# A Specific Increased Expression of Insulin Receptor Substrate 2 in Pancreatic β-Cell Lines Is Involved in Mediating Serum-Stimulated β-Cell Growth

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**Certain nutrients and growth factors can stimulate pancreatic β-cell growth. However, the appropriate mitogenic signaling pathways in β-cells have been relatively undefined. In this study, differential gene expression in NEDH rat insulinoma was compared with NEDH rat primary islet β-cells. Differential mRNA display analysis revealed an elevated expression in insulinoma of VL30 transposons, S24 ribosomal protein, and cytochrome-C oxidaseVIIc that is typical for cells undergoing mitosis. A gene candidate approach revealed that mRNA levels of the oncogenes c-*fos* and c-*jun* were equivalently expressed in insulinoma and islet cells, as was the mRNA for the mitogenic signal transduction molecule insulin receptor substrate (IRS)-1. However, in contrast to that of IRS-1, IRS-2 gene expression was 60- to 70-fold higher in the insulinoma tissue compared with islets, which was reflected at the protein as well as the mRNA level. The specific elevated IRS-2 expression was a consistent observation across all rodent pancreatic β-cell lines. To investigate whether IRS-2 was functional, serum-stimulated β-cell proliferation was examined in isolated insulinoma cells. After a 48-h period of serum withdrawal, 24 h of serum refeeding rendered an 8- to 10-fold increase in [$^3$H]thymidine incorporation into insulinoma cells. This serum-stimulated DNA synthesis was prevented by inhibitors of tyrosine protein kinase and phosphatidylinositol (PI) 3-kinase activities, as well as the activation of mitogen-activated protein (MAP) kinase and p70$^{S6K}$. Examination of IRS-mediated signal transduction pathways indicated that after 10–15 min of serum refeeding, there was increased tyrosine phosphorylation of IRS-2 and pp60, and PI 3-kinase recruitment to IRS-2. Serum also increased the association of growth factor–bound protein 2/ murine sons of sevenless 1 protein to a PI 3-kinase/IRS-2 protein complex. Moreover, serum also activated MAP-kinase (erk-1 and erk-2 isoforms) and 70 kD S6 kinase. Thus IRS-mediated signal transduction pathways are functional in pancreatic β-cells. It is conceivable that IRS-2 expression in β-cells contributes to maintaining the islet β-cell population, complementary to observations in the IRS-2 knockout mouse in which β-cell mass is markedly reduced.**
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Pancreatic islet β-cell growth can be mediated by two separate mechanisms (1,2). Either new islets can form from the budding of the pancreatic ductule epithelium (neogenesis) or the replication of existing islet β-cells (2). Neogenesis of islets primarily occurs during fetal and perinatal stages of development (1), but it has also been observed in the regenerating adult pancreas (3). Replication of pancreatic β-cells has been observed in late fetal stages, but it is thought to be the principal means of maintaining (or even increasing) β-cell mass in adults (1,2). The replication rate of existing β-cells appears to be determined by the number of cells able to enter into cell division cycle ($G_1$-, S-, $G_2$-, and M-phases) from the quiescent $G_0$ phase (1). However, in a population of normal adult pancreatic islet cells, the number of β-cells undergoing cell division is small (~0.5–2%) (4).

Several nutrient factors have been shown to increase the number of replicating β-cells within adult and fetal islets, including glucose and certain essential amino acids (1,2). However, in response to glucose, the number of β-cells entering mitogenesis within an islet increases to only ~5% of the total islet β-cell population. Glucose metabolism is required to induce β-cell mitogenesis (2), but it is not yet clear what secondary signals emanate from glucose metabolism to induce β-cell growth. One possibility is that glucose instigates a rise in intracellular cAMP that is concurrent with a modest increase in the islet β-cell growth effected by glucose (1). Indeed, glucagon and forskolin, which enhance β-cell cAMP levels, also modestly stimulate β-cell replication (1,5). Similarly, glucose-mediated increase in β-cell proliferation has been postulated to act via glucose activation of phospholipase C–γ and protein kinase C activities (6). Alternatively, glucose-

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BSA, bovine serum albumin; EGF, epidermal growth factor; FBS, fetal bovine serum; GH, growth hormone; Grb, growth factor–bound protein; HBSS, Hanks' balanced salt solution; IBMX, isobutylmethylxanthine; IRS, insulin receptor substrate; MAP, mitogen-activated protein; MEK, MAP-kinase kinase; mSOS, murine sons of sevenless 1 protein; p70$^{S6K}$, 70 kD S6 kinase; p85$^{PAN}$, p85 subunit of PI 3-kinase; PCR, polymerase chain reaction; PI, phosphatidylinositol; PMA, phorbol myristic acid; PPI, pre-proinsulin; PY, anti-phosphotyrosine; RITz cells, NEDH rat insulinoma tissue cells; RT, reverse transcription.

stimulated β-cell growth may be mediated via glucose-induced activation of mitogen-activated protein (MAP) kinase (erk-1 and erk-2 isoforms) that requires elevation of cytosolic $Ca^{2+}$ (7,8).

Several growth factors have also been shown to stimulate pancreatic β-cell replication (1,6). The best characterized are somatolactogeneic hormones (i.e., growth hormone [GH] and prolactin), which can increase the number of β-cells replicating within an islet to around 6% (9,10), reminiscent of that observed in vivo during pregnancy (11). Both GH and prolactin receptors are found on rodent pancreatic islet β-cells (12). It has been suggested that GH mediates its growth effect on pancreatic β-cells independently, or via stimulating local IGF-1 production, which in turn induces a paracrine or autocrine stimulation of β-cell replication (1). Indeed, IGF-1 has been shown to stimulate pancreatic β-cell growth in its own right (1,13). In addition, hepatocyte growth factor (14) and nerve growth factor (15) have also been implicated in pancreatic β-cell mitogenesis and differentiation. In contrast, epidermal growth factor (EGF) does not appear to affect β-cell replication, even though significant EGF-binding to β-cells has been observed (16). This may imply that elements of the EGF signal transduction mechanism are not appropriately active or expressed in pancreatic β-cells. However, the fact remains that mitogenic-signaling pathways in pancreatic β-cells have not been particularly well characterized.

In this study, we examined mitogenic-signaling pathways in pancreatic β-cells. A comparison of a model of rapidly growing β-cells, rat insulinoma cells (17,18), versus a model population of slow-growing β-cells, normal isolated pancreatic islet β-cells (1,2), was pursued to find elevated expression of certain genes in rapidly growing β-cells that could be implicated in the mechanism of β-cell mitogenesis. Both differential mRNA display and a candidate gene approach were applied. This approach indicated that insulin receptor substrate (IRS)-2 is markedly overexpressed in insulinoma cells; further characterization studies suggested that IRS-2 protein was functional in a mitogenic-signal transduction pathway. Serum-stimulated insulinoma cell growth could be mediated by increased tyrosine phosphorylation of IRS-2, increased IRS-2/phophatidylinositol (PI) 3-kinase association, and activation of both MAP-kinase and 70 kD S6 kinase ($p70^{S6K}$).

## RESEARCH DESIGN AND METHODS

**Materials.** L-[$^{35}$S]methionine (1,175 Ci/mmol) and [γ-$^{32}$P]cytidine 5′-triphosphate (800 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). Collagenase P (1.5 U/mmol) and fatty acid ultra-free bovine serum albumin (BSA; fraction V) were obtained from Boehringer Mannheim (Indianapolis, IN). Bovine insulin antiserum was obtained from Sigma (St. Louis, MO), antibodies against the 85-kD regulatory subunit of PI 3-kinase, murine sons of sevenless 1 protein (mSOS), growth factor–bound protein (Grb) 2, $p70^{S6K}$, activated phospho-MAP-kinase (erk-1 and erk-2), and total MAP-kinase (erk-1 and erk-2) were purchased from Upstate Biotechnology (Lake Placid, NY). Specific phosphotyrosine and IRS-1 and IRS-2 antisera were generated as previously described (19,20). Pansorbin, genistein, wortmannin, LY294002, PD98059, and rapamycin were purchased from Calbiochem (La Jolla, CA). Biotrans nylon membranes (0.2 μmol/l pore size) were purchased from ICN (Costa Mesa, CA). Prime-it II random primer labeling kit was obtained from Stratagene (La Jolla, CA). Histopaque and all other analytical-grade biochemicals were purchased from either Sigma or Fisher Scientific (Pittsburgh, PA).

**Islet isolation and insulinoma cell propagation.** Pancreatic islets of Langerhans were isolated from 150–200 g NEDH rats by collagenase digestion and Histopaque density gradient centrifugation, as described elsewhere (21). Batches of 200 islets were maintained in tissue culture for 16 h in RPMI-1640, 5.6 mmol/l glucose, and 10% dialyzed fetal bovine serum (FBS) (molecular weight cutoff <10 kD) at 37°C in 95% air/5% $CO_2$ before use. The X-ray–induced NEDH rat transplantable insulinoma, which contained >95% endocrine β-cells, was used as an ample tissue source of pancreatic β-cells (17). Insulinoma tissue was propagated and harvested as previously described (17). Isolated single insulinoma cells were isolated as described (22) and maintained as a polyclonal cell line in tissue culture in RPMI-1640 media containing 11.1 mmol/l glucose, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 50 μmol/l β-mercaptoethanol, and 10% (vol/vol) FBS as described (23).

**Growth analysis.** The incorporation of [$^3$H]thymidine into isolated insulinoma cells from the NEDH rat insulinoma tissue (RITz cells) was used to assess cell growth as described (24), using serum as a mitogenic stimulus. Essentially, RITz cells were plated at 20–30% confluence into 35-mm dishes in RPMI-1640 media and maintained for 48 h to reach 50% confluence when they were in a log phase of growth. The RITz cells were then washed twice with Hanks' balanced salt solution (HBSS) and incubated for 0–72 h at 37°C in fresh media with or without serum and with or without the indicated inhibitors. In RITz cells incubated without serum, 0.1% (vol/vol) dialyzed BSA (10 kD molecular weight cutoff) was used as a replacement. After incubation, 1 μCi/ml [$^3$H]thymidine was added, after which the RITz cells were incubated for a further 4 h at 37°C under the same conditions. The cells were then washed three times in ice-cold HBBS, and lyzed in 2 ml of 1 mg/ml SDS solution on ice and transferred to 12-ml tubes. Then 2.5 ml of ice-cold 20% (wt/vol) trichloroacetic acid was added and poured over a Whatman glass-fiber filter in a millipore filtration apparatus. The filter was then washed twice with ice-cold 10% (wt/vol) trichloroacetic acid and air dried. The [$^3$H]thymidine specifically incorporated into DNA trapped on the filter was then counted by liquid scintillation counting.

***Messenger RNA expression analysis.*** Northern blot analysis was carried out as previously described (25). Differential mRNA display was essentially followed as previously described (26). Briefly, total RNA was extracted from the NEDH rat transplantable insulinoma tissue (as a model of fast-growing dedifferentiated pancreatic β-cells), and primary pancreatic islets were isolated from normal NEDH rats (as a source of slow-growing differentiated pancreatic β-cells) by guanidinium thiocyanate, then resuspended in sterile water and DNase-treated. The RNA samples were diluted to 0.1 μg/ml and four reverse transcription (RT) reactions were performed using 1 μmol/l $T_{12}$NG, $T_{12}$NA, $T_{12}$NT, or $T_{12}$NC oligonucleotide (where N is the threefold degenerate for G, A, and C), as previously described (26). Polymerase chain reaction (PCR), using 10 units AmpliTaq DNA polymerase (Gibco, Grand Island, NY) per 20 μl final volume, was performed as described in the presence of 10 μCi [$^{35}$S]dATP (26), so that the four $T_{12}$NX oligonucleotide primers were screened against 12 different random 10-mer oligonucleotides during each of 40 thermocycling runs. It has been estimated that this would allow ~15,000 RNA species to be screened, representing >90% of the total cellular RNA pool (27). After PCR amplification, the samples were run on 6% (wt/vol) polyacrylamide sequencing gels, then dried without fixation and visualized by autoradiography. Equivalent samples obtained from isolated NEDH rat islets and NEDH rat insulinoma tissue were run on adjacent lanes for convenient analysis. Comparison was made only from the insulinoma to the isolated islet lanes (and not vice versa) to focus on β-cell differences in mRNA expression and minimize the contribution made by other minor islet cell types (i.e., α-, δ-, and PP-cells) that would be significant in the NEDH rat islet sample (28). Any band that was found to be specifically elevated in the insulinoma sample compared with the islet sample was considered to be of preliminary interest, and repeat RT-PCR reactions were performed to confirm this observation. Only bands showing reproducible differences were excised from the gel, recovered and cloned into a TA vector (Invitrogen), as previously described (26). Specific radiolabeled probes were generated from the TA vector to confirm the differential expression of a candidate cDNA in the insulinoma versus islet RNA by Northern blot analysis (25). The identity of genes that were elevated in the insulinoma versus the islets was confirmed by DNA sequencing performed directly from the TA vector, with subsequent comparison of obtained sequences to those in the Genebank databases at the National Institute for Biotechnology Information using the BLAST and FASTA network service.

**Protein immunoblot and co-immunoprecipitation analysis.** Analysis of protein expression, protein tyrosine phosphorylation, and stimulated protein-protein interactions for elements of mitogenic signal transduction pathways in rat insulinoma cells was performed by immunoblotting and co-immunoprecipitation, as previously described (19,20,30). Each individual analysis was performed on a 20-cm–diameter Petri dish of 50% confluent RITz cells.

**Pulse-chase radiolabeling experiments.** As a marker of differentiation state, proinsulin biosynthesis and processing were analyzed in RITz cells. Essentially, 50% confluent RITz cells in a 6-well plate were incubated in RPMI-1640 medium containing 11.1 mmol/l glucose, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 50 μmol/l β-mercaptoethanol, and either 10% (vol/vol) FBS or 0.5% (wt/vol) BSA (for serum-deprived cells). These cells were preincubated in methionine-free RPMI-1640 with or without serum containing 0.5 mmol/l glucose for 60 min at 37°C. RITz cells were then incubated for a further 60 min at 37°C in the same medium with or without serum, containing either 0.5 mmol/l glucose (treatment A), 16.7 mmol/l glucose (treatment B), or 16.7 mmol/l glucose, 1 mmol/l isobutylmethylxanthine (IBMX), 10 μmol/l forskolin, 1 μmol/l phorbol myristic acid (PMA), and 20 mmol/l KCl (treat-

TABLE 1
Differential mRNA display analysis of genes overexpressed in NEDH rat transplantable insulinoma tissue compared with NEDH rat isolated islets

| Clone | Fold increase insulinoma versus islet | | Sequence | Homology (accession number) | *P* value |
|---|---|---|---|---|---|
| I-4 | 3.5-fold | 1 | CACCCACCTT GATCCTGAAA GAAAAAACGAA GAGCAGTAAC AGCAGCTGAG | Rat S24 ribosomal protein (S78734) | $7 \times 10^{-38}$ |
| | | 51 | CGACTTCATC AGCTTTAAAC CCACAACTAT GCTTCTTCTT GTGATCACCA | | |
| | | 101 | TAAATTAAAC TCAAGTGTAT CAGTCCTC | | |
| I-9 | 5.8-fold | 1 | TAGGTGATTG TGTACTTTGG ATCTGGATTT GCTGCTCCTT TCTTTATAGT | Mouse cytochrome-C oxidase VIIc (X52940) | $8 \times 10^{-65}$ |
| | | 51 | AAGGCACCAG CTACTTAAAA AATAAGGATA TTTAATTCAT CCCATTAACA | | |
| | | 101 | GAATGAAGAA AGTTTAAGAG ATATGATCTGGAAATTGGAT TAAACTCTTG | | |
| | | 151 | AACTCTTATA CTAGAAAAAA ATGTAATAAA CTAATGATAT AAATATTTAA | | |
| | | 201 | TGCCTCTTCT CTGC | | |
| I-10 | >75-fold | 1 | TTTCGCTGTT ATGCTAGATG AAGCTGAGTT CTCCATGAAG TTCTAGGATT | Rat carboxypeptidase B (M23953) | $6 \times 10^{-15}$ |
| | | 51 | AGAACTATTA ACACCCCGAA GAAGTTGGGGA ATGAAGGATT AAAAATTAT | | |
| | | 101 | AATAAGCGCC TAGCTACC | mouse viral-like VL30 retrotransposon (M21123) | $6 \times 10^{-4}$ |
| I-13 | >75-fold | 1 | CAGCACCCAC TCCAACAAGG CCATACCTTC TCAAGTAGTG CCAGCCCCTG | Unknown | — |
| | | 51 | AGATTACCCA ATCAGATATA TGAGCCTGTG GGGGCCATTA TTACCCAAAC | | |
| | | 101 | CAACACAGGT GCCAACCCCA ACTGATACAT CTGCAATGTG GCTCCTGTAT | | |
| | | 151 | CAAGGCTCAT GGGACATTGA AGGAAGAGAA GGTAGAATAC TGTAGAGCAG | | |
| | | 201 | AGGATGAGCA CATCTTCTGT ATGTGTCAAG AGAACTGTAC CCGC | | |
| I-15 | >75-fold | 1 | AACCGACAAT TGTCGTAAAG ACTAGAAAAT CCGTCAATAC GTGATTCACA | Rat c-HA-ras (L06433) | $5 \times 10^{-30}$ |
| | | 51 | GTATGGCCGA CTTTGAGACG GGAATTCTAA GAAACGAGGG TTCGTTATAA | rat VL30 retrotransposon (M91235 and X04990) | $5 \times 10^{-29}$ |
| | | 101 | ATGAGTGAAG AGATCGAATA GAGTCGACCG ACGCTAGTG | | $2 \times 10^{-27}$ |

Identification of genes preferentially overexpressed in rapidly growing NEDH rat insulinoma tissue compared with slow-growing NEDH rat isolated pancreatic islets was by differential mRNA display analysis, with confirmation of the overexpression by Northern blot analysis as described in METHODS. The results are displayed as designated clones in which the fold overexpression in insulinoma by subsequent Northern blot analysis is stated. The nucleotide sequence of the clone is displayed, and its putative identity by a high probability of homology to existing sequences in the database is indicated.

ment C; formulated to gain a maximum secretory response) (31), with the final 30 min of this second incubation carried out in the presence of 100 μCi/ml [$^{35}$S]methionine. Cells were either collected (i.e., "pulse") or washed, then incubated for a further 180 min at 37°C in the same composition incubation media, except it contained 1 mmol/l methionine. At the end of the 180-min "chase" period, both cells and media were collected. The cells were lyzed and disrupted by sonication, as described previously (21). Specific immunoprecipitation of (pro)insulin and subsequent analysis by alkaline-urea gel electrophoresis, fluorography, and densitometric scanning were performed as previously described (21).

**Other procedures.** Protein assay was performed using the bicinchoninic acid method (Pierce, Rockford, IL). Data are means ± SE. Statistically significant differences between groups were analyzed using Student's *t* test, with $P < 0.05$ being considered significant.

## RESULTS

**Elevated expression of retroviral-like transposons (VL30 elements) in X-ray–induced insulinoma cells but not in differentiated primary islets.** Differential mRNA display analysis was applied to examine specific elevated expression of genes in NEDH rat insulinoma cells versus that in NEDH rat differentiated isolated pancreatic islet β-cells. We screened 25 upstream primers against 4 degenerate downstream primers to screen an estimated 30,000 mRNA species (27). Only species that were markedly elevated in the insulinoma versus the islet cells were considered, owing to the heterogeneity of the islet cell population (28). From the initial differential display analysis, 26 candidate clones were singled out by this criteria. Subsequent Northern blot analysis revealed that 13 of the 26 clones were undetectable, presumably because of low abundant mRNAs. One clone could not be recovered and another was composed of multiple species, making evaluation difficult. Despite the initial differential mRNA display screen, 6 of 26 clones were subsequently found not to be differentially regulated between insulinoma and islet RNA by Northern blot analysis, and thus were not considered any further. However, 5 of 26 candidate clones were confirmed to be elevated in insulinoma compared with differentiated islets by Northern blot analysis; these are described in Table 1. Clone I-4 was significantly elevated over threefold in insulinoma versus islet cells as determined by Northern blot analysis. Sequence analysis of clone I-4 revealed it to be identical to the ribosomal protein S24. Clone I-9 was revealed to be cytochrome-C oxidase VIIc, which was elevated almost sixfold in the insulinoma tissue (Table 1). Northern blot analysis revealed that clone I-13 had an approximate size of 15 S, and was expressed 75-fold more in insulinoma than in islet RNA. However, sequence analysis of I-13 revealed no significant homology to any known gene in the database, so its identity remained unknown. Northern blot analysis of clones I-10 and I-15 revealed that they were expressed 75-fold more in insulinoma tissue than in isolated pancreatic islets, with a single species mRNA size of around 30 S. Subsequent sequence analysis, for both I-10 and I-15 clones (Table 1), revealed a significant homology to endogenous retroviral-like VL30 transposon elements that would be appropriate for an mRNA size of 30 S (32). The I-10 clone sequence analysis also revealed significant homology to rat carboxypeptidase B (Table 1), but the Northern blot analysis revealed only an mRNA species in the insulinoma RNA equivalent to a VL30 transposon at 30 S, but not one for car-

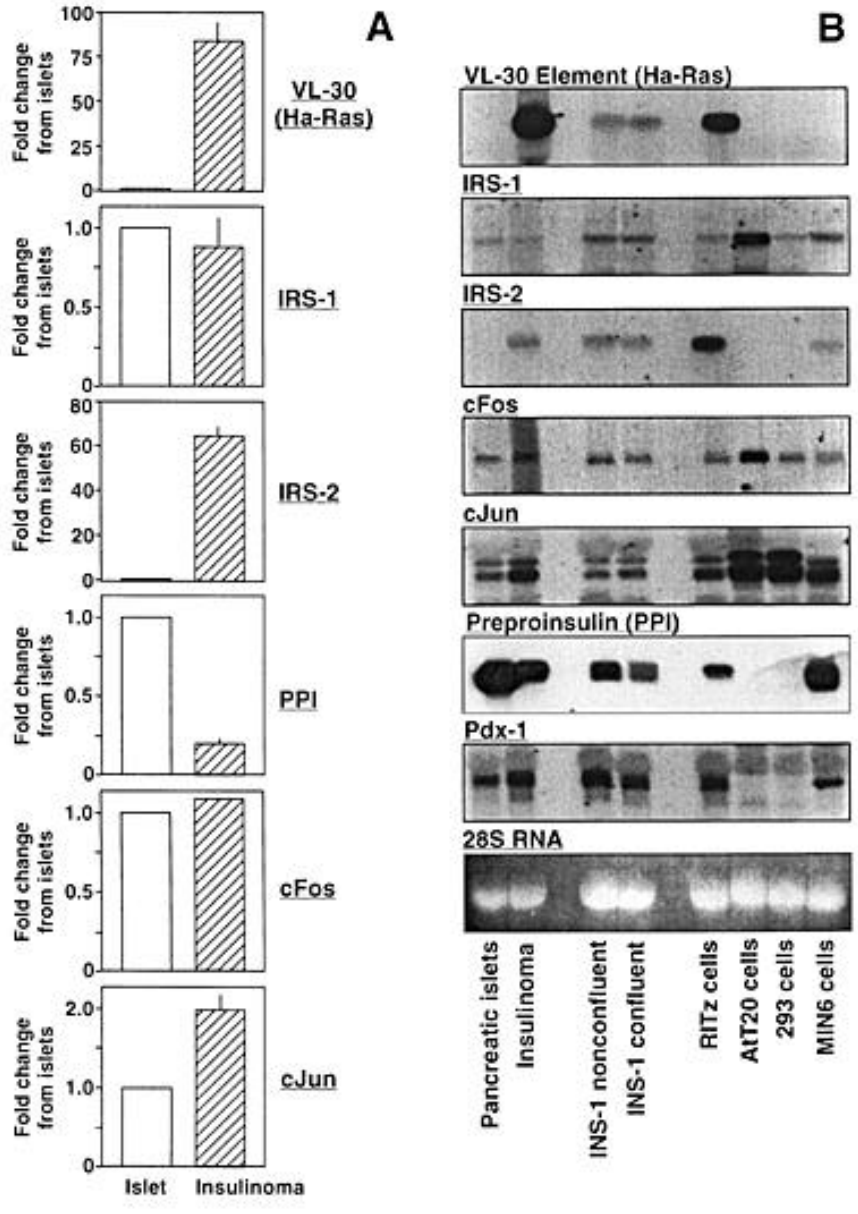


**FIG. 1. Differential gene expression in insulinoma cells versus rat pancreatic islets. The mRNA expression levels of VL30 transposon elements, mitogenic signal transduction pathway genes (IRS-1 and IRS-2), oncogenes (c-*jun* and c-*fos*), and β-cell specific genes (PPI and Pdx-1) were examined by Northern blot analysis in isolated NEDH rat islets, NEDH rat transplantable insulinoma, INS-1 cells (either 50% or fully confluent), RITz cells, mouse anterior pituitary AtT20 cells, human kidney 293 cells, and the mouse SV40 transformed insulinoma MIN6 cell-line as described in METHODS. Either 2.5 µg (for isolated islets and insulinoma tissue) or 5 µg (other cell lines) of total RNA were loaded per lane, with equal loading determined by ethidium bromide staining of 28S rRNA. *A*: Densitometric scan analysis (mean ± SE) of at least four individual experiments for mRNA levels in isolated NEDH rat pancreatic islets versus transplantable NEDH rat insulinoma tissue (17). *B*: Sample autoradiograph analysis of mRNA levels in NEDH rat pancreatic islets, NEDH rat insulinoma tissue, and the various cell lines indicated.**

boxypeptidase B mRNA, which would be expected to be smaller at around 2 kb. Likewise, clone I-15 revealed significant sequence homology to the rat c-HA-*ras* oncogene, but Northern blot analysis did not reveal an mRNA equivalent to c-HA-*ras* mRNA, but only an mRNA species in the insulinoma RNA equivalent to a VL30 transposon at 30 S. Furthermore, Northern blot and immunoblot analysis could not reveal any elevation of c-HA-*ras* expression at the mRNA or protein level in insulinoma compared with islet cells (data not shown). Thus the marked elevated mRNA expression of I-10 and I-15 clones is reflective of a specific increase in the expression of endogenous retroviral-like VL30 transposon elements in NEDH rat insulinoma cells and does not reflect any elevation in carboxypeptidase B or c-HA-*ras* expression.

**Differential expression of candidate mitogenic signaling proteins in rat insulinoma cells verses differentiated rat islet cells.** The mRNA levels of certain candidate genes were measured in RNA extracted from NEDH rat islets and the transplantable insulinoma tissue (17). Figure 1 indicates that the VL30 element containing the c-HA-*ras* sequence (clone I-15) mRNA was overexpressed by >80-fold in the insulinoma compared with in the islets ($P < 0.001$), in confirmation of the mRNA display analysis (Table 1). The elevated expression of this VL30-element mRNA was also present in insulin-producing cell lines that were derived from the X-ray–induced NEDH rat transplantable insulinoma (18), namely RIN-38, RIN-1042, RITz, and INS-1 cells (23) (Fig. 1). For INS-1 cells, the degree of confluence did not alter VL30 expression (Fig. 1). However, the mRNA expression of this particular VL30 did not appear to be elevated in other rodent insulinoma cell lines that were transformed by targeted expression of the SV40 large T-antigen, namely βTC3 cells (33) and MIN6 cells (Fig. 1) (34). Examples of proliferative non–β-cell lines, anterior pituitary AtT20 cells, or kidney 293 cells did not appear to express this VL30 element (Fig. 1).

The family of IRS molecules are key proteins in mitogenic signaling in cells (35–37). The expression of IRS-1 mRNA appeared to be relatively equivalent in insulinoma and islet

tissue, as well as in various β-cell lines, AtT20, and 293 cells (Fig. 1). In contrast, IRS-2 mRNA was markedly overexpressed by >65-fold ($P < 0.001$) in the insulinoma versus pancreatic islet tissue (Fig. 1). Furthermore, specific IRS-2 mRNA overexpression was found in all β-cell lines tested, including INS-1 (whether confluent or nonconfluent), RITz, and MIN6 cells (Fig. 1), as well as βTC3, βTC6-F7, RIN-38, RIN-1042, and RINm5f cells (data not shown). It was also found that IRS-2 mRNA expression in insulinoma cells was likely to be much higher than IRS-1 mRNA expression, since autoradiograph exposure of Northern blot analysis for IRS-1 was 6–7 days longer to gain a signal equivalent to that found for IRS-2 Northern blots.

The oncogenes c-*jun* and c-*fos* were found to be expressed in all the cell types examined (Fig. 1). Expression levels of c-*fos* were relatively equivalent, except for an approximate twofold to threefold elevation in mRNA level in AtT20 cells (Fig. 1). The mRNA levels were modestly increased twofold in insulinoma tissue, RITz and MIN6 cells ($P < 0.05$) compared with islet tissue (Fig. 1); however, INS-1 cell (whether confluent or nonconfluent) and islet mRNA levels of c-*jun* were not significantly different (Fig. 1).

As previously established, pre-proinsulin (PPI) mRNA levels were decreased by ~75% in the pancreatic β-cell lines compared with in primary islet tissue (Fig. 1). However, mRNA levels of the key β-cell transcription factor, Pdx-1 (38), were relatively equal in the β-cell lines versus the primary islet tissue (Fig. 1). As expected, pre-proinsulin and Pdx-1 mRNA were not present in AtT20 or 293 cells.

**Serum feeding of insulinoma cells increases DNA synthesis without significant effect on candidate gene expression.** [$^3$H]thymidine incorporation was used as an assay to indicate the degree of DNA synthesis and β-cell growth. The degree of [$^3$H]thymidine incorporation in 50% confluent RITz cells maintained in 10% (vol/vol) serum was taken at time zero. The cells were then made quiescent by withdrawal of serum for up to 48 h, then 10% (vol/vol) serum was added back and the cells were incubated in tissue culture for a further 48 h. After 24 h, the degree of [$^3$H]thymidine incorporation was decreased by twofold (NS) and then by fourfold by 48 h ($P < 0.05$) compared with time zero or serum fed RITz cells (Fig. 2*A*). The cells were then refed with 10% (vol/vol) serum after a 48-h period of serum withdrawal. After 24 h of serum refeeding, the degree of [$^3$H]thymidine incorporation was increased fourfold ($P < 0.05$), which reached a tenfold higher rate ($P < 0.01$) after 48-h serum refeeding compared with time zero (Fig. 2*A*). In the light of the IRS-2 overexpression in insulinoma cells, the effect of various inhibitors of IRS-mediated signal transduction pathways was examined. RITz cells were serum starved for 24 h and then refed with 10% (vol/vol) serum for a subsequent 24 h. In the serum refed cells, there was a ninefold increase in [$^3$H]thymidine incorporation over cells that were serum starved (Fig. 2*B*). In the added presence of the tyrosine kinase inhibitor, genistein (25 μmol/l; a concentration generally known to inhibit tyrosine kinase activity), serum-induced DNA synthesis was inhibited by >95% ($P < 0.01$; Fig. 2*B*). Inhibitors of PI 3-kinase activity—wortmannin (20 nmol/l), and LY249002 (10 μmol/l), used at concentrations known to inhibit PI 3-kinase activity in β-cells (39)—inhibited serum-stimulated DNA synthesis in RITz cells >95% ($P < 0.001$) (Fig. 2*B*). Inhibition of MAP-kinase kinase (MEK) by PD98059, which in turn inhibits MAP-kinase (erk-1 and erk-2) activation, inhibited serum-stimulated RITz cell DNA synthesis by 80% ($P < 0.01$) (Fig. 2*B*). The inhibitor of p70$^{S6K}$ activation, rapamycin (25 nmol/l), also significantly inhibited serum-induced RITz cell proliferation by 65% ($P < 0.05$) (Fig. 2*B*). This implied that tyrosine protein phosphorylation, PI 3-kinase activity, as well as a degree of downstream MAP-kinase and p70$^{S6K}$ activation were necessary for serum-stimulated insulinoma cell mitogenesis.

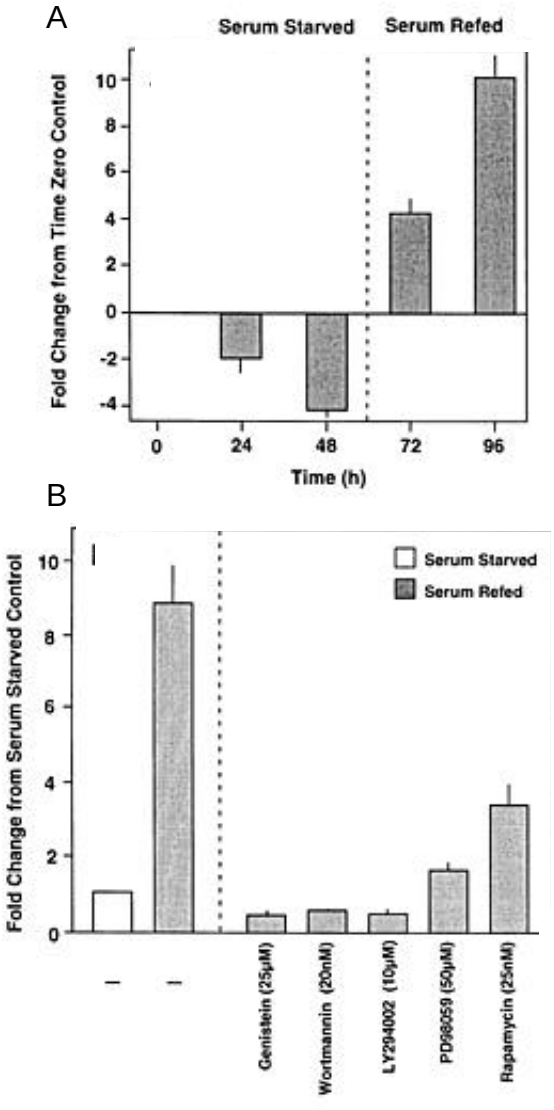


**FIG. 2. Serum refeeding stimulated DNA synthesis in NEDH rat insulinoma cells. *A*: RITz cells were deprived of serum for 24 h and 48 h, then refed with serum for a further 24 h or 48 h (96 h total incubation). At each time period, DNA synthesis was assessed by [$^3$H]thymidine incorporation, as described in METHODS. *B*: RITz cells were deprived of serum for 24 h then refed with serum for a further 24 h in the presence or absence of the various inhibitors indicated. After 24-h serum refeeding, DNA synthesis was assessed by [$^3$H]thymidine incorporation as described in METHODS. Data are means ± SE of three to five individual experiments, either as a fold change of [$^3$H]thymidine incorporation from time zero (*A*) or fold increase in serum refed cells with or without an inhibitor compared with serum-starved control cells (*B*).**

The mRNA expression levels of VL30, IRS-2, PPI, and c-*fos* and c-*jun* were assessed during the period of serum refeed-

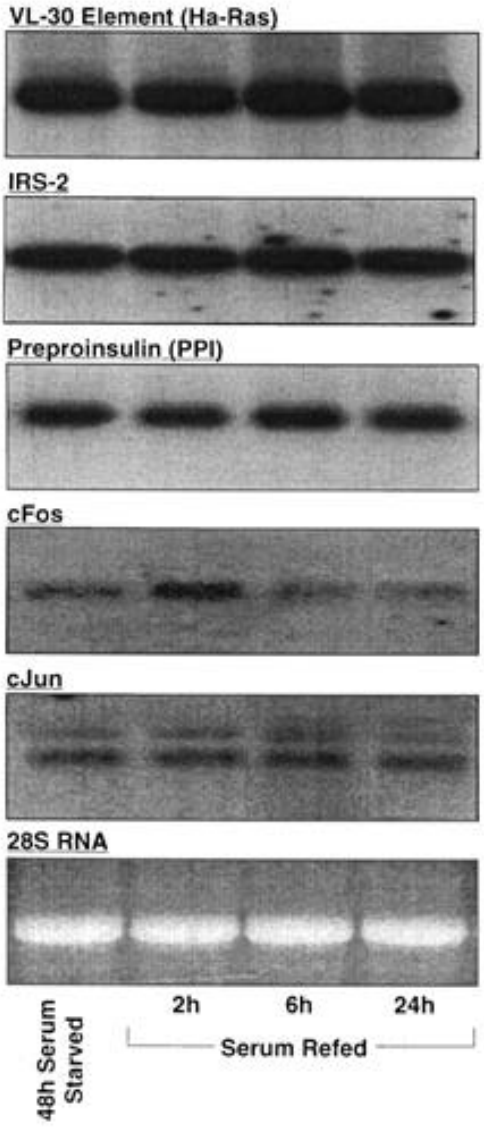


**FIG. 3. Serum refeeding does not adversely affect mRNA expression levels in NEDH rat insulinoma cells. RITz cells were serum starved for 48 h, then refed with 10% (vol/vol) FBS for 2, 6, and 24 h. Messenger RNA levels for VL30 elements, IRS-2 PPI, c-*fos* and c-*jun* were assessed by Northern blot analysis as described in METHODS. About 5 µg of total RNA was loaded per lane, with equal loading determined by ethidium bromide staining of 28S rRNA. A sample autoradiograph analysis of mRNA levels is shown.**

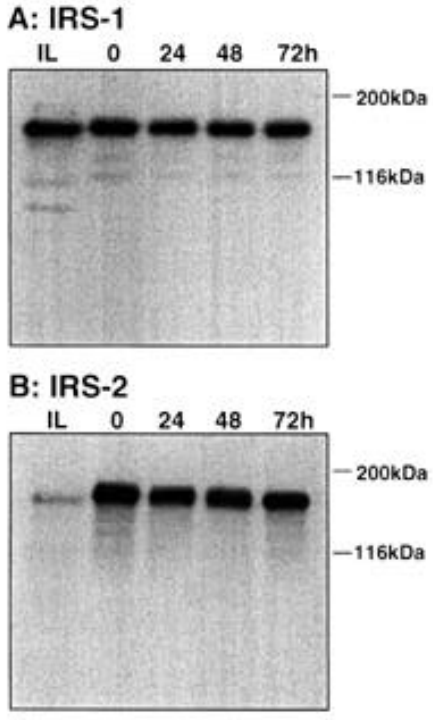


**FIG. 4. Serum deprivation does not alter IRS-1 and IRS-2 protein levels in NEDH rat insulinoma cells. RITz cells were serum starved for up to 72 h, then intracellular IRS-1 and IRS-2 levels were assessed by immunoblot analysis as described in METHODS. On the same immunoblot gel analysis, IRS-1 and IRS-2 protein levels were assessed in isolated NEDH rat pancreatic islets (IL). Sample immunoblot analyses for IRS-1 (*A*) and IRS-2 (*B*) are shown where 50 µg total protein was loaded per lane.**

ing (2–24 h) after a 48-h period of serum withdrawal (Fig. 3). Essentially, there was no change in mRNA expression levels of VL30, IRS-2, PPI, and c-*fos* in the 24 h period of refeeding compared with RITz cells deprived of serum for 48 h. However, c-*fos* mRNA levels increased twofold after 2 h of serum refeeding, but from 6 h onward reverted back to mRNA levels found in RITz cells deprived of serum for 48 h.

In considering the marked elevation of IRS-2 mRNA levels in insulinoma cells, the effect of serum withdrawal on IRS-1 and IRS-2 protein levels was examined in RITz cells. Immunoblot analysis revealed that IRS-1 levels did not alter over a 72-h period of serum withdrawal, and that IRS-1 levels were approximately equivalent in the insulinoma versus islet cells (Fig. 4*A*). In contrast, IRS-2 protein levels were approximately 50-fold higher in insulinoma cells compared to primary islet cells (Fig. 4*B*), complementary to IRS-2 mRNA levels (Fig. 1). IRS-2 protein levels did not alter during a 72-h period of serum withdrawal (Fig. 4*B*). Neither IRS-1 nor IRS-2 protein levels altered during a 60-min period of serum refeeding after a 48-h period of serum deprivation (data not shown).

**Serum activation of mitogenic signal transduction pathways in insulinoma cells.** Neither serum withdrawal nor refeeding appeared to significantly alter expression levels of IRS-2 (Fig. 3 and 4), whereas serum refeeding clearly stimulated DNA synthesis in insulinoma cells (Fig. 2). Moreover, inhibition of signaling pathways downstream of IRS resulted in a marked decrease in RITz cell DNA synthesis (Fig. 2*B*) (35). Thus the ability of serum feeding to activate IRS-mediated mitogenic signal transduction pathways in insulinoma cells was examined. Immunoprecipitation of RITz cell lysates with anti-phosphotyrosine (PY) antisera followed by PY immunoblot analysis revealed increased tyrosine phosphorylation of proteins at 180 and 60 kD (designated pp60) 10–30 min after 10% (vol/vol) serum addition, irrespective of the period of serum deprivation (Fig. 5). However, the magnitude of the increase in tyrosine phosphorylation of the 180 and 60 kD proteins at 10 and 30 min appeared greater in RITz cells deprived of serum for 24–72 h. This was mostly due to an increased degree of tyrosine phosphorylation of the 180

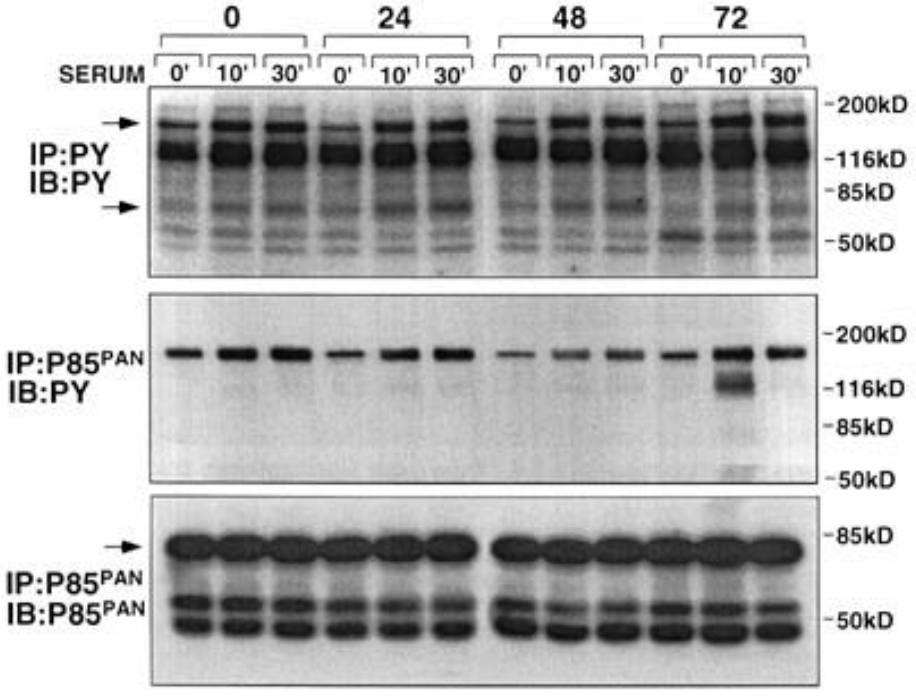


**FIG. 5. Serum-stimulated tyrosine phosphorylation in NEDH rat insulinoma cells. RITz cells (50% confluent on a 20-cm diameter Petri dish) were serum starved for 48 h, then refed with 10% (vol/vol) FBS for 0, 10, or 30 min, during which the specific IRS-associated tyrosine phosphorylation was examined in RITz cell lysates (~200 µg total protein) by immunoprecipitation (IP) with anti-phosphotyrosine antisera followed by anti-phosphotyrosine immunoblot (IB) analysis (IP:PY/IB:PY) or by immunoprecipitation with antisera against the p85 regulatory subunit of PI 3-kinase antisera followed by anti-phosphotyrosine immunoblot analysis (IP:P85$^{PAN}$/IB:PY) as described in METHODS section. A sample immunoblot for each tyrosine phosphorylation analysis is shown in which protein levels in the samples were confirmed by immunoprecipitation with antisera against the p85 regulatory subunit of PI 3-kinase antisera followed by p85/PI 3-kinase regulatory subunit immunoblot analysis (IP: P85$^{PAN}$/IB: P85$^{PAN}$).**

and 60 kD proteins at time zero, when cells had not been starved of serum (Fig. 5). It should be noted that there was a predominant tyrosine phosphorylated protein of about 125 kD. This 125-kD protein (designated pp125) was also modestly increased in its tyrosine phosphorylation state in response to serum, albeit not to the same extent as the 180 and 60 kD proteins (Fig. 5).

Growth factor activation of IRS-mediated mitogenic signal transduction pathways increases the association of SH2 domains in the regulatory subunit of PI 3-kinase with phosphotyrosine domains on IRS-1/IRS-2 (35). Immunoprecipitation of RITz cell lysates with antisera against the p85 subunit of PI 3-kinase (p85$^{PAN}$) followed by PY immunoblot analysis revealed the increased association of a tyrosine-phosphorylated 180 kD protein 10–30 min after 10% (vol/vol) serum addition, irrespective of the period of serum deprivation (Fig. 5). This likely reflected a serum-stimulated increase in association with an IRS-like molecule with PI 3-kinase. PI 3-kinase immunoblot analysis of RITz cell PI 3-kinase immunoprecipitates revealed that total levels of the PI 3-kinase 85-kD subunit did not alter during serum deprivation refeeding (Fig. 5), similar to that of IRS-1 and IRS-2 (Fig. 4). A further series of experiments was performed on the insulinoma cells that were serum starved for 24 h followed by another 24 h of continued serum starvation or serum refeeding. Subsequent immunoprecipitation of the cell lysates with antisera against p85$^{PAN}$ followed by immunoblot analysis with specific IRS-2 antisera revealed a serum-stimulated association of PI 3-kinase with IRS-2 (Fig. 6). This observation correlated with serum-stimulated tyrosine phosphorylation of the 180 kD protein present in the PI 3-kinase immunoprecipitate (Figs. 5 and 6). A similar immunoblot analysis of PI 3-kinase immunoprecipitates with IRS-1 antisera was unable to detect IRS-1. This was probably due to the low levels of IRS-1 in insulinoma cells versus IRS-2 (Figs. 1 and 4) rather than an inability of IRS-1 to associate with PI 3-kinase in β-cells, since it has been demonstrated that IRS-1 is capable of recruiting PI 3-kinase in a tyrosine phosphorylation–dependent manner in βTC3-cells (40). In addition to the serum-induced association of IRS-2 and PI 3-kinase, serum also induced recruitment of Grb2/mSOS to the PI 3-kinase/IRS-2–containing protein complex in RITz cells (Fig. 6). These serum-induced protein associations were specific, since the amount of p85$^{PAN}$ in the immunoprecipitate was not changed with or without serum (Fig. 6).

Phosphorylation activation of p70$^{S6K}$ occurs downstream of PI 3-kinase activation via its association with IRS (19,35). The phosphorylation activation of p70$^{S6K}$ can be evaluated by electrophoretic mobility shift on immunoblot analysis. There are multiple phosphorylation sites on p70$^{S6K}$, and the greater the phosphorylation state, the greater the retardation on gel electrophoresis (19,30). Immunoblot analysis indicated p70$^{S6K}$ activation in RITz cells, previously serum-deprived for 24–72 h, after 10 min of serum refeeding and even greater activation after 30 min of serum refeeding (Fig. 7). Phosphorylation activation of p70$^{S6K}$ was also observed in RITz cells not deprived of serum, but to a lesser extent (Fig. 7). Activation of p70$^{S6K}$ was slower than serum-induced tyrosine phosphorylation of IRS and IRS-2/PI 3-kinase association (Figs. 5 and 6), in line with p70$^{S6K}$ activation being downstream of PI 3-kinase activation (30,35).

There are several branches of possible mitogenic signal transduction pathways mediated via IRS (35). As well as a signaling pathway mediated via PI 3-kinase and p70$^{S6K}$ activation, there is an alternative route via Grb2/mSOS recruitment to an IRS-signaling protein complex (Fig. 6), followed by subsequent activation of the *ras-raf* signaling pathway that results in MAP-kinase activation (35). We have investigated serum-induced phosphorylation activation of MAP-kinase in

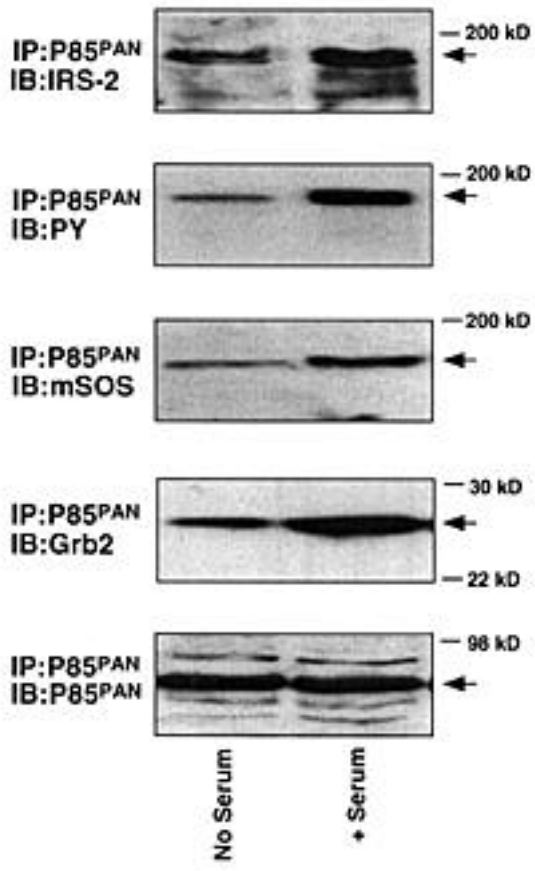


**FIG. 6. Serum stimulation induces PI 3-kinase association with IRS-2 and Grb2/mSOS in NEDH rat insulinoma cells. RITz cells (50% confluent on a 20-cm diameter Petri dish) were serum starved for 24 h, then refed with 10% (vol/vol) FBS for 0 or 15 min, during which the specific protein association to PI 3-kinase was examined in RITz cell lysates (~200 µg total protein) by immunoprecipitation with antisera against the p85 regulatory subunit of PI 3-kinase antisera followed by immunoblot analysis with IRS-2 antisera (IP:P85$^{PAN}$/IB:IRS-2), anti-phosphotyrosine antisera (IP:P85$^{PAN}$/IB:PY), mSOS antisera (IP:P85$^{PAN}$/IB:mSOS), and Grb2 antisera (IP:P85$^{PAN}$/IB:Grb2) as described in METHODS. A sample immunoblot for each immunoblot analysis is shown in which equal protein levels in the samples were confirmed by immunoprecipitation with antisera against the p85 regulatory subunit of PI 3-kinase antisera followed by p85/PI 3-kinase regulatory subunit immunoblot analysis (IP:P85$^{PAN}$/IB:P85$^{PAN}$).**

insulinoma cells by immunoblot analysis using antisera that recognizes only the phosphorylated active form of MAP-K (erk-1 and erk-2 isoforms) (8). In RITz cells serum starved for 24 h yet not deprived of 15 mmol/l glucose in the culture medium, MAP-kinase was found to be already activated despite refeeding with serum and glucose (Fig. 8*A*). However, in RITz cells deprived of serum and incubated in only 0.5 mmol/l glucose for 24 h, adding back 15 mmol/l glucose alone (Fig. 8*B*) or 10% (vol/vol) serum alone (Fig. 8*C*) resulted in activation of MAP-kinase. Activation of MAP-kinase by 15 mmol/l glucose appeared to be more rapid than that by 10% (vol/vol) serum (Figs. 8*B* and 8*C*), but the effect of glucose and serum did not appear to be additive (Fig. 8*A*). Glucose-induced activation of MAP-kinase complements previous observations in pancreatic β-cells (7,8). Deprivation of serum and/or glucose for 24 h followed by serum and/or glucose refeeding did not affect total levels of these MAP-kinase isoforms in these insulinoma cells (Figs. 8*A* and 8*C*).

**Serum starvation/refeeding did not further affect the differentiation state of insulinoma cells.** Proinsulin biosynthesis appeared lower in cells starved of serum for 48 h; however, this was due to there being fewer cells present because of a lower cell growth rate (Fig. 2). However, the characteristics of proinsulin biosynthesis were similar in the 30-min pulse-radiolabeling period, whether RITz cells were incubated at 0.5 mmol/l glucose (treatment A) (Fig. 9), 16.7 mmol/l glucose (treatment B) (Fig. 9), or in a maximal stimulation cocktail of 16.7 mmol/l glucose 1 mmol/l IBMX, 10 µmol/l forskolin, 1 µmol/l PMA, and 20 mmol/l KCl (treatment C) (Fig. 9). It is characteristic of β-cell lines derived from the NEDH rat insulinoma to show no nutrient-induced translational regulation of proinsulin biosynthesis (22,23). Nonetheless, proinsulin was efficiently converted to insulin during the 180-min chase period, but most of the newly synthesized (pro)insulin was not retained in the RITz cells, and secreted into the chase media (Fig. 9). Although RITz cells indicated no glucose-stimulated insulin release, the amount of insulin release was greater in RITz cells incubated with the maximal stimulation cocktail, which correlated with less insulin being retained in the cells under these conditions (Fig. 9). Nonetheless, proinsulin biosynthesis, processing, and subsequent secretion of newly synthesized (pro)insulin-related material was unaffected in RITz cells, whether they were deprived of serum or not (Fig. 9).

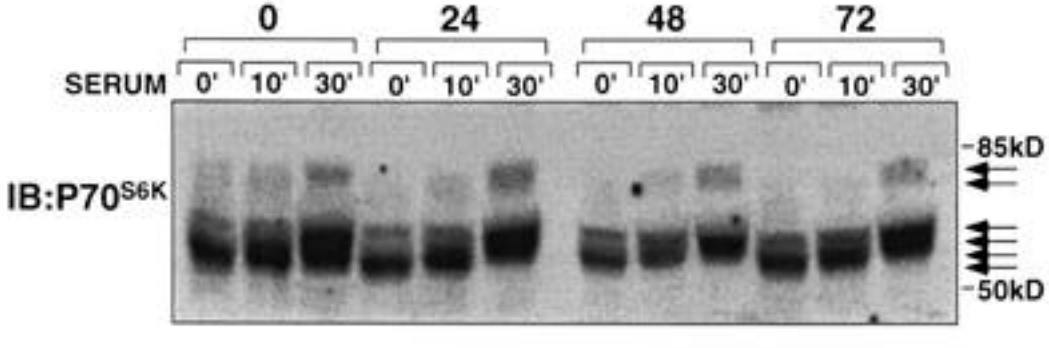


**FIG. 7. Serum-stimulated phosphorylation activation of p70$^{S6K}$ in NEDH rat insulinoma cells. RITz cells (50% confluent on a 20-cm diameter Petri dish) were serum starved for 48 h, then refed with 10% (vol/vol) FBS for 0, 10, or 30 min during which the specific phosphorylation activation of p70$^{S6K}$ was examined in RITz cell lysates (75 µg total protein) by immunoblot analysis as described in METHODS. A sample immunoblot for p70$^{S6K}$ is shown.**

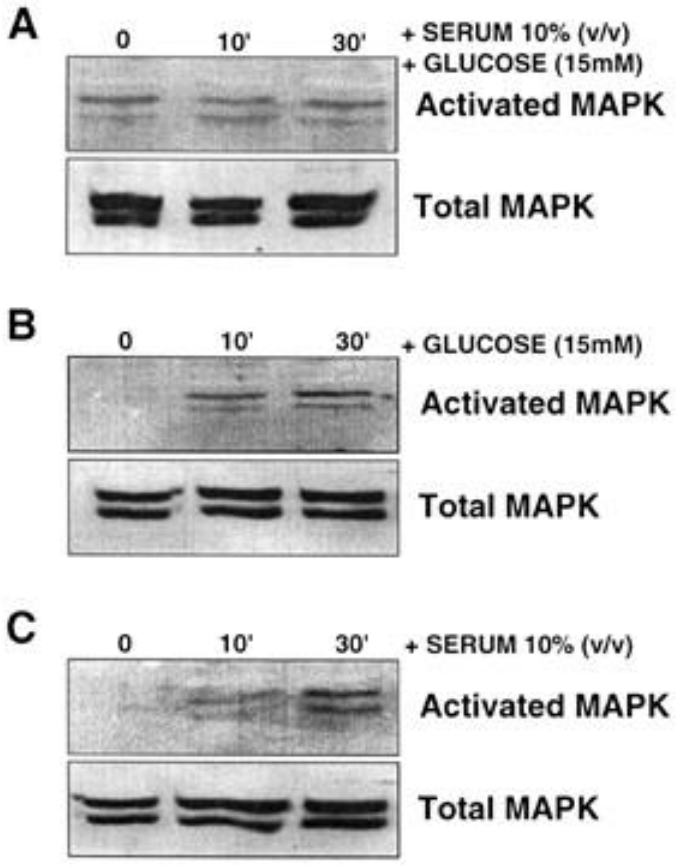


**FIG. 8. Serum- and glucose-stimulated phosphorylation activation of MAP-kinase (MAPK; erk-1 and erk-2 isoforms) in NEDH rat insulinoma cells. RITz cells (50% confluent on a 10-cm diameter Petri dish) were either serum- or serum- and glucose-starved for 24 h then refed with 10% (vol/vol) FBS or 15 mmol/l glucose, for 0, 10, or 30 min in which the specific immunoblot analysis of phosphorylation-activated MAPK and total MAPK was examined in RITz cell lysates (75 µg total protein) as described in METHODS. A sample immunoblot analysis of "activated" MAPK and total MAPK is shown. *A*: RITz cells deprived of serum for 24 h then refed with 10% (vol/vol) FBS and 15 mmol/l glucose. *B*: RITz cells deprived of serum and glucose for 24 h then refed with 15 mmol/l glucose only. *C*: RITz cells deprived of serum and glucose for 24 h then refed with 10% (vol/vol) FBS only.**

## DISCUSSION

Several growth factors have been implicated in the stimulation of β-cell replication; however, the downstream signal transduction pathways in pancreatic β-cells for the majority of these growth factors remain relatively undefined (1). In this study, we attempted to identify elements in mitogenic signal transduction pathways that may be important components for the mechanism behind the control of pancreatic β-cell replication. In this regard, we have taken the somewhat simple notion that primary pancreatic β-cells in isolated rat islets represent a model of slow-growing, well-differentiated β-cells, and that rat insulinoma cells depict a model of rapidly growing, relatively dedifferentiated β-cells. The expression of certain genes has been compared in these two cell models to implicate certain elements that might be involved in mechanisms of β-cell growth and/or differentiation.

Differential mRNA display identified several genes that were significantly overexpressed in NEDH rat insulinoma cells, compared with in NEDH rat isolated islets. A modest elevation of S24 ribosomal protein in insulinoma cells may well complement the increased demand on protein synthesis required for increased cell replication (41,42). Increased expression of cytochrome-C oxidase VIIc was likely associated with the increased mitochondrial biogenesis required during cell replication (43). A >75-fold elevated expression of certain genes in the insulinoma tissue compared with in the primary islet cells by Northern blot analysis likely implied that 3 of 5 clones were expressed only in the insulinoma. This was corroborated by the identification of two of these clones as VL30 retrotransposon elements that are commonly expressed in tumor cells (32,44,45) and are involved in gene rearrangement during mitogenesis (32). VL30 retrotransposon expression therefore might be secondary to tumorogenesis, rather than be a primary instigator of β-cell proliferation. In this light, it should be noted that the preferential expression of VL30 elements in insulinoma appeared to be relevant only to β-cell lines derived from the X-ray–induced NEDH rat insulinoma (i.e., RIN, RITz, and INS-1 cells) (18,23), and not to other β-cell lines that were transformed by SV40 large T-antigen overexpression (i.e., βTC3 and MIN6 cells) (33,34) or in AtT20 and 293 cells. The I-13 insulinoma clone was unknown because no significant homology was found in the database; however, it was unlikely that it was also a VL30 retrotransposon element since its mRNA was too small as assessed by Northern blot (~2.5 kb; data not shown). Thus the identity of I-13 will remain unknown until a more primary sequence is generated. The differential mRNA display technique was able to reveal some genes that were elevated in the insulinoma cells and were relevant to an increased cell replication rate (Table 1) (41–45). However, this technique is limited in that it requires many rounds of differential mRNA display (using different random primer oligonucleotides) for a thorough analysis of genes elevated in insulinoma cells versus those in primary β-cells. Therefore, not all differentially expressed genes in insulinoma cells versus primary β-cells could be detected by differential mRNA display with a single RT-PCR amplification round. This was illustrated by the increased expression of IRS-2 in insulinoma cells being found not by differential mRNA display but by using a candidate gene approach.

It was found that IRS-2 was markedly overexpressed in all the β-cell lines examined, whether they were generated by exposure to X-ray radiation (e.g., RIN cell lines) (18,23) or SV40 large T-antigen expression (e.g., βTC3 or MIN6 cells) (33,34). This appeared to be a specific effect, since mRNA levels of IRS-1 or the oncogenes c-*fos* and c-*jun* were essentially unchanged in insulinoma cells compared with in primary islets. The IRS-2 expression in insulinoma cells appeared to be functional, in line with having a contributing role for the regulation of β-cell mitogenesis. Serum addition to insulinoma cells, after a period of serum-starvation, rapidly increased the tyrosine phosphorylation state of a 180-kD protein that was likely IRS-2 because of its over expression in insulinoma cells compared with IRS-1. This was supported by the observed serum-induced IRS-2/PI 3-kinase association that correlated with the downstream activation of $p70^{S6K}$ (35) (Figs. 5–7). Furthermore, inhibition of PI 3-kinase activity (by wortmannin and LY294002 ) or $p70^{S6K}$ activation (by rapamycin) resulted in a significant inhibition of serum-stimulated insulinoma cell proliferation (Fig. 2*B*). There are at

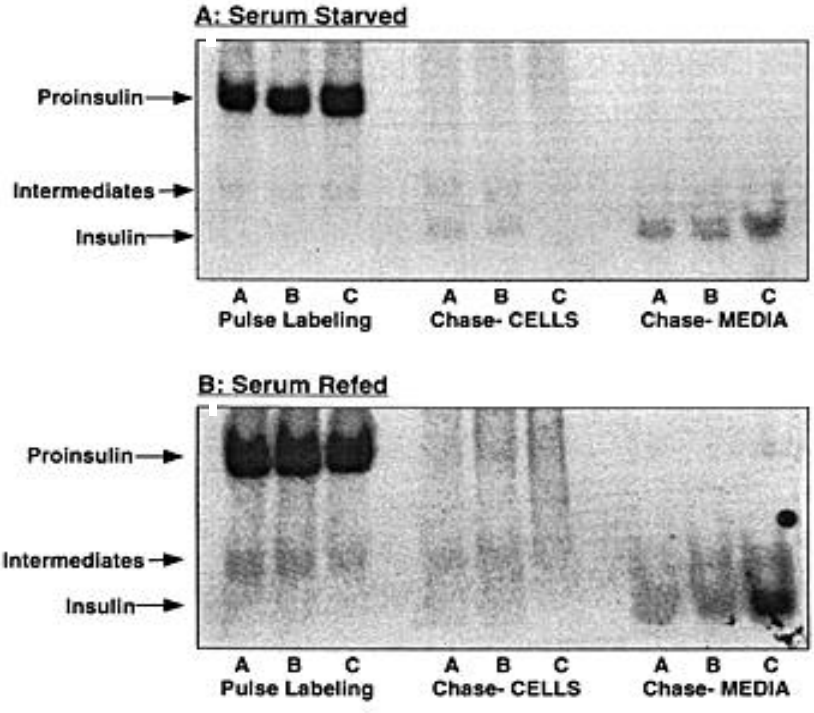


**FIG. 9. Serum starvation/refeeding does not affect proinsulin biosynthesis, processing, or secretion of newly synthesized insulin in NEDH rat insulinoma cells. RITz cells (50% confluent on a six-well plate) were serum starved for 24 h then further deprived of serum for another 24-h period (serum starved cells) or refed with 10% (vol/vol) FBS for 24 h (serum-refed cells). The cells were then incubated for a further 60 min at 37°C in the same medium with or without serum, containing either 0.5 mmol/l glucose (treatment A), 16.7 mmol/l glucose (treatment B), or 16.7 mmol/l glucose, 1 mmol/l IBMX, 10 µmol/l forskolin, 1 µmol/l PMA, and 20 mmol/l KCl (treatment C; formulated to gain a maximum secretory response) (31), with the final 30 min of this second incubation carried out in the presence of 100 µCi/ml [$^{35}$S]methionine as described in METHODS. RITz cell lysates from "pulsed" cells and cell lysates and media from "chased" cells were immunoprecipitated for [$^{35}$S](pro)insulin; the immunoprecipitates were then analyzed by alkaline gel electrophoresis and fluorography as previously described (see METHODS). Sample fluorograph analyses are shown for serum-starved RITz cells (*A*) and serum-refed RITz cells (*B*).**

least two possible mitogenic signal transduction pathways downstream of IRS-2: one via activation of p70$^{S6K}$ and the other via MAP-kinase activation (35). In insulinoma cells starved of both serum and glucose, subsequent addition of 15 mmol/l glucose activated MAP-kinase as previously observed (7,8). However, serum was also able to activate MAP-kinase in insulinoma cells independently of glucose (Fig. 8*C*). Serum stimulation of insulinoma cells resulted in recruitment of Grb2/mSOS to a protein complex containing IRS-2/PI 3-kinase (Fig. 6). This was indicative of a serum-mediated Grb2/mSOS association with IRS-2 that would lead to downstream activation of MAP-kinase (erk-1 and erk-2 isoforms) (Fig. 8) via the *ras*/*raf*/MEK/MAP-kinase pathway (35). Inhibition of MAP-kinase activation in RITz cells by the MEK inhibitor PD98059 resulted in a significant reduction in insulinoma cell proliferation (Fig. 2*B*). Therefore, it appears that serum-stimulated β-cell growth required downstream activation of both the *ras*/*raf*/MEK/MAP-kinase and PI 3-kinase/p70$^{S6K}$ branches of IRS-mediated signal transduction pathways (19,35).

It should be noted that there are likely several signal transduction pathways that can lead to β-cell mitogenesis (1). This study has implicated that a signaling pathway via IRS-2 mediated activation of MAP-kinase (erk-1 and erk-2 isoforms) and PI 3-kinase/p70$^{S6K}$ activation could be playing a contributory role for committing a β-cell into the growth phase of the cell cycle. However, other signaling elements and mitogenic signal transduction pathways in β-cells that were not addressed in this study cannot be ruled out (e.g., the JAK/STAT pathway) (6,46). Furthermore, it should be noted that there are numerous members of the IRS family (47), and it is unlikely that β-cell mitogenesis is exclusively regulated by IRS-2–mediated signaling pathways. In this regard it should be noted that a 60-kD (pp60) and a 125-kD protein (pp125) were also tyrosine phosphorylated in insulinoma cells in response to serum, which might also contribute to regulation of β-cell proliferation. For the moment, the identity of pp60 and pp125 is unknown. However, it is conceivable that pp60 might correspond to IRS-3 (48,49), and pp125 might be equivalent to a tyrosine-phosphorylated 125-kD protein previously observed in β-cells (50), although further studies will be required to substantiate this. In addition, further experiments will be required to unveil the appropriate growth factor in serum that increases β-cell proliferation, and activation of IRS-mediated signal transduction pathways. It will also be important to establish whether this experimental model of serum-stimulated β-cell growth was due to stimulated mitogenesis (35), inhibition of apoptosis (51), or both.

However, this study further established that certain elements of IRS-mediated signal transduction pathways are present in pancreatic β-cells (40,52), and that their activation correlates with increased mitogenesis (35). The differential expression of IRS-1 and IRS-2 in insulinoma cells could indicate that they are mediating different signaling pathways in β-cells (53). Increased IRS-2 expression in insulinoma cells raises the question as to whether IRS-2 is associated with increased β-cell growth in vivo. In this light, it is interesting to note the different pancreatic islet phenotype in the IRS-1 and IRS-2 knockout mice (54,55). In IRS-1 knockout mice, in which a degree of insulin signaling is rerouted via IRS-2, insulin resistance is compensated for by β-cell hyperplasia and hyperinsulinemia (20). IRS-1–deficient knockout mice, although glucose intolerant, do not develop diabetes (54). However, in IRS-2 knockout mice, insulin resistance is not compensated for by any β-cell hyperplasia, and the mice consequently develop diabetes (55). Indeed, the β-cell mass is remarkably low in IRS-2 knockout mice compared with wild-type control mice and IRS-1 knockout mice (55). It should be noted that expression of IRS-2 may not necessarily be a primary instigator of β-cell growth, since its acti-

vated increased tyrosine phosphorylation state is dependent on appropriate growth factor/growth factor receptor interaction (35). Nonetheless, the results of this study suggesting that IRS-2–mediated signaling pathways contribute to the regulation of insulinoma cell proliferation complement observations in IRS-2 knockout mice in which the pancreatic β-cell mass is reduced.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Swenne I: Pancreatic beta-cell growth and diabetes mellitus. *Diabetologia* 35:193–201, 1992
2. Bonner-Weir S: Two pathways of β-cell growth in the regenerating rat pancreas: implications for islet transplantation. *Diab Nutr Metab* 5 (Suppl.1):1–3, 1992
3. Smith FS, Rosen KM, Villa-Komaroff L, Weir GC, Bonner-Weir S: Enhanced insulin-like growth factor I gene expression in the regenerating rat pancreas. *Proc Natl Acad Sci U S A* 88:6152–6156, 1991
4. Swenne I, Andersson A: Effect of genetic background on the capacity for islet cell replication in mice. *Diabetologia* 27:464–467, 1984
5. Chick WL: Beta cell replication in pancreatic monolayer cultures: effects of glucose, tolbutamide, glucocorticoid, growth hormone and glucagon. *Diabetes* 22:687–693, 1973
6. Welsh M, Mares J, Öberg C, Karlsson T: Genetic factors of importance for β-cell proliferation. *Diabet Metab Rev* 9:25–36, 1993
7. Frodin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB, Van Obberghen E: Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting beta-cell line, INS-1. *J Biol Chem* 270:7882–7889, 1995
8. Khoo S, Cobb MH: Activation of mitogen-activating protein kinase by glucose is not required for insulin secretion. *Proc Natl Acad Sci U S A* 94:5599–5604, 1997
9. Nielsen JH, Linde S, Welinder BS, Billestrup N, Madsen OD: Growth hormone is a growth factor for the differentiated β-cell. *Mol Endocrinol* 13:165–173, 1989
10. Billestrup N, Nielsen JH: The stimulatory effect of growth hormone, prolactin and placental lactogen on beta-cell proliferation is not mediated by insulin-like growth factor 1. *Endocrinol* 129:883–888, 1991
11. Brelje TC, Sorenson RL: Role of prolactin versus growth hormone on islet B-cell proliferation in vitro: implications for pregnancy. *Endocrinol* 128:45–57, 1991
12. Moldrup A, Billestrup NB, Nielsen JH: Rat insulinoma cells express both a 115-kDa growth hormone receptor and a 95-kDa prolactin receptor structurally related to the hepatic receptors. *J Biol Chem* 265:8686–8690, 1990
13. Swenne I, Hill DJ, Strain AJ, Milner RDG: Growth hormone regulation of somatomedin S/insulin-like growth factor 1 production and DNA replication in fetal rat islets in tissue culture. *Diabetes* 36:288–294, 1987
14. Beattie GM, Rubin JS, Mally MI, Otonkoski T, Hayek A: Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes* 45:1223–1228, 1996
15. Polak M, Scharfmann R, Seilheimer B, Eisenbarth G, Dressler D, Verma IM, Potter H: Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line. *Proc Natl Acad Sci U S A* 90:5781–5785, 1993
16. Nielsen JH: Mechanisms of pancreatic B-cell growth and regeneration: studies in rat insulinoma cells. *Exp Clin Endocrinol Diabetes* 93:277–285, 1989
17. Chick WL, Warren S, Chute RN, Like AA, Lauris V, Kitchen KC: A transplantable insulinoma in the rat. *Proc Natl Acad Sci U S A* 74:628–632, 1977
18. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC, Lauris V: Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. *Proc Natl Acad Sci U S A* 77:3519–3523, 1980
19. Myers MGJ, Grammer TC, Wang LM, Sun XJ, Pierce JH, Blenis J, White MF: Insulin receptor substrate-1 mediates phosphatidylinositol 3'-kinase and p70S6K signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. *J Biol Chem* 269:28783–28789, 1994
20. Patti ME, Sun XJ, Bruening JC, Araki E, Lipes MA, White MF, Kahn CR: 4PS/insulin receptor substrate (IRS)-2 is the alternative substrate of the insulin receptor in IRS-1–deficient mice. *J Biol Chem* 270:24670–24673, 1995
21. Alarcón C, Lincoln B, Rhodes CJ: The biosynthesis of the subtilisin-related proprotein convertase PC3, but not that of the PC2 convertase, is regulated by glucose in parallel to proinsulin biosynthesis in rat pancreatic islets. *J Biol Chem* 268:4276–4280, 1993
22. Hutton JC, Davidson HW, Grimaldi KA, Peshavaria M: Biosynthesis of betagranin in pancreatic β-cells: identification of a chromogranin-A like precursor and its parallel processing with proinsulin. *Biochem J* 244:449–456, 1987
23. Asfari M, Janjic D, Meda P, Guodong L, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178, 1992
24. Hofmann C, White MF, Whittaker J: Human insulin receptors expressed in insulin-insensitive mouse fibroblasts couple with extant cellular effector systems to confer insulin sensitivity and responsiveness. *Endocrinology* 124:257–264, 1989
25. Schuppin GT, Rhodes CJ: Specific co-ordinated regulation of PC3 and PC2 gene transcription with that of pre-proinsulin in insulin-producing βTC3 cells. *Biochem J* 313:259–268, 1995
26. Aiello LP, Robinson GS, Lin Y-W, Nishio Y, King GL: Identification of multiple genes in bovine retinal pericytes altered by exposure to elevated levels of glucose by using mRNA differential display. *Proc Natl Acad Sci U S A* 91:6231–6235, 1994
27. Laing P, Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971, 1992
28. Orci L: The insulin factory: a tour of the plant surroundings and a visit to the assembly line. *Diabetologia* 28:528–546, 1985
29. Newgard CB: Cellular engineering for the treatment of metabolic disorders: prospects for therapy in diabetes. *Biotechnology* 10:1112–1120, 1992
30. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR: Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* 17:4902–4911, 1994
31. Olszewski S, Deeney JT, Schuppin GT, Williams KP, Corkey BE, Rhodes CJ: Rab3A effector domain peptides induce insulin exocytosis via a specific interaction with a cytosolic protein doublet. *J Biol Chem* 269:27987–27991, 1994
32. Hodgson CP, Elder PK, Ono T, Foster DN, Getz MJ: Structure and expression of VL30 genes. *Mol Cell Biol* 3:2221–2231, 1983
33. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, Hanahan D, Baekkeskov S: Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A* 85:9037–9041, 1988
34. Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y: Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 36:1139–1145, 1993
35. Myers MG, White MF: Insulin signal transduction and IRS proteins. *Annu Rev Pharmacol Toxicol* 36:615–658, 1996
36. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MGJ, Glasheen E, Lane WS, Pierce JH, White MF: Role of IRS-2 in insulin and cytokine signaling. *Nature* 377:173–177, 1995
37. Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352:73–77, 1991
38. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
39. Gao Z, Konrad RJ, Collins H, Matchinsky FM, Rothenburg PL, Wolf BA: Wortmannin inhibits insulin secretion in pancreatic islets and βTC3- cells independent of its inhibition of phosphatidylinositol-3-kinase. *Diabetes* 45:854–862, 1996
40. Rothenberg PL, Willison LD, Simon J, Wolf BA: Glucose-induced insulin receptor tyrosine phosphorylation in insulin-secreting beta-cells. *Diabetes* 44:802–809, 1995
41. Kar S, Carr BI: Differential display and cloning of messenger RNAs from the late phase of rat liver regeneration. *Biochem Biophys Res Commun* 212:21–26, 1995
42. Morley SJ: Signal transduction mechanisms in the regulation of protein synthesis. *Mol Biol Rep* 19:221–231, 1994
43. Kim K, Lecordier A, Bowman LH: Both nuclear and mitochondrial cytochrome c oxidase mRNA levels increase dramatically during mouse postnatal development. *Biochem J* 306:353–358, 1995
44. Islam TC, Bugge TH, Bohm S: The long terminal repeat of VL30 retrotransposons contains sequences that determine retinoic acid-induced transcription in cultured keratinocytes. *J Biol Chem* 268:3251–3259, 1993
45. Han KA, Rothberg P, Kulesz-Martin M: Altered levels of endogenous retroviral-like sequence (VL30) RNA during mouse epidermal cell carcinogenesis. *Mol Carcinog* 3:75–82, 1990

46. Hansen LH, Wang X, Kopchick JJ, Bouchelouche P, Nielsen JH, Galsgaard ED, Billestrup N: Identification of tyrosine residues in the intracellular domain of the growth hormone receptor required for transcriptional signaling and Stat5 activation. *J Biol Chem* 271:12669–12673, 1996
47. Yenush L, White MF: The IRS-signaling system during insulin and cytokine action. *Bioessays* 19:491–500, 1997
48. Smith-Hall J, Pons S, Patti ME, Burks DJ, Yenush L, Sun XJ, Kahn CR, White MF: The 60 kDa insulin receptor substrate functions like an IRS protein (pp60IRS3) in adipose cells. *Biochem* 36:8304–8310, 1997
49. Lavan BE, Lane WS, Lienhard GE: The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem* 272:11439–11443, 1997
50. Konrad RJ, Dean RM, Young RA, Billings PC, Wolf BA: Glucose-induced tyrosine phosphorylation of p125 in beta cells and pancreatic islets: a novel proximal signal in insulin secretion. *J Biol Chem* 271:24179–24186, 1996
51. Parrizas M, Saltiel AR, LeRoith D: Insulin-like growth factor-1 inhibits apoptosis using the phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways. *J Biol Chem* 272:154–161, 1997
52. Harbeck MC, Louie DC, Howland J, Wolf BA, Rothenberg PL: Expression of insulin receptor mRNA and insulin receptor substrate 1 in pancreatic islet beta-cells. *Diabetes* 45:711–717, 1996
53. Bruning JC, Winnay J, Cheatham B, Kahn CR: Differential signaling by insulin receptor substrate 1 (IRS-1) and IRS-2 in IRS-1 deficient cells. *Mol Cell Biol* 17:1513–1521, 1997
54. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, Johnson RS, Kahn CR: Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186–190, 1994
55. Whithers DJ, Gutierres JS, Towery H, Ren J-M, Burks DJ, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type-2 diabetes in mice. *Nature* 391:900–904, 1998