2, growth factor bound protein 2; MEK, MAP-kinase kinase; IGF-I, insulin-like growth factor I; IRS, insulin receptor substrate; PI 3′-kinase, phosphatidylinositol 3-kinase; $p70^{S6K}$, 70-kDa S6 kinase; Shc, SH2-containing protein; mSOS, murine sons of sevenless-1 protein; BSA, bovine serum albumin; GH, growth hormone; PKA, protein kinase A.

protein (mSOS), a guanine nucleotide exchange factor which converts inactive Ras-GDP into active Ras-GTP (17–19). Activated GTP-bound Ras then recruits the Raf serine kinase that phosphorylates MAP kinase kinase (MEK), resulting in MEK-mediated phosphorylation activation of the MAPK (erk-1 and -2 isoforms) (18, 19, 22). Furthermore, IGF can also activate the Ras/MAPK branch of the pathway independently of IRS, via IGF-I receptor kinase tyrosine phosphorylation of the SH2-containing protein (Shc) which then directly binds Grb-2/mSOS resulting activation of Ras/MAPK (17–20). Notwithstanding, by which ever signal transduction pathway it is mediated, activation of MAPK and $p70^{S6K}$ are known to be a requirement for induction of a mitogenesis in most mammalian cell types (17–20).

In the pancreatic β-cell, although several peptide growth factors and nutrients have been shown to increase β-cell proliferation (2), the intracellular signal transduction pathway(s) involved in induction of β-cell mitogenesis have not been well defined. Moreover, the regulation of β-cell growth may actually be uniquely different from other cell types, since β-cell function is exquisitely related to its metabolic state (23). In these studies we have used the β-cell line INS-1 as a model to better characterize glucose and IGF-I-mediated signal transduction pathways that specially influence β-cell proliferation. Compared with other β-cell lines, despite a higher mitotic index than primary β-cells, INS-1 cells are relatively well differentiated and respond to glucose in terms of insulin secretion in a physiologically relevant glucose concentration range (24).

EXPERIMENTAL PROCEDURES

Materials—The [*methyl*-^{3}H]thymidine (20 Ci/mmol) was from NEN Life Science Products Inc. (Boston, MA). Anti-"active-MAPK" antiserum was purchased from Promega Corp. (Madison, WI), the "total MAPK" antiserum (erk1/erk2) was a gift from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX), and the IRS-1 and IRS-2 antisera were generated as described previously (20). All other antisera were purchased from Upstate Biotechnology (Lake Placid, NY). Transblot nitrocellulose membrane (0.45 μm pore size) was from Bio-Rad, immunoblot chemiluminescence detection kit from NEN Life Science Products. IGF-I and protein kinase/phosphatase inhibitors were purchased from Calbiochem-Novabiochem (La Jolla, CA). The (*R*p)-2′-*O*-monobutyryl-cAMP and (*S*p)-2′-*O*-monobutyryl-cAMP were from Biolog Life Sciences Institute (La Jolla, CA). All the other biochemicals were purchased from either Sigma or Fisher Scientific (Pittsburgh, PA) and were of the highest purity available.

Cell Culture—The glucose-sensitive pancreatic β-cell line, INS-1 (24), was used in the experiments. INS-1 cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, and 11.2 mM glucose, and incubated at 37 °C, 5% CO_2 as described (24). Cells were subcultured at 80% confluence.

[^{3}H]Thymidine Incorporation—Incorporation of [^{3}H]thymidine was used as an indicator of DNA synthesis and INS-1 cell proliferation (7, 25). INS-1 cells were cultured on 96-well plates (10^5 cells/well) and incubated for 2 days at 37 °C in INS-1 medium. The medium was removed and the cells made quiescent by serum and glucose deprivation for 24 h in RPMI 1640 containing 0.1% BSA instead of serum and no glucose. The INS-1 cells were then incubated for a further 24 h in RPMI 1640, 0.1% BSA at different glucose concentrations (0–24 mM glucose) with or without IGF-I (0.1–100 nM), ± various inhibitors. The last 4 h of this latter incubation period was carried out in the additional presence of 5 μCi/ml [^{3}H]thymidine to monitor the degree of DNA synthesis and gain an assessment of the β-cell proliferation rate. After this final incubation period, the cells were collected and lysed using a semi-automatic cell harvester (Cambridge Technology Inc.) and the cell lysates transferred to Whatmann glass fiber micropore filters. The [^{3}H]thymidine specifically incorporated into the INS-1 cell DNA trapped on glass fiber filters was counted by liquid scintillation counting.

Protein Immunoblot and Co-Immunoprecipitation Analysis—INS-1 cells were subcultured on 10-cm plates to about 50% confluence as described previously (24). The cells were then subjected to a 24-h period of quiescence by serum and glucose deprivation in RPMI 1640 medium containing 0.1% BSA instead of serum and no glucose. After the quiescent period, INS-1 cells were then incubated in fresh RPMI 1640 medium containing 0, 3, 6, 9, or 18 mM glucose ± 10 nM IGF-I for between 5 and 60 min as indicated. The cells were then lysed in 0.5 ml of ice-cold lysis buffer consisting of 50 mM Hepes (pH 7.5), 1% (v/v) Nonidet P-40, 2 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 4 mM EDTA, 10 μM leupeptin, 10 μg/ml aprotonin, and 100 μM phenylmethylsulfonyl fluoride.

Immunoblot analysis of mitogenic signal transduction protein expression and protein tyrosine phosphorylation was as described previously, using horseradish peroxidase based chemiluminescence reaction as a secondary detection method (21, 26). Examination of stimulated protein-protein interactions between mitogenic signal transduction pathway proteins was by co-immunoprecipitation analysis as described previously (21, 26). For immunoblot analysis, 50–75 μg of INS-1 cell total protein lysate was used, and for immunoprecipitation 750 μg of INS-1 cell total protein.

Other Procedures—Protein assay was by the bicinchoninic acid method (Pierce, Rockford, IL). Data are presented as a mean ± S.E. Statistically significant differences between groups were analyzed using Student's t test, where $p < 0.05$ was considered statistically significant.

RESULTS

IGF-I Only Stimulates INS-1 Cell Proliferation in the Presence of Physiological Glucose Concentrations—The effect of glucose (0.1–24 mM) ± IGF-I (10 nM) on INS-1 cell DNA synthesis was determined by [^{3}H]thymidine incorporation as an index of β-cell proliferation. Glucose independently increased [^{3}H]thymidine incorporation into INS-1 cells (Fig. 1). In the absence of IGF-I, no change in [^{3}H]thymidine incorporation in INS-1 cells was observed between 0 and 0.5 mM glucose (Fig. 1), but at 1 mM glucose a modest increase in INS-1 cell proliferation was observed (1.4-fold increase compared with no glucose, $p < 0.001$; Fig. 1). However, the most effective glucose concentration range on INS-1 cell proliferation occurred in the physiologically relevant range between 6 and 18 mM glucose (4–19-fold above that in the absence of glucose; $p < 0.001$; Fig. 1). Maximum [^{3}H]thymidine incorporation into INS-1 cells above "zero glucose" occurred at 18 mM glucose (19-fold increase, $p < 0.0001$; Fig. 1), and declined above this glucose concentration (Fig. 1). Notwithstanding, a 14-fold higher INS-1 cell proliferation rate at 24 mM glucose was significantly higher than that observed in the absence of glucose ($p < 0.001$; Fig. 1).

It was apparent that IGF-I only exerted a notable effect on [^{3}H]thymidine incorporation into INS-1 cells when glucose was present at physiologically relevant concentrations (6–18 mM glucose; Fig. 1). Below 1 mM glucose, IGF-I modestly increased [^{3}H]thymidine incorporation into INS-1 cells (1.9-fold increase above zero glucose in the absence of IGF-I, $p < 0.001$; Fig. 1), however, this effect was rather small compared with the synergistic effects of IGF-I and glucose in the physiologically relevant range (6–18 mM; Fig. 1). At 6 mM glucose, IGF-I instigated a 10-fold increase in [^{3}H]thymidine incorporation above that in the absence of glucose and IGF-I ($p < 0.001$; Fig. 1), which was 2.5-fold higher than the rate of INS-1 cell proliferation at 6 mM glucose alone ($p < 0.01$; Fig. 1). IGF-I instigated a maximum increase in INS-1 cell [^{3}H]thymidine incorporation at 15 mM glucose (52-fold above that in the absence of glucose and IGF-I glucose, $p < 0.001$; Fig. 1), which was 4.2-fold higher than that at 15 mM glucose alone ($p < 0.01$; Fig. 1). Above 18 mM glucose the synergistic effect of IGF-I and glucose on [^{3}H]thymidine incorporation into INS-1 cells was significantly diminished ($p < 0.001$ above 15 mM glucose + 10 nM IGF-I; Fig. 1), so that at 24 mM glucose addition of IGF-I only surpassed the glucose effect by 1.4-fold (Fig. 1). Notwithstanding, this was 21-fold higher than the proliferation rate in the absence of glucose and IGF-I ($p < 0.001$; Fig. 1).

Both IGF-I and glucose-stimulated INS-1 cell proliferation

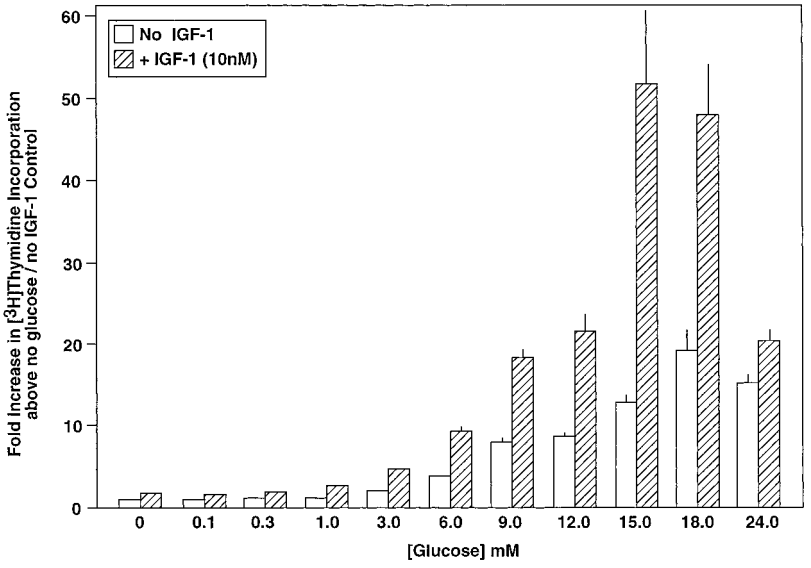

FIG. 1. **[^{3}H]Thymidine incorporation in INS-1 cells with different glucose concentrations.** Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 0–24 mM glucose ± 10 nM IGF-I, then assessed for proliferation rate by [^{3}H]thymidine incorporation as described under "Experimental Procedures." All experiments were done in triplicate on at least eight independent occasions. The data are expressed as a fold increase above the control observation in the absence of glucose and IGF-I (*i.e.* 500–1200 cpm/10^5 cells), and depicted as a mean ± S.E. ($n \geq 8$).

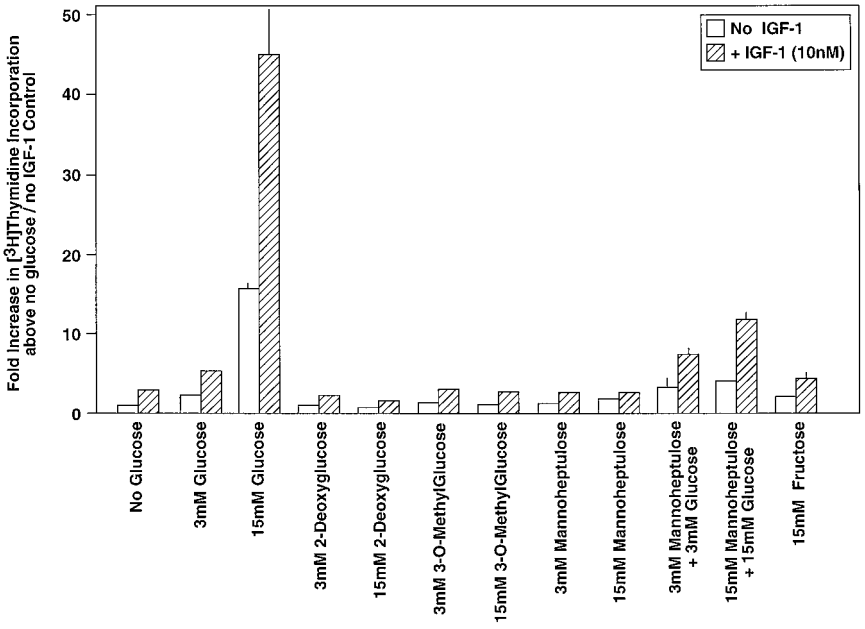

FIG. 2. **[^{3}H]Thymidine incorporation in INS-1 cells with different monosaccharides.** Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 3 or 15 mM glucose, 3 or 15 mM 2-deoxyglucose, 3 or 15 mM 3-*O*-methylglucose, 3 or 15 mM mannoheptulose, mannoheptulose ± 3 or 15 mM glucose or 15 mM fructose, ± 10 nM IGF-I, then assessed for proliferation rate by [^{3}H]thymidine incorporation as described under "Experimental Procedures." All experiments were done in triplicate on three independent occasions. The data are expressed as a fold increase above the control observation in the absence of glucose and IGF-I (*i.e.* 500–1200 cpm/10^5 cells), and depicted as a mean ± S.E. ($n = 3$).

required glucose metabolism (Fig. 2). Mannoheptulose (15 mM), a competitive inhibitor of glycolysis (27), completely inhibited 15 mM glucose-stimulated INS-1 cell [^{3}H]thymidine incorporation in the presence or absence of IGF-I ($p < 0.001$; Fig. 2). Not surprisingly, mannoheptulose (3 or 15 mM) had no independent effect on INS-1 cell proliferation whether IGF-I was present or not (Fig. 2). Likewise, the non-metabolizable glucose analogues, 2-deoxyglucose or 3-*O*-methyl glucose (at concentrations of 3 or 15 mM), had no effect on [^{3}H]thymidine incorporation into INS-1 cells (Fig. 2), and could not provide a suitable platform for IGF-I instigated INS-1 cell proliferation (Fig. 2). Fructose (15 mM), which is not efficiently metabolized in pancreatic β-cells (27), did not significantly increase INS-1 cell proliferation in the presence or absence of IGF-I (Fig. 2). These observations further emphasize the requirement of physiologically relevant concentrations of glucose for IGF-I to stimulate INS-1 cell proliferation.

The effect of increasing IGF-I concentrations on [^{3}H]thymidine incorporation in INS-1 cells at various glucose concentrations was examined (Fig. 3). In the absence of IGF-I there was an incremental increase in [^{3}H]thymidine incorporation with increasing glucose between 3 and 18 mM as observed previously (Fig. 1). IGF-I above 1 nM increased [^{3}H]thymidine incorporation >2-fold compared with cells treated with glucose alone, with a maximal increase at 10 nM IGF-I, 18 mM glucose (49-fold above zero glucose, $p < 0.001$; Fig. 3). This effect was not exceeded above a concentration of 10 nM IGF-I (Fig. 3), and subsequently 10 nM IGF-I was used as an optimal IGF-I concentration in following experiments.

The effect of insulin to compete for IGF-I (10 nM)-stimulated [^{3}H]thymidine incorporation into INS-1 cells was also examined at various glucose concentrations (Fig. 4). Only at very high insulin concentrations (>10 μM) was any significant competition for IGF-I-stimulated INS-1 cell proliferation observed, which then rendered only a 20–30% inhibition ($p < 0.02$; Fig. 4). In the absence of IGF-I, only high concentrations of insulin (>10 μM) could instigate a modest increase in [^{3}H]thymidine incorporation into INS-1 cells at 15 mM glucose (1.3-fold above 15 mM glucose without IGF-I, $p < 0.05$; Fig. 4). Thus, the observations found in this study were predominately attributable to IGF-I working through the IGF-I receptor.

The Effect of Various Protein Phosphorylation Inhibitors on

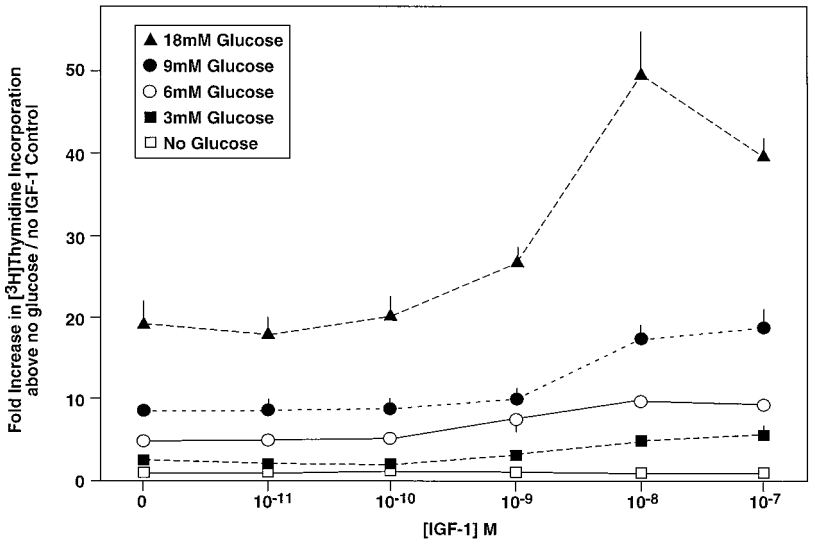

FIG. 3. **[^{3}H]Thymidine incorporation in INS-1 cells with different IGF-I concentrations.** Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 0–100 nM IGF, 0–18 mM glucose, then assessed for proliferation rate by [^{3}H]thymidine incorporation as described under "Experimental Procedures." All experiments were done in triplicate on six independent occasions. The data are expressed as a fold increase above the control observation in the absence of glucose and IGF-I (*i.e.* 500–1200 cpm/10^5 cells), and depicted as a mean ± S.E. (n = 6).

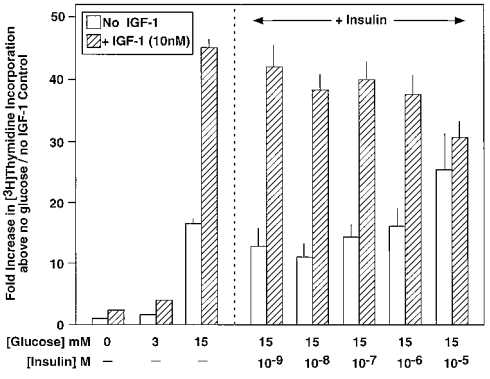

FIG. 4. **The effect of exogenous insulin on glucose/IGF-I-stimulated [^{3}H]thymidine incorporation in INS-1 cells.** Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 0, 3, or 15 mM glucose, or 15 mM glucose + 10^{-5}-10^{-9} M bovine insulin, then assessed for proliferation rate by [^{3}H]thymidine incorporation as described under "Experimental Procedures." All experiments were done in triplicate on three independent occasions. The data are expressed as a fold increase above the control observation in the absence of glucose, IGF-I, and insulin (*i.e.* 500–1200 cpm/10^5 cells), and depicted as a mean ± S.E. (n = 3).

Glucose and IGF-I-stimulated INS-1 Cell Proliferation—The effect of specific protein kinase and phosphatase inhibitors on stimulation of [^{3}H]thymidine incorporation in INS-1 cells by 15 mM glucose ± 10 nM IGF-I was examined (Table I). In these series experiments 15 mM glucose instigated a 20-fold increase in [^{3}H]thymidine incorporation above that in the absence of glucose ($p < 0.001$), and the combination of 15 mM glucose and 10 nM IGF-I gave a 52-fold increase above that in the absence of glucose and IGF-I ($p < 0.001$) similar to that observed previously (Fig. 1). Addition of (*S*p)-2′-*O*-monobutyryl-cAMP (5 μM), a cell permeable PKA agonist (28), did not show any significant changes in the rate of INS-1 cell [^{3}H]thymidine incorporation at either 15 mM glucose alone or in the additional presence of IGF-I (Table I). However, in the presence of (*R*p)-$O^{2'}$-monobutyryl-cAMP (5 μM), a cell permeable cAMP analogue which inhibits protein kinase-A (PKA) activity (28), 15 mM glucose-induced [^{3}H]thymidine incorporation in INS-1 cells was inhibited by 87% ($p < 0.001$; Table I), whereas it was not significantly affected in the additional presence of IGF-I (Table I). This suggested a possible role for PKA in glucose-induced INS-1 cell mitogenesis (29), but this was overcome by addition of IGF-I.

In the presence of sphingosine (10 μM), a selective inhibitor of protein kinase C (30), both 15 mM glucose-stimulated and IGF-I + 15 mM glucose-stimulated [^{3}H]thymidine incorporation in INS-1 cells were significantly inhibited (50–60% inhibition; $p < 0.02$; Table I). This suggested that certain protein kinase C isoform(s) could be involved in regulating glucose and IGF-I-mediated INS-1 cell proliferation (29). Staurosporine (20 nM) an inhibitor of protein kinase C, PKA, and protein kinase G (30), inhibited 15 mM glucose-stimulated [^{3}H]thymidine incorporation into INS-1 cells (50% inhibition, $p < 0.001$; Table I),

TABLE I
The effect of protein phosphorylation inhibitors on glucose/IGF-I stimulated [^{3}H]thymidine incorporation in INS-1 cells

Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPM1 1640 medium containing 0.1% BSA, 15 mM glucose ± 10 nM IGF-I, ± various inhibitors of protein kinases, phosphoprotein phosphatases, or tyrosine kinase signaling cascades as indicated, then assessed for proliferation rate by [^{3}H]thymidine incorporation as outlined under "Experimental Procedures." The data are presented as a percentage of either the control [^{3}H]thymidine incorporation at 15 mM glucose in the absence of IGF-1, or the control [^{3}H]thymidine incorporation at 15 mM glucose + 10 nM IGF-1, as appropriate. A mean ± S.E. are depicted of at least five experiments done in triplicate.

Inhibitor	Percentage [^{3}H]thymidine incorporation compared to 15 mM glucose control	Percentage [^{3}H]thymidine incorporation compared to 15 mM glucose + 10 nM IGF-1 control
Protein kinase A		
(*R*p)-2′-*O*-monobutyryl-cAMP (5 μM)	6.3 ± 0.7[a]	94.2 ± 4.9
(*S*p)-2′-*O*-monobutyryl-cAMP (5 μM)	78.7 ± 8.4	88.6 ± 11.3
Protein kinase C		
Sphingosine (10 μM)	37.7 ± 4.6[a]	42.9 ± 3.5[a]
Staurosporin (20 nM)	48.8 ± 3.1[a]	81.1 ± 2.8
Ca^{2+}/calmodulin kinase		
Calmidozolium (50 nM)	20.9 ± 1.8[a]	81.4 ± 3.7
KN-93 (1 μM)	91.0 ± 3.7	94.4 ± 4.3
Phosphoprotein phosphatase		
Okadaic acid (50 nM)	76.5 ± 5.0	97.6 ± 8.4
Cyclosporin A (5 μM)	88.6 ± 8.1	101.8 ± 1.1
Orthovanadate (0.5 mM)	3.3 ± 0.2[a]	2.5 ± 0.2[a]
Tyrosine kinase cascades		
Genistein (25 μM)	5.7 ± 0.6[a]	4.7 ± 0.8[a]
Wortmannin (10 nM)	1.9 ± 0.4[a]	3.6 ± 1.0[a]
LY294002 (5 μM)	1.6 ± 0.2[a]	5.4 ± 0.5[a]
PD98059 (50 μM)	82.6 ± 6.4	16.5 ± 1.9[a]
Rapamycin (10 nM)	50.5 ± 3.8[a]	45.6 ± 1.8[a]

[a] Statistically significant difference from the equivalent control at $p \leq 0.02$.

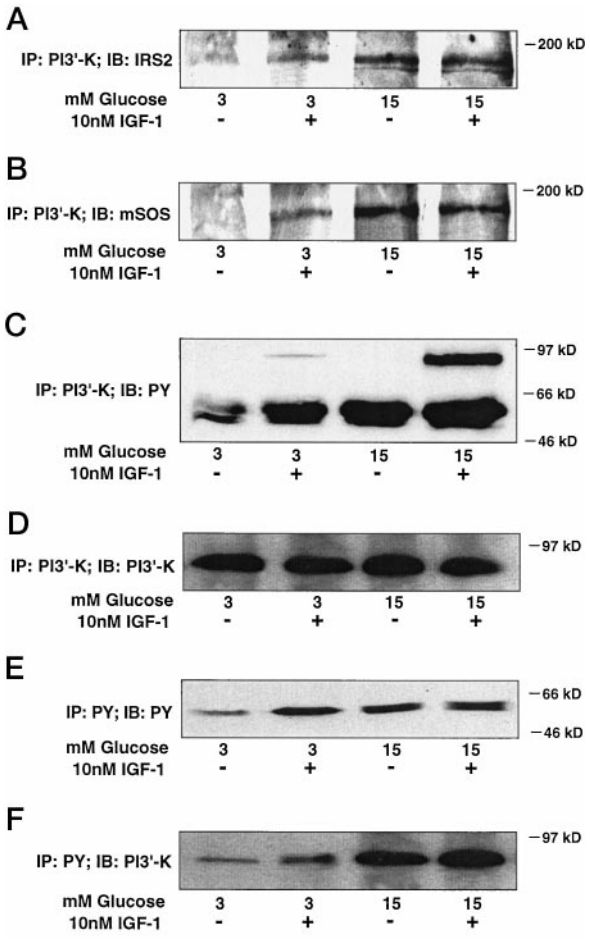

FIG. 5. **IGF-I and glucose both increase protein phosphotyrosine phosphorylation of IRS-2 and pp60, resulting in increased association of PI 3′-kinase and mSOS.** INS-1 cells (50% confluent on a 15-cm diameter dish) were stimulated with 3 or 15 mM glucose ± 10 nM IGF-I for 10 min, and cell lysates generated as described under "Experimental Procedures." INS-1 cell lysates were then subjected to immunoprecipitation (*IP*) with antiserum against the p85 regulatory subunit of PI 3′-kinase (*panels A-D*) or anti-phosphotyrosine (*PY*, *panels E* and *F*) antibody. Immunoprecipitates were then subjected to immunoblot (*IB*) analysis with IRS-2 (*panel A*), mSOS (*panel B*), anti-phosphotyrosine (*panels C* and *E*), and p85 PI 3′-kinase (*panels D* and *F*) antibodies, as described under "Experimental Procedures." An example blot for such co-immunoprecipitation analysis is shown.

but not that in the additional presence of IGF-I (Table I). This was similar to the effect of (*R*p)-$O^{2'}$-monobutyryl-cAMP on INS-1 cell proliferation (Fig. 5), and suggested that staurosporine may be mediating its effect via inhibition of PKA.

It has been implicated that intracellular [Ca^{2+}] might be involved in signaling pathways that lead to glucose-induced β-cell mitogenesis (7, 8), that may be mediated by activation of Ca^{2+}/calmodulin-dependent proteins. Calmidazolium (50 nM), a calmodulin antagonist, significantly reduced 15 mM glucose-stimulated [^{3}H]thymidine incorporation into INS-1 cells by 86% ($p < 0.002$; Table I), but did not inhibit IGF-I-stimulated INS-1 cell proliferation at 15 mM glucose (Table I). KN-93 (1 μM), an inhibitor of calmodulin kinase II activity in β-cells (31), showed no significant decrease in 15 mM glucose-stimulated INS-1 cell proliferation whether IGF-I was present or not (Table I). These data suggested a role for Ca^{2+}/calmodulin (but not necessarily calmodulin kinase II) for glucose-induced INS-1 cell proliferation (29), however, this was averted by the addition of IGF-I.

The possible role of phosphoprotein phosphatase activities on glucose/IGF-I-induced INS-1 cell proliferation was investigated. Okadaic acid (50 nM), an inhibitor of phosphoprotein phosphatase 1 and phosphoprotein phosphatase 2A, and cyclosporin A (5 μM), an inhibitor of phosphoprotein phosphatase 2B, had no significant effect on 15 mM glucose or glucose + IGF-I- stimulated INS-1 cells proliferation (Table I). However, the protein tyrosine phosphatase inhibitor, orthovanadate (0.5 mM), markedly inhibited 15 mM glucose and IGF-I + 15 mM glucose-stimulated [^{3}H]thymidine incorporation into INS-1 cells by >95% ($p < 0.001$; Table I). This was indicative of protein tyrosine phosphorylation as an important aspect of glucose/IGF-I mitogenic signal transduction pathway(s) in INS-1 cells.

Protein tyrosine phosphorylation cascades have been strongly implicated in mitogenic signal transduction pathways (19, 20). Genistein (25 μM), an inhibitor of protein tyrosine kinase activity, markedly inhibited both 15 mM glucose and IGF-I-stimulated [^{3}H]thymidine incorporation into INS-1 cells by >95% ($p < 0.001$; Table I). Specific inhibitors of PI 3′-kinase activity (wortmannin (10 nM) and LY294002 (5 μM)) also markedly inhibited by both 15 mM glucose and IGF-I-induced INS-1 cell proliferation by >90% ($p < 0.001$; Table I). Activation of $p70^{S6K}$ lies downstream of PI 3′-kinase activation in mitogenic signaling pathways, and correspondingly specific inhibition of $p70^{S6K}$ by rapamycin (10 nM) resulted in a significant 50% inhibition of both 15 mM glucose ($p < 0.02$) and IGF-I ($p < 0.001$)-induced [^{3}H]thymidine incorporation into INS-1 cells incubated (Table I). The MEK inhibitor, PD98059 (50 μM), inhibited INS-1 cell proliferation as stimulated by the combination of 10 nM IGF-I + 15 mM glucose by >80% compared with the equivalent control ($p < 0.001$; Table I). In contrast, PD98059 had no significant effect on [^{3}H]thymidine incorporation into INS-1 cells incubated with 15 mM glucose in the absence of IGF-I (Table I). These data implicate that the PI 3′-kinase branch of mitogenic signal transduction pathways was important for both glucose and IGF-I-stimulated INS-1 cell proliferation, whereas the MAPK branch might only be relevant for IGF-I-mediated INS-1 cell mitogenesis.

IGF-I and Glucose Activate IRS-mediated Mitogenic Signal Transduction Pathways in INS-1 Cells—Protein phosphorylation activation of mitogenic signal transduction pathways by 15 mM glucose ± 10 nM IGF-I in INS-1 cells was investigated using co-immunoprecipitation and immunoblot analysis. Immunoprecipitation of the 85-kDa regulatory subunit of PI 3′-kinase followed by immunoblot analysis with IRS-2 antiserum, revealed a specific increased association of PI 3′-kinase and IRS-2 instigated by both 15 mM glucose and IGF-I. At a basal 3 mM glucose, 10 nM IGF-I increased the amount of IRS-2 associated with PI 3′-kinase within 10 min (Fig. 5*A*). Increasing the glucose concentration to 15 mM further increased IRS-2/PI 3′-kinase association, which was not particularly affected by the additional presence of IGF-I (Fig. 5*A*). The specific nature of this glucose/IGF-I-induced IRS-2/PI 3′-kinase interaction was illustrated in that PI 3′-kinase immunoblot analysis of PI 3′-kinase immunoprecipitates revealed that an equivalent amount of PI 3′-kinase present in each sample (Fig. 5*D*). In contrast to the PI 3′-kinase/IRS-2 interaction in INS-1 cells, no detectable difference in the PI 3′-kinase/IRS-1 interaction could be found at 15 mM glucose ± IGF-I in INS-1 cells (data not shown). However, immunoblotting of the PI 3′-kinase immunoprecipitates with antiserum recognizing the C-terminal region of mSOS (Fig. 5*B*) indicated an increased association of mSOS within 10 min in INS-1 cells stimulated with IGF-I at 3 mM glucose (presumably via increased Grb2 association with tyrosine-phosphorylated IRS (19, 20)). The PI 3′-kinase/mSOS association in INS-1 cells was further increased by 15 mM glucose alone, but this was not substantially increased by the

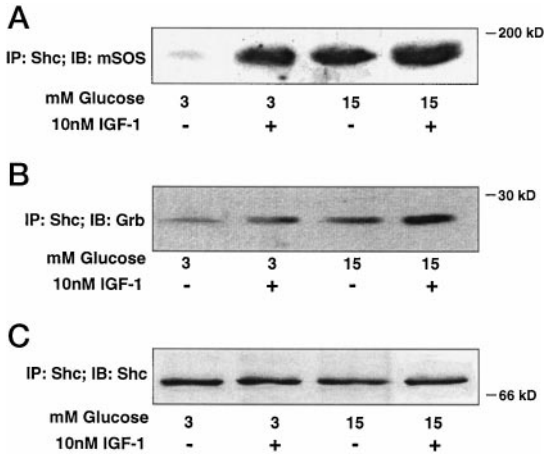

FIG. 6. **IGF-I and glucose both increase the association of Grb2 and mSOS with Shc.** INS-1 cells (50% confluent on a 15-cm diameter dish) were stimulated with 3 or 15 mM glucose ± 10 nM IGF-I for 10 min, and cell lysates generated as described under "Experimental Procedures." INS-1 cell lysates were then subjected to immunoprecipitation (*IP*) with antiserum Shc. The Shc immunoprecipitates were then subjected to immunoblot (*IB*) analysis with mSOS (*panel A*), Grb2 (*panel B*), and Shc (*panel C*) antibodies, as described under "Experimental Procedures." An example blot for such co-immunoprecipitation analysis is shown.

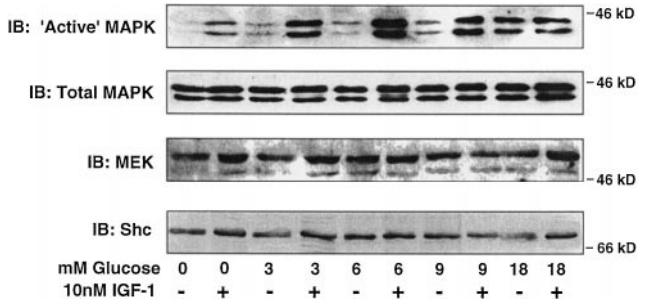

FIG. 7. **IGF-I and glucose both stimulated phosphorylation activation of MAPK (erk-1/erk-2 isoforms) in INS-1 cells.** INS-1 cells (50% confluent on a 10-cm diameter dish) were stimulated with 3, 6, 9, or 18 mM glucose ± 10 nM IGF-I for 10 min, and cell lysates generated as described under "Experimental Procedures." Specific immunoblot analysis for phosphorylation activated MAPK, total MAPK, MEK, and Shc was examined in the INS-1 cell lysates as described under "Experimental Procedures." A representative immunoblot (*IB*) analysis of "activated" MAPK, total MAPK, MEK, and Shc is shown.

additional presence of IGF-I at 15 mM glucose (Fig. 5*B*).

Immunoprecipitation of the p85 PI 3′-kinase regulatory subunit followed by anti-phosphotyrosine (PY) immunoblot analysis revealed an increase in the presence of tyrosine-phosphorylated 95-kDa β-subunit of the IGF-I receptor only in the presence of IGF-I (Fig. 5*C*). In the same analysis a prominent 60-kDa tyrosine-phosphorylated protein (pp60) showed increased association to the PI 3′-kinase immunoprecipitate after a 10-min exposure to 15 mM glucose compared with that at a basal 3 mM glucose (Fig. 5*C*). The association of tyrosine-phosphorylated pp60 with PI 3′-kinase was further increased by IGF-I at both 3 and 15 mM glucose within 10 min (Fig. 5*C*). Correspondingly, immunoprecipitation with an anti-phosphotyrosine antibody followed by anti-phosphotyrosine immunoblot analysis indicated an increase in the tyrosine phosphorylated state of pp60 by IGF-I at a basal 3 mM glucose, and at 15 mM glucose ± IGF-I (Fig. 5*E*). Immunoblot analysis of anti-phosphotyrosine immunoprecipitates with p85 PI 3′-kinase antisera revealed an increased association of PI 3′-kinase with tyrosine-phosphorylated proteins instigated by IGF-I at a basal 3 mM glucose (Fig. 5*F*). The p85 PI 3′-kinase association with phosphotyrosine proteins was increased at 15 mM glucose, compared with that at 3 mM glucose ± IGF-I, and further enhanced by the addition of IGF-I (Fig. 5*F*).

IGF-I was also able to activate the Ras/Raf/MEK/MAPK mitogenic signal transduction pathway independent of IRS by IGF-I receptor tyrosine kinase-mediated phosphorylation of Shc (18–20). Immunoprecipitation of Shc followed by immunoblot analysis of mSOS (Fig. 6*A*) and Grb2 (Fig. 6*B*) from INS-1 cells incubated for 10 min at 3 or 15 mM glucose ± IGF-I, indicated that IGF-I at a basal 3 mM glucose could promote the association of mSOS/Grb2 to Shc (Fig. 6, *A* and *B*). Furthermore, 15 mM glucose alone was found to promote the association of Grb2/mSOS with Shc, that was further enhanced by the additional presence of IGF-I (Fig. 6, *A* and *B*). The specific nature of glucose/IGF-I-induced Grb2-mSOS interaction with Shc in INS-1 cells was indicated in that an equivalent amount of Shc was detected by immunoblot analysis of Shc immunoprecipitates (Fig. 6*C*).

The mSOS activation, by its Grb2-mediated association with tyrosine-phosphorylated IRS and/or Shc, results in downstream activation of Ras which in turn stimulates Raf-1 serine kinase activity (18–20). Raf-1 then activates MEK which in turn activates MAPK (erk-1 and -2 isoforms) by serine phosphorylation (7, 8, 18–20). Activated MAPK can be detected with specific antiserum that only recognizes the phosphorylation activated forms of erk-1 and -2 (7, 8). Immunoblot analysis of glucose ± IGF-I-stimulated INS-1 cells with "activated phospho-MAPK" antiserum showed little activated MAPK in the absence of glucose or IGF-I (Fig. 7). However, an increase in active phospho-MAPK after 10 min exposure to 3 mM glucose was detected which reached a maximum at 18 mM glucose independently of IGF-I (Fig. 7). IGF-I treatment of INS-1 cells for 10 min in the absence of glucose activated MAPK (Fig. 7). In the additional presence of glucose (3–9 mM), IGF-I instigated a further increase in activation of MAPK in INS-1 cells above that of glucose alone (Fig. 7). The IGF-I activation of MAPK reached a maximum at 6 mM glucose, but thereafter decreased so that at 18 mM glucose IGF-I could instigate an activation of MAPK above that of 18 mM glucose alone (Fig. 7). The amount of total MAPK in INS-1 cells, as ascertained by immunoblot analysis with antisera recognizing both active and inactive forms of MAPK, was not significantly altered by glucose and IGF-I treatment (Fig. 7). Similarly immunoblot analysis of total MEK and Shc in INS-1 cells treated with various glucose concentrations ± IGF-I indicated that levels of these proteins did not noticeably alter (Fig. 7).

In the IGF-I mediated signal transduction pathway, $p70^{S6K}$ is activated downstream of PI 3′-kinase activation (18). Phosphorylation activation of $p70^{S6K}$ occurs on multiple sites so that $p70^{S6K}$ phosphorylation can be detected on immunoblot analysis by an apparent electrophoresis mobility retardation (26). INS-1 cells were incubated for 5–60 min in the presence of 3–18 mM glucose ± 10 nM IGF-I. Maximal $p70^{S6K}$ phosphorylation was observed at 30 min (data not shown). Immunoblot analysis with $p70^{S6K}$-specific antiserum indicated phosphorylation activation of $p70^{S6K}$ in response to both glucose and IGF-I (Fig. 8). In the absence of IGF-I, glucose $p70^{S6K}$ phosphorylation above 6 mM glucose reached a maximum at 18 mM glucose (Fig. 8). In the added presence of IGF-I no activation of $p70^{S6K}$ was observed below 3 mM glucose, however, above 3 mM glucose IGF-I increased $p70^{S6K}$ activation by glucose alone, reaching a maximum potentiating effect of IGF-I at 18 mM glucose (Fig. 8). A p85 PI 3′-kinase immunoblot analysis of the same INS-1 cell lysates used for $p70^{S6K}$ analysis indicated that there was little change in the total amount of PI 3′-kinase protein per sample, that emphasized a specific activation of $p70^{S6K}$ instigated by glucose and IGF-I (Fig. 8).

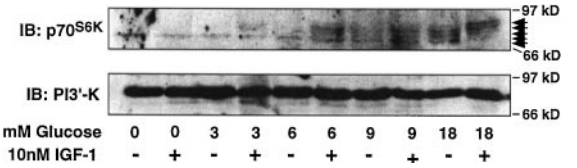

FIG. 8. **IGF-I and glucose both stimulated phosphorylation activation of p70^{S6K} in INS-1 cells.** INS-1 cells (50% confluent on a 15-cm diameter dish) were stimulated with 3, 6, 9, or 18 mM glucose ± 10 nM IGF-I for 30 min, and cell lysates generated as described under "Experimental Procedures." Specific phosphorylation activation of p70^{S6K} was examined in the INS-1 cell lysates by immunoblot (*IB*) analysis as described under "Experimental Procedures." A representative immunoblot for p70^{S6K} is shown. Phosphorylated forms of p70^{S6K} become retarded on SDS-polyacrylamide gel electrophoresis analysis, and these multi-phosphorylated p70^{S6K} forms are indicated by the *arrows*.

DISCUSSION

Although adult pancreatic β-cells have a relatively low mitotic index (1), both IGF-I and glucose have been shown to stimulate pancreatic β-cell proliferation (2, 15, 32). However, little has been revealed about the mitogenic signal transduction pathways in β-cells that are activated by glucose and certain growth factors, which irreversibly leads to committing a β-cell into a growth phase of the cell cycle (33). Moreover, the regulation of mitogenesis in pancreatic β-cells is likely to be more complicated than in other eukaryotic cells due to the unique characteristic of the β-cell's stimulus-coupling mechanisms being tightly linked to its metabolic state (23, 34). Indeed, glucose has been shown to induce adult β-cell replication both *in vitro* and *in vivo* (2, 3), although the concentration dependence has not been previously established. In this study glucose-induced β-cell proliferation has been examined in the relatively well differentiated INS-1 cell line. INS-1 cells respond to glucose in terms of insulin secretion in the physiologically relevant range (5–20 mM) (24). Likewise, it was found that glucose-induced [^{3}H]thymidine incorporation into INS-1 cells was only apparent at glucose concentrations between 6 and 24 mM. Similar to glucose-induced insulin secretion (24), the threshold glucose concentration to instigate an increase in INS-1 cell proliferation was between 3 and 6 mM that reached a maximum at 18 mM glucose. Glucose metabolism was required to provoke a mitogenic response in INS-1 cells, as previously established in adult and fetal pancreatic β-cells (2), however, it remains unclear what the appropriate signaling elements are immediately downstream of glucose metabolism required for a glucose-induced mitogenic response (2).

IGF-I has been shown to be an effective stimulus for inducing differentiated pancreatic β-cell growth (2). Furthermore, IGF-I has been implicated to play a prominent role for increasing the population of pancreatic islets in the regenerating pancreas (3). In this study, it was found that IGF-I (1–10 nM) could markedly increase INS-1 cell proliferation, but only in the physiologically relevant glucose concentration range between 6 and 18 mM (Figs. 1 and 3). Thus, unlike the mitogenic effect of IGF-I on other eukaryotic cells (17), in pancreatic β-cells IGF-I was dependent on glucose being present to provoke a mitogenic response. The additional presence of IGF-I at 6–18 mM glucose increased INS-1 cell proliferation 2–3-fold above that at glucose alone. Thus, there was a degree of synergy between IGF-I and glucose (6–18 mM) to increase the pancreatic β-cell proliferation rate. A further indication of the glucose-dependent nature of IGF-I-induced INS-1 cell replication was that metabolism of glucose was required for the IGF-I mitogenic effect (Fig. 2). However, at higher glucose concentrations (24 mM), the effect of IGF-I on INS-1 cell [^{3}H]thymidine incorporation was reduced, perhaps indicative of the adverse effects of elevated glucose concentrations on β-cell function (35). Nonetheless, at 15 mM glucose, it was apparent that it was a specific effect of IGF-I working through IGF-I receptors (previously shown to be present on pancreatic β-cells (36)), and not a secondary effect of insulin secreted from INS-1 cells working via insulin and/or IGF-I receptors or IGF-I operating via insulin receptors (36, 37). Insulin could only slightly inhibit 10 nM IGF-I-induced β-cell proliferation at very high concentrations (>10 μM insulin; Fig. 4). Likewise, in the absence of IGF-I, insulin only modestly potentiated glucose-induced INS-1 cell growth at very high unphysiological concentrations (>10 μM insulin; Fig. 4).

A degree of insight for signaling requirements of glucose-induced and glucose-dependent IGF-I-stimulated β-cell proliferation were gained from inhibitor studies (Table I). Glucose-induced INS-1 cell proliferation appeared to require both PKA and Ca^{2+}/calmodulin as previously suggested (2, 38), but this requirement was vanquished in the additional presence of IGF-I. Notably, inhibition of protein phosphotyrosine phosphatases or tyrosine protein kinases resulted in complete inhibition of glucose and IGF-I-induced INS-1 cell replication, implicating certain protein tyrosine phosphorylation/dephosphorylation signaling cascades were an important ingredient in INS-1 cell mitogenic signaling. Activation of PI 3′-kinase occurs along IRS-mediated tyrosine phosphorylation signaling pathways (19, 20), and PI 3′-kinase activity appeared essential for both glucose or IGF-I to provoke a mitogenic response. This was substantiated by the finding that rapamycin, an inhibitor of p70^{S6K} activation that occurs downstream of PI 3′-kinase activation (19), also inhibited glucose and IGF-I-induced INS-1 cell proliferation. However, it should be noted that rapamycin only partly inhibited the glucose/IGF-I-induced mitogenic response in INS-1 cells, perhaps suggesting that alternative factors downstream of PI 3′-kinase activation (*e.g.* other protein substrates of phosphatidylinositide 3,4,5-triphosphate-activated protein kinase B (39)), ought to be considered. Intriguingly, inhibition of MEK and consequential MAPK activation had no effect on glucose-induced INS-1 cell proliferation. Thus, whereas the PI 3′-kinase branch of mitogenic signaling pathways may be required for glucose-induced INS-1 cell replication, that via MAPK may not, despite glucose-induced activation of MAPK in β-cells (7, 8) (Fig. 7). However, this did not appear to be the case for IGF-I where inhibition of MEK/MAPK significantly inhibited glucose-dependent IGF-I-induced INS cell mitogenesis. It follows that these inhibitor studies revealed that there are differences between glucose and IGF-I signaling to induce β-cell mitogenesis. Nonetheless, a degree of caution should be taken in interpreting such inhibitor studies, since these pharmacological reagents often act via secondary mechanisms and as such can be misleading. Thus, such inhibitor experiments are better supported with alternative biochemical evidence, and hence in this study IRS/Shc-mediated signal transduction pathways were directly examined in INS-1 cells.

Increasing the glucose concentration from a basal 3 mM to a stimulatory 15 mM in the absence of IGF-I resulted in activation of IRS-mediated signaling pathways independent of a growth factor stimulus. Glucose induced an increased association of PI 3′-kinase with IRS-2 in INS-1 cells, but that with IRS-1 could not be detected. This was supportive of recent observations of the key role for IRS-2 in β-cell mitogenesis, in that there is a specific increase in IRS-2 expression but not that of IRS-1 in pancreatic β-cell lines (40), and that β-cell mass *in vivo* is markedly reduced in IRS-2 knockout mice yet increased in IRS-1-deficient mice (41). Glucose-stimulated IRS-2/PI 3′-kinase association in INS-1 cells correlated with an increase in tyrosine phosphorylation (19, 20), as indicated by glucose-induced increase of PI 3′-kinase in anti-phosphotyrosine immunoprecipitates (Fig. 5*F*). However, it is unlikely that glucose-

induced PI 3′-kinase activation in INS-1 cells is exclusively mediated via IRS-2. Glucose also increased an association of PI 3′-kinase with a 60-kDa tyrosine-phosphorylated protein (pp60; Fig. 5*C*). The identity of pp60 in INS-1 cells has yet to uncovered, however, it is quite possible that this may represent the truncated member of the IRS family, IRS-3, that associates with PI 3′-kinase in a tyrosine phosphorylation-dependent manner resulting in PI 3′-kinase activation (42, 43). Notwithstanding, it was found that glucose could induce an activation of $p70^{S6K}$ which lies downstream of PI 3′-kinase activation (19–21). The $p70^{S6K}$ activation correlated with the glucose concentration dependence for stimulation of [^{3}H]thymidine incorporation in INS-1 cells (Fig. 1 *verses* Fig. 8). Thus, these data are consistent with the notion that glucose can activate the PI 3′-kinase/$p70^{S6K}$ branch of IRS-mediated signaling pathways in pancreatic β-cells, which is required for glucose increased β-cell proliferation.

Glucose induced recruitment of mSOS to p85 PI 3′-kinase immunoprecipitates (Fig. 5*B*). This was not necessarily due to a direct association of p85 PI 3′-kinase and mSOS, but rather via Grb2 interaction with tyrosine-phosphorylated IRS which co-immunoprecipitated with p85 PI 3′-kinase as components of an activated IRS signaling complex (19, 20). MAPK is activated downstream of Grb2/mSOS association with IRS, so this complemented the observation of glucose-induced activation of MAPK (7, 8) (Fig. 7). Notwithstanding, MAPK can also be activated via an IRS-independent pathway involving tyrosine phosphorylation of the adaptor molecule Shc (17, 19, 20). Glucose independently induced an increase in Grb2/mSOS association with Shc (Fig. 6), that would also contribute to downstream activation of MAPK (7, 8) (Fig. 7). However, it should be noted that the concentration dependence for glucose-stimulated activation of MAPK (Fig. 7) did not correlate with glucose stimulation of [^{3}H]thymidine incorporation in INS-1 cells (Fig. 1). This suggested that MAPK activation was not necessarily required for glucose-induced β-cell proliferation, in agreement with the finding that MEK inhibition did not affect glucose-induced [^{3}H]thymidine incorporation into INS-1 cells.

IGF-I-induced INS-1 cell mitogenesis was glucose-dependent. For the moment, it is uncertain what the critical signaling factor(s) downstream of glucose metabolism might be that provides a platform for IGF-I to provoke a mitogenic response in β-cells. However, the addition of IGF-I tended to potentiate glucose-induced activation of IRS/Shc signal transduction pathways in INS-1 cells. Phosphotyrosine immunoblot analysis of PI 3′-kinase immunoprecipitates revealed that IGF-I induced tyrosine phosphorylation of a 95-kDa protein (Fig. 5*C*). This phosphotyrosine protein was not detectable in the absence IGF-I, and as such, it was likely the 95-kDa β-subunit of the IGF-I receptor (17). At a stimulatory 15 mM glucose, an increase in tyrosine phosphorylation of the IGF-I receptor β-subunit was detected in PI 3′-kinase immunoprecipitates, compared with that at a basal 3 mM glucose. This may be due to either increased association of the IGF-I receptor with a PI 3′-kinase immunoprecipitated signaling complex or increased tyrosine phosphorylation of the IGF-I receptor at a stimulatory glucose concentration. However, increases in extracellular glucose concentration are unlikely to affect binding of IGF-I to its receptor. Thus, in considering that glucose is required for IGF-I-induced INS-1 cell proliferation, it is more likely that glucose facilitates recruitment of IRS/PI 3′-kinase to an activated tyrosine-phosphorylated IGF-I receptor during formation of an activated IRS signaling complex (17, 19, 20). This notion was supported in that, IGF-I further increased the association of PI 3′-kinase with IRS-2, mSOS (presumably via Grb2 (17, 19, 20)), and pp60 (Fig. 5, *A-C*), as well as Shc with Grb2/mSOS (Fig. 6), especially at a basal 3 mM glucose. Furthermore, downstream activation of $p70^{S6K}$ was enhanced by IGF-I at physiological glucose concentrations between 6 and 18 mM (Fig. 8), which correlated with the extent of IGF-I-induced INS cell proliferation rate (Fig. 1). However, unlike $p70^{S6K}$ activation, IGF-I-induced MAPK activation did not correlate with IGF-I-stimulated INS-1 cell proliferation (Fig. 7 *verses* Fig. 1). Nonetheless, inhibition of MAPK activation led to a marked inhibition of IGF-I-induced INS-1 cell proliferation in contrast to that by glucose alone (Table I). Thus, some MAPK activity was likely required in order to facilitate IGF-I increased β-cell proliferation, but activation of MAPK alone was not sufficient to provoke a mitogenic response.

In summary, this study establishes that certain elements of the mitogenic signal transduction pathway are present in pancreatic β-cells, and can be stimulated by glucose ± IGF-I leading to downstream activation of MAPK and $p70^{S6K}$. Although, MAPK activity was likely required, activation of PI 3′-kinase was an essential element for glucose/IGF-I-induced β-cell proliferation. It will be important in future studies to identify the appropriate transcription factors relevant to β-cell mitogenesis that are activated downstream of MAPK and $p70^{S6K}$, and the "signaling factor" which renders IGF-I signaling in β-cells dependent on glucose metabolism. Notwithstanding, only a limited number of mitogenic signal transduction elements have been examined in this study, and other factors should not be ruled out (36, 44, 45). This is an important consideration, especially as there is likely interaction between certain mitogenic signal transduction pathways in β-cells as in other mammalian cells (46). In the light of the apparent tight regulation of adult β-cell mitogenesis (2–4, 6), it is probably synergy between different signaling pathways that irreversibly commits the β-cell into a growth phase of the cell cycle.

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