

Hepatocyte growth factor stimulates proliferation of pancreatic β -cells particularly in the presence of subphysiological glucose concentrations

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Abstract

We investigated the role of hepatocyte growth factor (HGF) in β -cell growth and its complex intracellular signal transduction pathways. Cell proliferation was measured in the β -cell line INS-1 using [3 H]thymidine incorporation. Activation of mitogenic signaling proteins was assessed using co-immunoprecipitation, immunoblot analysis and specific protein activity inhibitors in proliferation assays. HGF (1.375 nM) increased INS-1 cell proliferation in the presence of 3–24 mM glucose up to 45-fold vs unstimulated controls. HGF exceeded the effect of glucose alone (2.2-fold at 3 mM glucose and 1.7-fold in the presence of 15 mM glucose). The HGF-induced INS-1 cell proliferation was further increased by addition of IGF-I or GH. Stimulation with HGF activated the JAK-2/STAT-5 pathway with a subsequent activation of phosphatidylinositol-3'-kinase (PI3'K). PI3'K activation was necessary for HGF- and glucose-stimulated INS-1 cell proliferation. The effect of PI3'K was mediated via 70 kDa S6 kinase and protein kinase B, which showed maximum activation in the presence of 3–6 mM glucose. Protein kinase C was essential for HGF-induced INS-1 cell proliferation. The HGF effect was also mediated at low glucose concentrations via insulin receptor substrate 4 (IRS-4) whereas other IRS proteins did not show any activation. High glucose concentrations also showed an increased IRS-4/PI3'K binding and therefore activation. In conclusion, β -cell proliferation is mediated via complex interacting signal transduction pathways. HGF, in contrast to other growth factors, seems to be of importance particularly in the presence of low glucose concentrations and therefore takes a special role in this complex concert.

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Introduction

The insulin-producing β -cells of the pancreatic islets are well-differentiated and consequently have a low mitotic index (Brockenbrough *et al.* 1988, Swenne 1992). Under normal conditions only 0.5% are undergoing mitosis (Brockenbrough *et al.* 1988). However, in certain clinical states, e.g. pregnancy and obesity, the β -cell proliferation rate can be considerably higher. Recent studies have revealed that nutrients such as amino acids or glucose in a physiologically relevant range (6–18 mM glucose) or changes in electrolyte concentrations (e.g. calcium ions) can instigate a marked increase in β -cell mitosis (Chick 1973, Swenne 1992, Hügl *et al.* 1998), whereas an inhibitory effect on β -cell mitosis has been seen in the presence of free fatty acids

(Cousin *et al.* 2001). The regulation of mitogenesis in pancreatic β -cells seems to be even more complex than in other eukaryotic cells due to the unique characteristic that the stimulus-coupling mechanisms of the β -cell are tightly linked to its metabolic state (Newgard & McGarry 1995, Prenki 1996).

Certain growth factors, in particular insulin-like growth factor (IGF-I) and growth hormone (GH), have been shown to enhance the number of replicating β -cells in rodent islets from 0.5% up to 6% (Nielsen *et al.* 1989, Billestrup & Nielsen 1991, Swenne 1992). Both IGF-I and GH stimulate β -cell proliferation glucose-dependently, but are using different, not completely understood signal transduction pathways mediating β -cell proliferation (Hügl *et al.* 1998, 1999). On the other hand,

glucose itself is able to induce activation of the signaling proteins Erk1/2 and 70 kDa S6 kinase (p70^{S6K}) independently of growth factors (Hügl *et al.* 1998). IGF-I stimulates β -cell proliferation mainly via tyrosine autophosphorylation activation of the IGF-I-receptor tyrosine kinase, which results in downstream tyrosine phosphorylation of insulin receptor substrate-2 (IRS-2) and SH2-containing protein (Shc), and a consecutive activation of phosphatidylinositol-3'-kinase (PI3'K), protein kinase B (PKB), the mammalian target of rapamycin, and p70^{S6K} (Hügl *et al.* 1998, Dickson *et al.* 2001). The proliferative effect of GH is mediated via the activation of the Janus kinase-2 (JAK-2) and the subsequent activation of the signal transducer and activator of transcription-5 (STAT-5) (Hügl *et al.* 1999).

Both growth factors, IGF-I and GH, stimulate β -cell growth independently and show a synergistic effect. There is no proof for any crosstalk between the JAK-2/STAT-5 and the IRS-2 pathway. Apparently, glucose metabolism is necessary to obtain the proliferative effect of IGF-I and GH (Hügl *et al.* 1998, 1999). Glucose mediates hereby the activation of protein kinase C (PKC) (Welsh *et al.* 1993, Hügl *et al.* 1998) and the elevation of Ca²⁺ and cAMP (Chick 1973, Swenne 1992, Frodin *et al.* 1995). Experiments with IRS-1 and IRS-2 knockout mice (IRS-1^{-/-}, IRS-2^{-/-}) support these findings: IRS-1^{-/-} mice showed no significant decrease in β -cell mass and β -cell proliferation, whereas IRS-2^{-/-} mice developed a severe lack of β -cell mass and stimulation of β -cell growth by IGF-I was not possible (Withers *et al.* 1998, 1999).

Intriguingly, another important mitogen signal transduction protein, the mitogen-activated protein kinase (MAPK), seems to be of minor importance in pancreatic β -cell proliferation. Only in the presence of low, subphysiological glucose concentrations was some activation detectable (Hügl *et al.* 1998, 1999, Dickson *et al.* 2001). But still the signal transduction pathways involved and their complex interactions are not fully resolved.

Besides IGF-I and GH, hepatocyte growth factor (HGF) is known to induce cell growth. HGF hereby activates the PI3'K and the MAPK signal transduction pathways (Boylan & Gruppuso 1998, Jehle *et al.* 1998, To & Tsao 1998). HGF is also known to stimulate islet cell proliferation, but most of the experiments were done in fetal islets

(Otonkoski *et al.* 1994, 1996, Beattie *et al.* 1996, 1997). But also in adult islet cells increased proliferation in the presence of HGF was seen (Hayek *et al.* 1995, Vila *et al.* 1995). Even more overexpression of HGF showed a marked increase in β -cells (Garcia-Ocana *et al.* 2000), but the effect of HGF on mitogen signal transduction pathways in pancreatic β -cells is still not completely understood.

In our experiments we used the well-differentiated β -cell line INS-1. INS-1 cells secrete insulin, glucose-dependently (Asfari 1992), and show a somewhat higher mitotic index than primary islets cells. The proliferation of this cell line is stimulated by nutrients and several growth factors (Hügl *et al.* 1998, 1999). So the INS-1 cell is a well-defined, convenient model to study the proliferation of pancreatic β -cells. The aim of the following experiments was to get a deeper insight in the complex mechanisms which activate the proliferation of pancreatic β -cells and to find out how different growth factors like HGF, IGF-I and GH interact in terms of mitogen transduction pathways.

Materials and methods

Materials

[Methyl-³H]thymidine (20 Ci/mmol) was from NEN (Boston, MA, USA). IGF-I, HGF and all protein activity inhibitors were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Anti-phospho-MAPK antiserum was from Promega Corp. (Madison, WI, USA), anti-IRS-1 and -2 antisera were a gift from Dr M Myers (Joslin Diabetes Center, Boston, MA, USA), all the other antisera were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Transblot nitrocellulose membrane (0.45 μ m pore size) was from Biorad (Hercules, CA, USA), and the chemiluminescence detection kit (ECL+) from Amersham International (Amersham, Bucks, UK). All the other chemicals were purchased from Sigma Chemical Co. (Deisenhofen, Germany) or Merck (Darmstadt, Germany) and were of the highest purity available.

Cell culture

All experiments were carried out using the glucose-sensitive pancreatic cell line INS-1 (Asfari

1992). The INS-1 cells were maintained at 37 °C in 5% CO₂ in RPMI 1640 medium containing 50 µM β-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% fetal calf serum, 11.2 mM glucose, 100 units/ml penicillin and 100 µg/ml streptomycin as described (Asfari 1992) and were subcultured at 80% confluence.

[³H]Thymidine incorporation

Thymidine incorporation was used to quantify INS-1 cell proliferation (Hofmann *et al.* 1989, Frodin *et al.* 1995). [³H]Thymidine incorporation was more suited for INS-1 cell proliferation than other methods such as cell number counting because INS-1 cells grow adherently and are not easy to separate to get quantitatively representative data. INS-1 cells (10⁵/well) were subcultured in 96-well plates and incubated for 48 h at 37 °C in 5% CO₂ in RPMI 1640 medium containing 50 µM β-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% fetal calf serum, 11.2 mM glucose, 100 units/ml penicillin and 100 µg/ml streptomycin as described. The medium was then replaced by RPMI 1640 with 0.1% BSA instead of serum and no glucose and the cells starved for 24 h. Cell growth was then stimulated for 24 h at 37 °C in 5% CO₂ by different glucose concentrations (0–24 mM) with or without HGF and with or without various protein activity inhibitors. [³H]Thymidine (5 µCi/ml) was added for the last 4 h of this incubation period to assess the proliferation rate of the INS-1 cells. The cells were then lysed using a semiautomatic cell harvester (Inotech, Basel, Switzerland) and the lysates transferred to glass fiber micropore filters (Packard, Meriden, CT, USA). The INS-1 cell DNA was trapped on the filters and the incorporated [³H]thymidine counted by liquid scintillation counting. All experiments were done in triplicates; data are presented as means ± S.E. of at least five independent experiments. Statistically significant differences between groups were analyzed using Student's *t*-test; *P* < 0.05 was considered statistically significant.

Protein preparation

INS-1 cells were subcultured in 10 cm dishes to about 50% confluence and made quiescent by glucose and serum deprivation as described before.

The cells were then incubated in fresh RPMI 1640 medium containing 0–24 mM glucose with or without 1.375 nM HGF for 5–60 min and the cells then lysed in 0.5 ml of ice-cold lysis buffer (50 mM Hepes, pH 7.5, 1% (v/v) Nonidet P40, 2 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 4 mM EDTA, 10 µM leupeptin, 10 µg/ml aprotinin and 100 µM phenylmethylsulfonyl fluoride) as described previously (Cheatham *et al.* 1994, Myers *et al.* 1994). Cell lysates were stored at –80 °C. Protein assays were done using the bicinchoninic acid method (Pierce, Rockford, IL, USA).

Protein immunoblot and co-immunoprecipitation analysis

Mitogenic signal transduction protein expression and protein tyrosine phosphorylation were tested using immunoblot analysis, stimulated protein–protein interactions between mitogenic signal transduction proteins using co-immunoprecipitation analysis as described previously (Cheatham *et al.* 1994, Myers *et al.* 1994). A horseradish peroxidase-based chemiluminescence reaction was used as the secondary detection method. Fifty to seventy-five micrograms of INS-1 cell total protein lysate were used for immunoblot analysis, 750 µg for co-immunoprecipitation analysis.

Results

HGF stimulates INS-1 cell proliferation in the presence of physiological and especially of subphysiological glucose concentrations

The effect of different glucose concentrations (0–24 mM) and HGF (1375 nM) on INS-1 cell proliferation was determined by [³H]thymidine incorporation. INS-1 cells were used as a model to examine pancreatic β-cell proliferation as they respond, unlike the majority of other pancreatic β-cell lines, to glucose in the physiologically relevant range (6–18 mM glucose) in terms of insulin secretion (Asfari 1992) and cell proliferation (Hügl *et al.* 1998, 1999). INS-1 cells show a somewhat higher mitotic index than pancreatic β-cells and other slower-growing glucose-responsive β-cell lines and are therefore a convenient tool to

generate information on the proliferation of differentiated pancreatic β -cells, even if the results should be interpreted with some reservation as insulinoma-derived cell lines always show a degree of aberrant growth control. As demonstrated previously (Hügl *et al.* 1998, 1999) glucose induced INS-1 cell proliferation up to 30-fold measured by [3 H]thymidine incorporation, compared with unstimulated controls, in a dose-dependent manner. Maximal stimulation was seen at 8 mM glucose (Fig. 1). Addition of HGF showed an increase in INS-1 cell proliferation up to 45-fold compared with unstimulated controls (Fig. 1). HGF was hereby able to stimulate INS-1 cell proliferation even in the presence of subphysiological glucose concentrations and augmented the effect of glucose alone 2.2-fold at 3 mM glucose. The maximum effect of HGF-induced INS-1 cell proliferation was seen in the presence of 8 mM glucose + 1.375 nM HGF, but the additional effect of HGF over stimulation with glucose alone was only 1.5-fold. In a series of dose-finding experiments (data not shown), HGF showed the optimum effect at very low concentrations. The best results were seen at 1.375 nM HGF. Higher concentrations (up to 275 nM HGF) did not yield a better proliferation rate (data not shown). So subsequently 1.375 nM HGF was used as the standard concentration in all following experiments. Insulin secretion in these cells was thereby increased (1532 ng/mg total protein in HGF+glucose-stimulated cells vs 997 ng/mg in glucose-only-stimulated INS-1 cells), demonstrating that HGF-stimulated cells show no sign of dedifferentiation.

The glucose- and dose-dependent stimulating effect of HGF showed similarities to that instigated by IGF-I or GH (Hügl *et al.* 1998, 1999). However, coinubation of INS-1 cells with HGF+IGF-I and HGF+GH revealed distinct differences; INS-1 cells instigated with HGF+IGF-I showed an increase of [3 H]thymidine incorporation up to 1.7-fold, which was significant, especially in the presence of glucose concentrations >12 mM ($P < 0.05$, Fig. 2). Coinubation of INS-1 cells with HGF and GH as well showed an increase in cell proliferation up to 1.7-fold, but this time the increase above HGF alone was significant only in the presence of low glucose concentrations up to 9 mM ($P < 0.05$, Fig. 2). In the presence of higher glucose concentrations this effect was still seen as a trend, but it was not significant ($P > 0.05$, Fig. 2).

The effects of various protein phosphorylation inhibitors on glucose- and HGF-stimulated INS-1 cell proliferation

The effect of different specific protein kinase and phosphatase inhibitors on INS-1 cell proliferation in the presence of 15 mM glucose with or without 1.375 nM HGF was examined using [3 H]thymidine incorporation (Table 1). In these series of experiments 15 mM glucose increased [3 H]thymidine incorporation about 20-fold compared with equally treated cells in the absence of glucose ($P < 0.001$), whereas the combination of 15 mM glucose and 1.375 nM HGF augmented [3 H]thymidine incorporation in INS-1 cells about 35-fold ($P < 0.001$) compared with unstimulated controls as described previously (Fig. 1).

Addition of wortmannin (10 nM), a specific inhibitor of PI3'K activity (Ogreid *et al.* 1994), significantly reduced 15 mM glucose-stimulated INS-1 cell proliferation by 90% ($P < 0.001$, Table 1) and proliferation stimulated by 15 mM glucose + 1.375 nM HGF by 75% ($P < 0.001$, Table 1).

Rapamycin is a specific inhibitor of p70^{S6K}, which is activated downstream of PI3'K (Kardalidou *et al.* 1994). Rapamycin (10 nM) added to INS-1 cells stimulated with 15 mM glucose reduced [3 H]thymidine incorporation by 60% ($P < 0.001$), whereas INS-1 cells stimulated with 15 mM glucose + 1.375 nM HGF showed a reduction by 50% ($P < 0.001$) (Table 1).

Apparently, PI3'K-mediated proliferation in INS-1 cells uses different signal transduction pathways. Inhibition of MEK, the activator of MAPK, by PD98059 (50 μ M) (Pang *et al.* 1995) did not change the proliferation rate of INS-1 cells significantly either in the presence of glucose ($P = 0.3$) or in the presence of glucose+HGF ($P = 0.4$) (Table 1). This suggests that the MAPK pathway plays no important role in the HGF-mediated INS-1 cell proliferation.

Inhibition of PKC by sphingosine (10 μ M) (Liu 1996) resulted in about 57% reduction of HGF-stimulated [3 H]thymidine incorporation ($P < 0.001$) (Table 1). Besides inhibiting PKC, staurosporine also inhibits protein kinase G (PKG) and protein kinase A (PKA) (Sjoholm 1997) but had no additional inhibitory effect compared with sphingosine (Table 1). These findings give some hint that certain PKC isoforms might be involved in the regulation of HGF-mediated INS-1 cell

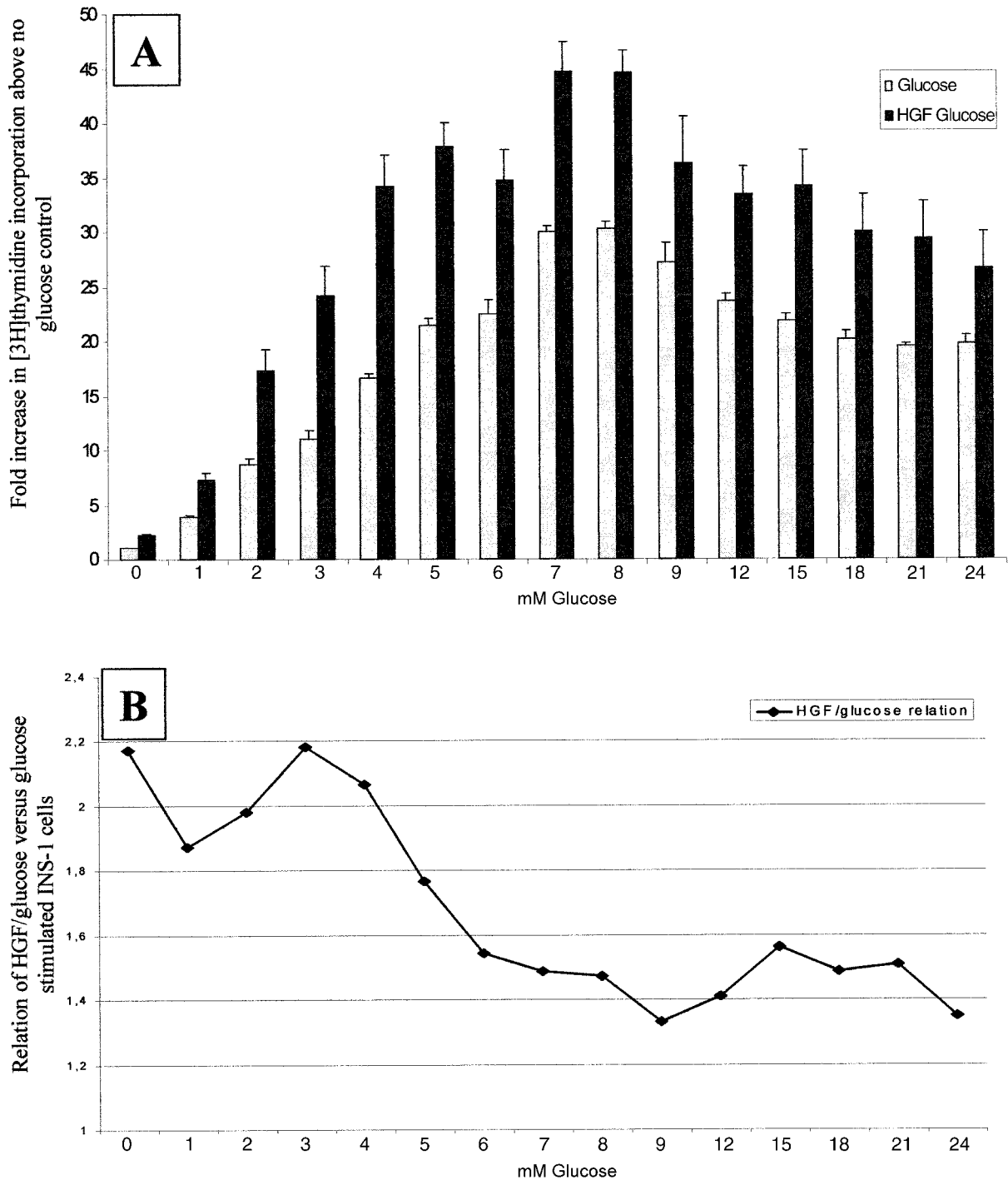


Figure 1 [³H]Thymidine incorporation in INS-1 cells with different glucose concentrations. (A) Approximately 10⁵ quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA and 0–24 mM glucose with or without 1.375 nM hepatocyte growth factor (HGF), then assessed for proliferation rate by [³H]thymidine incorporation as described in Materials and methods. All experiments were done in triplicates on at least eight independent occasions. Data are presented as x-fold increase above the control observation in the absence of glucose and HGF (i.e. 3000–4000 c.p.m./10⁵ cells), depicted as means ± s.e. (n ≥ 8). (B) Relative increase in [³H]thymidine incorporation in HGF-stimulated INS-1 cells at different glucose concentrations vs glucose-only-stimulated cells.

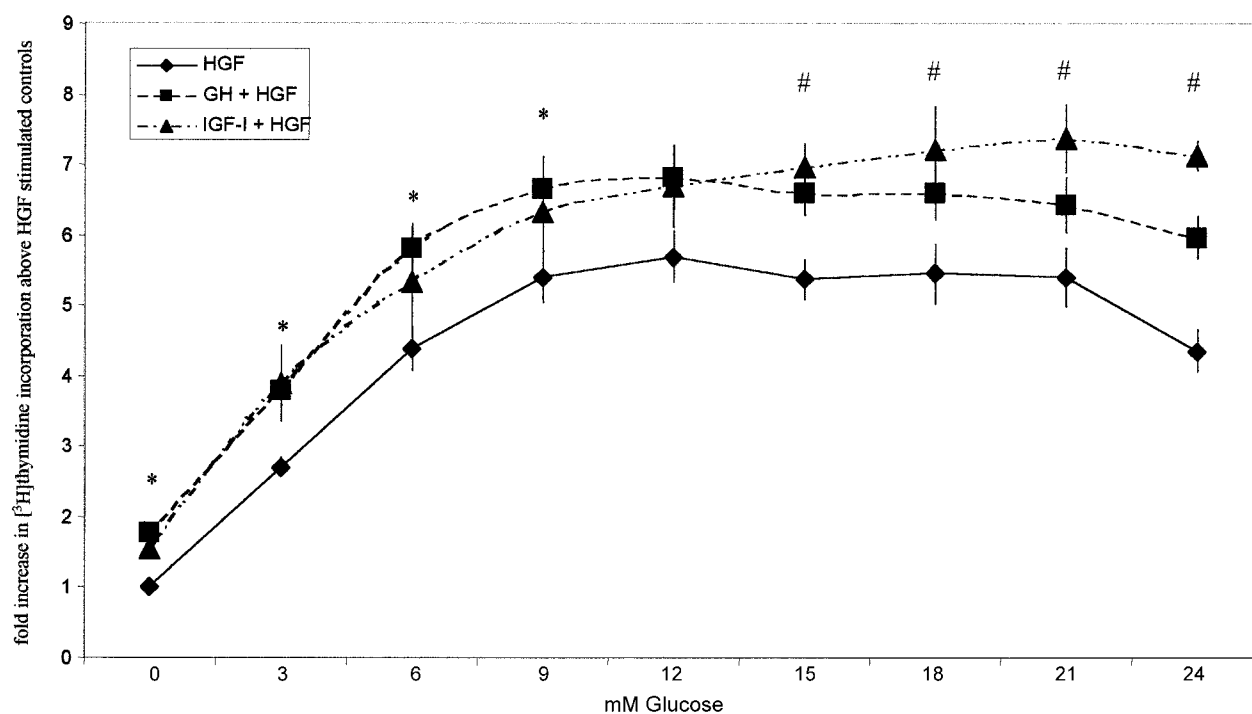


Figure 2 [^3H]Thymidine incorporation in INS-1 cells stimulated with 1.375 nM HGF with or without additional growth factors. Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 0–24 mM glucose and 1.375 nM HGF with or without 10 nM IGF-I or 1.375 nM HGF with or without 10 nM GH, then assessed for proliferation rate by [^3H]thymidine incorporation as described in Materials and methods. All experiments were done in triplicate on at least five independent occasions. Data are presented as x-fold increase above the control observation in the presence of 1.375 nM HGF, depicted as means \pm s.e. ($n \geq 5$). *Significant increase in proliferation of cells stimulated with GH+HGF vs HGF-stimulated controls; #significant increase in proliferation of cells stimulated with IGF-I+HGF ($P < 0.05$).

proliferation as well as in the glucose-stimulated cell proliferation.

Intracellular Ca^{2+} concentration is essential for cell proliferation (Frodin *et al.* 1995, Khoo & Cobb 1997). In our experiments we used calmidazolium (50 nM) to inhibit Ca^{2+} /calmodulin-dependent proteins, which reduced [^3H]thymidine incorporation in INS-1 cells stimulated with HGF+glucose by 80% ($P < 0.001$). These data underline the essential role of Ca^{2+} /calmodulin for the growth of pancreatic β -cells (Table 1).

Protein phosphatases seem to play an important role in HGF-stimulated INS-1 cell proliferation as well. Inhibition of protein tyrosine phosphatases by orthovanadate (0.5 mM) completely inhibited INS-1 cell proliferation, and cyclosporin A (5 μM) showed the same effect, completely inhibiting phosphoprotein phosphatase B2 (Table 1).

HGF-mediated mitogenic signal transduction pathways in INS-1 cells

Protein phosphorylation activation of mitogenic signal transduction pathways by 1.375 nM HGF with or without 3, 6 or 15 mM glucose in INS-1 cells was investigated using co-immunoprecipitation and immunoblot analysis. Immunoprecipitation with an anti-phosphotyrosine (PY) antibody followed by immunoblot analysis with anti-PKC antiserum showed an increase in PY phosphorylation of PKC after a 10 min exposure to HGF (1.375 nM) in the presence of glucose concentrations up to 6 mM compared with stimulation with glucose alone (Fig. 3). Above 6 mM glucose no additional increase in PKC activation was seen.

Immunoprecipitation of the 85 kDa regulatory subunit of PI3'K (PI3'K p85) followed by immunoblot analysis with antiserum to recognize PKB showed a maximum binding in the presence

Table 1 The effect of protein phosphorylation inhibitors on glucose- and HGF-stimulated [³H]thymidine incorporation in INS-1 cells. Approximately 10⁵ quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 15 mM glucose, 1.375 nM HGF, various inhibitors of protein kinases, phosphoprotein phosphatases, or tyrosine kinases as indicated, then assessed for proliferation rate by [³H]thymidine incorporation as described in Materials and methods. All experiments were done in triplicate on at least five independent occasions. The data are presented as a percentage of either the [³H]thymidine incorporation at 15 mM glucose in the absence of HGF or the [³H]thymidine incorporation at 15 mM glucose+1.375 nM HGF, and are means±s.e. (n=5). Significant changes are marked (*)

Inhibitor	Inhibited protein	[³ H]thymidine incorporation (%)	
		In 15 mM glucose	In 15 mM glucose+ 1.375 nM HGF
Control	—	100.0±4.4	100.0±2.6
Wortmannin (10 nM)	PI3'K	9.90±0.7*	25.9±6.2*
PD98059 (50 mM)	MAPK	94.5±5.7	98.7±3.1
Rapamycin (10 nM)	p70 ^{S6K}	33.4±2.1*	50.1±5.2*
Sphingosine (10 mM)	PKC	32.4±3.6*	42.9±5.4*
Staurosporine (20 nM)	PKC, PKA, PKG	52.8±3.2*	58.3±3.1*
Cyclosporin A (5 mM)	Phosphoprotein phosphatase 2B	27.0±3.5*	31.1±5.0*
Orthovanadate (0.5 mM)	Protein tyrosine phosphatase	6.5±0.7*	11.8±3.0*
Calmidazolium (50 nM)	Calmodulin	4.6±0.7*	17.9±6.1*

of 1.375 nM HGF and 3 mM glucose. Above this glucose concentration less association was seen in the presence of HGF but still there was an increased activity compared with 0 mM glucose (Fig. 4). Immunoprecipitation with PI3'K p85 and subsequent immunoblot analysis with the upstream IRS-1 showed a slight increase in IRS-1/PI3'K binding in the presence of HGF at low glucose concentrations up to 6 mM (Fig. 4), whereas IRS-2/PI3'K binding was not augmented in the presence of HGF (data not shown). Intriguingly, IRS-4 showed an increased binding to PI3'K in the presence of higher glucose concentrations (maximum binding at 15 mM glucose), whereas in the presence of HGF an increased PI3'K binding was seen only at low glucose concentrations (Fig. 4).

Immunoprecipitation with anti-PY antibody followed by immunoblot analysis with anti-STAT-5 antisera showed an increased binding after stimulation with HGF and 0 or 3 mM glucose, which diminished at higher glucose concentrations (Fig. 3). Immunoprecipitation with anti-PY and immunoblotting with anti-STAT-5 showed as well an increased association in the presence of HGF and low glucose concentrations (Fig. 3).

p70^{S6K} is activated downstream of PI3'K (Kadowaki *et al.* 1996). Phosphorylation activation of p70^{S6K} occurs at multiple sites and can be

detected on immunoblot analysis by electrophoresis mobility retardation (Cheatham *et al.* 1994). Maximal activation occurs 30 min after stimulation (Hügl *et al.* 1998, 1999). Immunoblot analysis with p70^{S6K}-specific antiserum showed phosphorylation activation in the presence of HGF with a maximum activation at 3 mM glucose. Above 6 mM glucose, HGF-induced phosphorylation activation of p70^{S6K} diminished (Fig. 5) whereas stimulation with glucose alone showed maximum activation at 15 mM glucose.

Immunoblot analysis with specific antibodies against phosphorylated MAPK did not show a significant difference after stimulation with HGF (data not shown). Neither did the upstream proteins mSOS (murine sons of sevenless-1 protein) or growth factor-bound protein-2 show an increased binding to Shc and subsequent activation in the presence of HGF (data not shown), indicating that the MAPK pathway does not play an important role in the HGF-instigated proliferation of INS-1 cells.

Discussion

HGF has been shown to stimulate pancreatic β -cell proliferation and differentiation (Otonkoski *et al.* 1994, 1996, Hayek *et al.* 1995, Vila *et al.* 1995, Beattie *et al.* 1996, 1997, Garcia-Ocana *et al.* 2000).

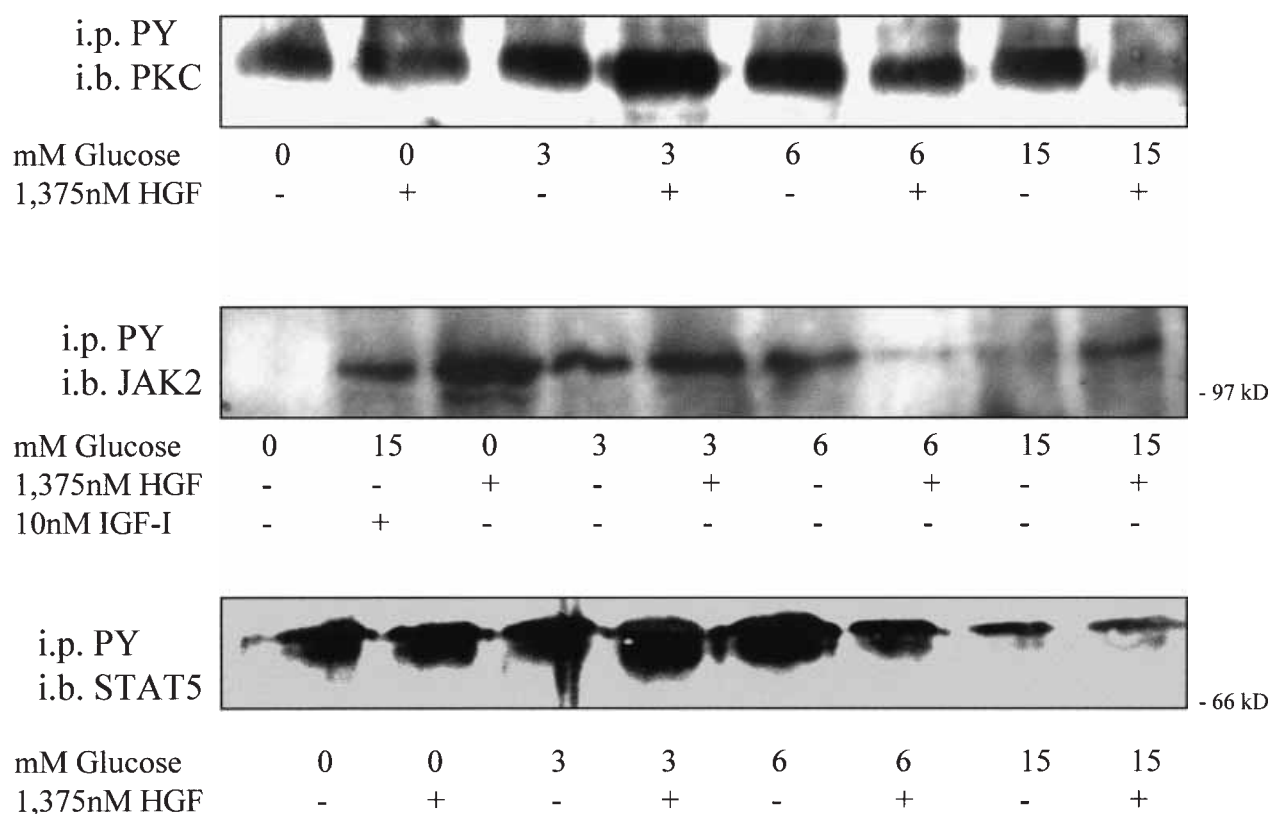


Figure 3 HGF and glucose both increase protein PY phosphorylation of PKC, JAK-2 and STAT-5. INS-1 cells (50% confluent in a 15 cm diameter dish) were stimulated with 3–15 mM glucose with or without 1.375 nM HGF for 10 min, and cell lysates generated as outlined in Materials and methods. INS-1 cell lysates were then subjected to immunoprecipitation (i.p.) with antiserum against PY. Immunoprecipitates were then subjected to immunoblot (i.b.) analysis with anti-PKC (upper panel), anti-JAK-2 (middle panel) and anti-STAT-5 (lower panel) antibodies as described in Materials and methods. A representative blot for such co-immunoprecipitation analysis is shown.

However, the HGF-induced mechanisms still remain unclear. In this study, we show that HGF stimulates proliferation of the glucose-dependent β -cell line INS-1 particularly in the presence of low glucose concentrations (Fig. 1). At higher glucose concentrations the proliferative effect of HGF above glucose alone diminished from 2.2-fold at 3 mM glucose to 1.3-fold at 24 mM glucose. This differs from the effects of other growth factors like IGF-I or GH where the stimulatory effect was maximal at 15 mM glucose (about 4-fold in IGF-I-stimulated cells) and only about 1.5-fold at low glucose concentrations (Hügl *et al.* 1998, 1999). Costimulation of INS-1 cells with HGF and IGF-I showed a synergistic increase in [3 H]thymidine incorporation, which was significant in the presence of glucose concentrations above 12 mM glucose (Fig. 2). It can be speculated whether

different growth factors cover different nutritional ranges to secure β -cell proliferation and that HGF is mostly active in the presence of physiological or subphysiological glucose conditions whereas IGF-I or GH are more important in the case of high glucose concentrations. But as these experiments were done in INS-1 cells, further experiments are required before transferring the results to primary cells. For example it might be possible that INS-1 cells show a glucose optimum different from human primary islet cells and that activation of the shown signal transduction pathways occurs at a different glucose range in human cells. But the results suggest that the effect of IGF-I and HGF at least in part activate different signal transduction mechanisms. In contrast, costimulation of HGF and GH shows a different pattern (Fig. 2). Here the increase in cell proliferation was only significant above the

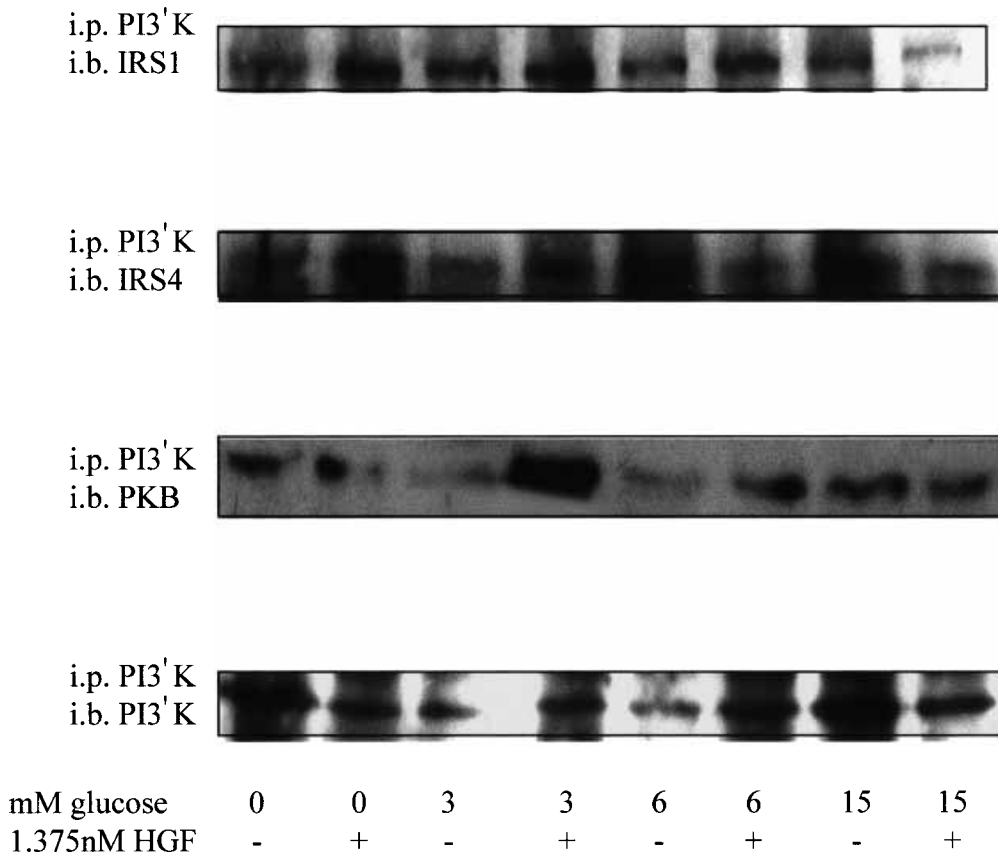


Figure 4 HGF and glucose both increase the association of PI3'K with PKB and IRS-4. INS-1 cells (50% confluent in a 15 cm diameter dish) were stimulated with 3–15 mM glucose with or without 1.375 nM HGF for 10 min, and cell lysates generated as outlined in Materials and methods. INS-1 cell lysates were then subjected to immunoprecipitation (i.p.) with antiserum against the p85 regulatory subunit of PI3'K. Immunoprecipitates were then subjected to immunoblot (i.b.) analysis with antibodies to IRS-1 (upper panel), IRS-4 (second panel), PKB (third panel) and PI3'K (lower panel), as described under Materials and methods. An example blot for such co-immunoprecipitation analysis is shown.

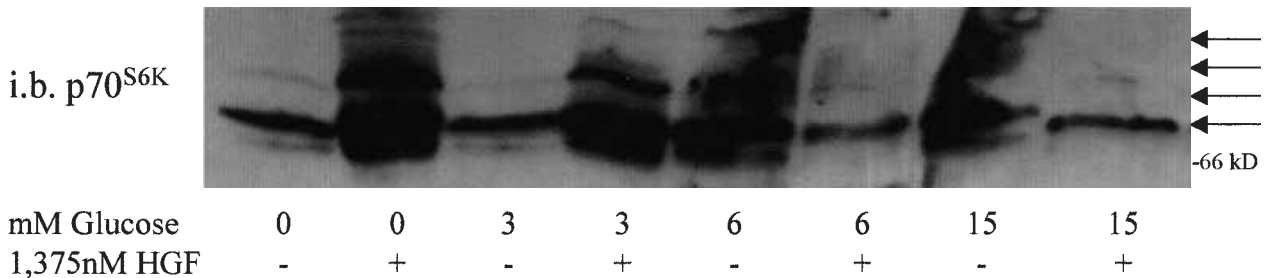


Figure 5 HGF and glucose both stimulate phosphorylation activation of p70^{S6K} in INS-1 cells. INS-1 cells (50% confluent in a 15 cm diameter dish) were stimulated with 3–15 mM glucose with or without 1.375 nM HGF for 30 min, and cell lysates generated as outlined in Materials and methods. Specific phosphorylation activation of p70^{S6K} was examined in the INS-1 cell lysates by immunoblot (i.b.) analysis, as described under Materials and methods. A representative immunoblot for p70^{S6K} is shown. Phosphorylated forms of p70^{S6K} become retarded on SDS-PAGE analysis, and these multi-phosphorylated p70^{S6K} forms are indicated by arrows.

stimulation with HGF and glucose alone in the presence of low glucose concentrations up to 9 mM glucose. An explanation might be the activation of IRS-4 which is induced by HGF at low glucose concentrations and at high glucose concentrations, but at high glucose concentrations apparently there is no additional activation of IRS-4 (Fig. 4) and therefore no additional increase in proliferation occurs. The other activation seemed to be mediated via activation of the JAK-2/STAT-5 signal transduction pathway, which is the same as seen in GH-instigated cell proliferation (Hügl *et al.* 1999). In contrast, IGF-I-induced mitogen signal transduction is mediated via IRS-2 (Hügl *et al.* 1998), which is not activated by HGF and explains the increase in [³H]thymidine incorporation after costimulation of INS-1 cells with HGF and IGF-I.

HGF-induced β -cell proliferation was hereby dependent on PI3'K activation; inhibition of PI3'K activity by the specific protein activity inhibitor wortmannin abolished any INS-1 cell proliferation (Table 1). This was an effect similar to that seen in experiments with IGF-I or GH (Hügl *et al.* 1998, 1999). Inhibition of PI3'K completely abolished INS-1 cell proliferation, which showed no significant differences from non-stimulated β -cells ($P=0.08$ for glucose-stimulated, $P=0.2$ for cells stimulated with HGF+glucose). Therefore PI3'K seems to have a central role in glucose- and growth factor-stimulated pancreatic β -cell proliferation. But PI3'K activation by different growth factors occurs via different signal transduction pathways (Krasilnikov 2000, Reddy *et al.* 2000, Vanhaesebroeck & Alessi 2000). HGF and GH hereby use the JAK-2/STAT-5 pathway to activate PI3'K whereas IGF-I-induced stimulation is mediated via IRS-2. Interestingly, HGF showed the most effective signal transduction protein activation in the presence of 3–6 mM glucose, whereas GH showed an optimum effect in the presence of 6–15 mM glucose (Hügl *et al.* 1999). This explains why coincubation of HGF and GH only showed a slight but not significant increase in [³H]thymidine incorporation, while HGF and IGF-I had a synergistic effect on INS-1 cell proliferation (Fig. 2). Downstream of PI3'K these growth factors all use at least in part similar pathways mediating their mitogenic effects via activation of p70^{S6K} and PKB (Figs 3–5) (Cheatham *et al.* 1994, Myers *et al.* 1994, Hügl *et al.* 1998, Dickson *et al.* 2001).

The MAPK pathway seems to be of less importance in HGF-mediated INS-1 cell proliferation. Inhibition of MAPK activity by PD98059 showed no significant decrease in HGF-stimulated β -cell proliferation (Table 1, $P=0.4$). HGF did not change the activity of MAPK nor did the upstream signaling proteins (Shc, mSOS, MEK) (data not shown). But still MAPK activation seems to be involved in the activation of IGF-I-mediated INS-1 cell proliferation as a completion in the complex concert of the mitogen signal transduction pathways where a balanced activation is necessary (Dickson *et al.* 2001). In GH-mediated proliferation, MAPK seems to be of less importance, as seen in HGF-activated cell proliferation (Hügl *et al.* 1999).

Another important factor in HGF- and glucose-mediated β -cell proliferation is the intracellular Ca²⁺ level. Inhibition of the calmodulin kinase by calmidazolium leads to a complete inhibition of HGF-stimulated (88%, $P<0.001$) and glucose-stimulated (95%, $P<0.001$) INS-1 cell proliferation (Table 1). In contrast, IGF-I-stimulated cell proliferation showed no significant decrease in the presence of calmidazolium (Hügl *et al.* 1998) nor did that of GH (Hügl *et al.* 1999). These findings provide evidence that calmodulin-dependent proteins are of importance only for glucose- and HGF-stimulated INS-1 cell proliferation. PKC as well seems to be an important factor in β -cell proliferation. PKC activation occurred most in the presence of 3–6 mM glucose in the presence of HGF (Fig. 3) while inhibition of PKC by sphingosine significantly decreased INS-1 cell proliferation mediated by glucose (68%, $P<0.001$), HGF (57%, $P<0.001$) (Table 1), IGF-I (58%, $P<0.001$) (Hügl *et al.* 1998) and GH (52%, $P<0.001$) (Hügl *et al.* 1999). PKA and PKG activation also seems to be necessary in some way for glucose- and HGF-instigated cell proliferation, as demonstrated by our inhibition studies (Table 1), whereas GH- and IGF-induced cell proliferation does not significantly decrease after inhibition of PKA and PKG by staurosporine (Hügl *et al.* 1998, 1999) even if these inhibitor experiments should be interpreted with some precautions since these inhibitors are not sufficiently specific. The activation of these signal transduction pathways explains the proliferative effect of HGF at low glucose concentrations but fails to explain that there is still a proliferation of INS-1 cells in the presence of high glucose concentrations (Fig. 1).

Further experiments are required to identify the missing links in the mitogenic signal transduction cascades.

In summary this study establishes that HGF-induced signaling transduction via IRS-4 and JAK-2/STAT-5 and subsequent PI3'K activation is present in the pancreatic β -cell line INS-1. The HGF effect is glucose-dependent and occurs even in the presence of subphysiological glucose concentrations. The MAPK pathway seems to be of less importance for HGF-stimulated β -cell proliferation. HGF activates similar pathways to glucose, which explains the reduced effect of HGF in the presence of high glucose concentrations. It will be important in future studies to identify the appropriate transcription factors which enable the crosstalk between the different pathways and the signal transduction factors which are activated downstream of PI3'K, PKB and p70^{S6K}. Finally, the results have to be transferred to primary cells to find the optimum conditions for human β -cells.

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