Endocrine Pancreas in Insulin Receptor–Deficient Mouse Pups

Malene Jackerott, Anne Baudry, Betty Lamothe, Danielle Bucchini, Jacques Jami, and Rajiv L. Joshi

Insulin receptor (IR)-deficient pups rapidly become hyperglycemic and hyperinsulinemic and die of diabetic ketoacidosis within a few days. Immunocytochemical analysis of the endocrine pancreas revealed that IR deficiency did not alter islet morphology or the number of β-, α-, δ-, and pancreatic polypeptide (PP) cells. The lack of IR did not result in major changes in the expression of islet hormone genes or of β-cell–specific marker genes encoding pancreas duodenum homeobox–containing transcription factor-1 (PDX-1), glucokinase (GCK), and GLUT2, as shown by reverse transcriptase–polymerase chain reaction analysis. The serum glucagon levels in IR-deficient and nondiabetic littermates were comparable. Finally, total insulin content in the pancreas of IR-deficient pups was gradually depleted, indicating sustained insulin secretion, not compensated for by increased insulin biosynthesis. These findings are discussed in light of recent results suggesting a role of IR in β-cell function. *Diabetes* 50 (Suppl. 1):S146–S149, 2001

Several studies using transgenic and knockout mice have established that the development and function of pancreatic β-cells are controlled by a number of genes encoding specific transcription factors such as pancreas duodenum homeobox–containing transcription factor-1 (PDX-1), hormones, growth factors such as IGF-I and IGF-II and their receptors, or proteins involved in glucose sensing such as glucokinase (GCK) and the glucose transporter GLUT2 (1,2).

The biological effects of insulin are mediated by the insulin receptor (IR), a heterotetrameric ($\alpha_2\beta_2$) membrane tyrosine kinase. After insulin binding, the activated IR phosphorylates docking proteins such as insulin receptor substrate (IRS)-1 or IRS-2. These adaptor proteins subsequently recruit intracellular mediators, which in turn lead to the activation of various signaling pathways (3). Although muscle, liver, and fat tissue represent the major target tissues for the metabolic action of insulin, IR is thought to be present in most cells and its expression in the β-cell has also been documented (4). Despite a number of in vivo studies of humans or animal models, the question as to whether IR expression in β-cells has any functional significance has remained a matter of debate for several years. It has emerged from some recent work using purified β-cells or β-cell lines that insulin action through IR might play an important autocrine role in normal β-cell function. This conclusion is based on the observations that insulin can stimulate insulin gene transcription as well as insulin secretion in β-cells (5–7). It was further shown that the signaling pathways activated by IR that lead to the transcriptional upregulation of the insulin gene involve the IRS-2/phosphatidylinositol 3-kinase/p70 S6 kinase and the calcium/calmodulin-dependent protein (CaM) kinase cascades (5).

We and others have previously reported that targeted disruption of the IR gene (*Insr*) in the mouse resulted in diabetic ketoacidosis and neonatal lethality (8,9). IR deficiency did not preclude the development of a pancreas with both exocrine and endocrine components. IR-deficient pups were normal at birth, but rapidly became hyperglycemic and hyperinsulinemic. To gain further insight into the role of IR in β-cell growth and function, we analyzed in this work the endocrine pancreas in IR-deficient pups.

RESEARCH DESIGN AND METHODS

Animals and genotyping. The generation of mice with targeted disruption of the IR gene has been described previously (8). IR-deficient mice produced by intercrossing heterozygous null mutants could be identified by their diabetic phenotype. The nondiabetic littermates were genotyped by polymerase chain reaction (PCR) performed on tail DNA using Taq DNA polymerase (Gibco) and primers specific for the neomycin phosphotransferase gene (5′-GTGTTCCGG CTGTCAGCGCA-3′/5′-GTCCTGATAGCGGTCCGCCA-3′). The following reaction cycles were used: 95°C for 5 min, 65°C for 1 min, and 72°C for 1 min, followed by 36 cycles of 95°C for 20 s, 65°C for 30 s, and 72°C for 1 min and finally 72°C for 7 min. A PCR product of 620 bp was detected in heterozygous mutant pups.

Immunocytochemistry and quantification of islet cells. The splenic part of the pancreas of newborn mice was fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Dewaxed sections (5 µm) were first blocked for endogenous peroxidase activity by incubation in a methanol/H_2O_2 mixture, then rehydrated and treated with proteinase K (1–4 µg/ml) at 37°C. After preincubation in goat serum, the sections were exposed to guinea pig anti-insulin (Sigma) or rabbit anti-glucagon (Dako), anti-somatostatin (Eurodiagnostica), or anti–human pancreatic polypeptide (PP) (provided by Dr. L.-I. Larsson) antibodies. Detection was by biotin-labeled goat anti–guinea pig IgG (Jackson ImmunoResearch Labs) or goat anti-rabbit IgG (Immunotech), followed by incubation with peroxidase-labeled streptavidin (DAKO) and development in diaminobenzidine-H_2O_2. The sections were counterstained with hematoxylin. Islet cells were quantified by counting immunopositive cells on six sections separated by more than 50 µm, followed by measurement of the surface area of the sections using Biocom VisioL@b 1000 software.

Reverse transcriptase–PCR analysis of pancreatic RNAs. Total pancreatic RNA from pools of four pancreata were prepared using SV Total RNA Iso-

From the Department of Genetics, Development and Molecular Pathology, ICGM, Institut National de la Santé et de la Recherche Médicale Unit 257, Paris, France.

Address correspondence and reprint requests to Dr. Rajiv L. Joshi, INSERM U257, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France. E-mail: joshi@cochin.inserm.fr.

Received for publication 22 May 2000 and accepted 14 August 2000.

This article is based on a presentation at a symposium. The symposium and the publication of this article were made possible by an unrestricted educational grant from Les Laboratoires Servier.

CaM, calcium/calmodulin-dependent protein; GCK, glucokinase; IGF-1R, type 1 receptor for IGFs; IR, insulin receptor; IRS, insulin receptor substrate; PCR, polymerase chain reaction; PP, pancreatic polypeptide; RIA, radioimmunoassay; RT, reverse transcriptase; TBP, TATA binding protein.

lation System (Promega). RNA samples (100 ng) were subjected to reverse transcriptase (RT)-PCR analysis using Access RT-PCR System (Promega) according to the manufacturer's instructions. The primers and probes used to analyze insulin 1/insulin 2, glucagon, somatostatin, PP, and β-actin mRNA have been specified previously (10). The primer sets and oligonucleotide probes used to analyze the transcripts for PDX-1, GCK, GLUT2, and TATA binding protein (TBP) were as follows: PDX-1, 5′-TCGCTGGGATCACTGGAGCA-3′/5′-GGTTCCGCTGTGTAAGCACC-3′ and 5′-[^{32}P]-GACCTTTCCCGAATGGA ACC-3′; GCK, 5′-CACCCAACTGCGAAATCACC-3′/5′-CATTTGTGGGGTGTG GAGTC-3′ and 5′-[^{32}P]-GGGCCAGTGAAATCCAGGCA-3′; GLUT2, 5′-GAGCC AAGGACCCCGTCCTA-3′/5′-GTGAAGACCAGGACCACCCC-3′ and 5′-[^{32}P]-GCCCTCTGCTTCCAGTACAT-3′; and TBP, 5′-AAGAGAGCCACGGACAACTG-3′/5′-TACTGAACTGCTGGTGGGTC-3′ and 5′-[^{32}P]-GAGTTGTGCAGAAGTTG GGC-3′. PCR reactions were run for 20 cycles for insulin 1, insulin 2, glucagon, somatostatin, PP, and β-actin mRNAs or 24 cycles for transcripts of PDX-1, GCK, GLUT2, and TBP, which allowed exponential coamplification at equal rate of β-actin or TBP mRNAs with the various gene transcripts. PCR products were run on agarose gels, transferred onto Hybond membranes, and hybridized using ^{32}P-labeled oligonucleotide probes. For insulin 1/insulin 2, a unique primer pair was used for RT-PCR, and the product was digested with *Msp*1 before Southern blot analysis using a single ^{32}P-labeled oligonucleotide probe, which revealed a fragment of 71 bp for insulin 1 and of 112 bp for insulin 2. Quantification of PCR products was performed using a Phosphorimager equipped with ImageQuant software (Molecular Dynamics) and was expressed in relation to internal control.

Measurement of pancreatic insulin content and serum glucagon levels. Pancreatic insulin was extracted using acid/ethanol and quantified by radioimmunoassay (RIA) (ICN Pharmaceutical). Each extract was prepared from a pool of five pancreata from newborn mice. Serum glucagon levels were measured using a glucagon RIA kit (Linco Research).

RESULTS AND DISCUSSION

Islet morphology in IR-deficient mice. Pancreatic sections from IR-deficient and wild-type animals showed similar islet morphology (Fig. 1*A*). In addition, quantitative analysis revealed that the numbers of the different islet cell types were also comparable (Fig. 1*B*). Thus, IR is not indispensable for islet formation and development of its various cell types.

IR and the type 1 receptor for IGFs (IGF-1R) are structurally very similar and activate common signaling pathways (11). Recent analysis of the endocrine pancreas in IGF-1R–deficient mice revealed that the numbers of β- and α-cells were significantly reduced (12). Thus, IGF-1R, in contrast to IR, plays a major role in the growth of the various endocrine cells. It was suggested that this growth-promoting function of IGF-1R could be achieved by IRS-2–mediated inhibition of apoptosis, because this was mimicked in mice lacking IRS-2 (12,13).

We previously reported that insulin deficiency in *Ins1/Ins2* double knockout mice resulted in β-cell hyperplasia (10). Thus, the lack of insulin or of IR does not have the same consequence on islet cell proliferation. In adult rats, short exposure to high glucose can lead to increase in β-cell mass (14,15). However, hyperglycemia per se cannot be responsible for β-cell hyperplasia, since both insulin- and IR-deficient mice become hyperglycemic. An attractive hypothesis is that lack of insulin could favor IGF-II action through IR, which might stimulate cell proliferation to a greater extent than insulin itself (16). More recently, it was reported that overexpression of IGF-II in β-cells in transgenic mice resulted in increased β-cell mass (17); it was proposed that more efficient IR activation by IGF-II could partly contribute to this effect. That IGF-II action through IR can indeed stimulate cell proliferation has been clearly demonstrated using a fibroblast cell line derived from IGF-1R–deficient mice (16). It is also conceivable that in the absence of insulin, the existence of empty IRs might result in an increased number of IR–IGF-1R hybrid receptors that can efficiently bind IGF-I. One wonders whether these hybrid receptors, which cannot form in IR-deficient mice, may have a greater mitogenic potential (18).

IR deficiency and gene expression in endocrine pancreas. Expression of genes encoding the four islet hormones was studied by RT-PCR. The results show that IR deficiency did not lead to major changes in the expression of these genes (Fig. 2*A*). Recent studies using purified β-cells or β-cell lines provided evidence that IR is involved in glucose regulation of insulin gene transcription, the secreted insulin acting on IR present on the β-cell (5). This mechanism of glucose action can account for the finding that IR tyrosine phosphorylation was induced in β-cell lines stimulated with either glucose or insulin (19). Also, the effect of glucose on insulin gene transcription in β-cell lines was abolished in the presence of insulin-signaling inhibitors that block phosphatidylinositol 3-kinase, CaM kinase, and p70 s6k (5). Furthermore, overexpression of IR in β-cell lines resulted in increased cellular levels of insulin mRNAs (6). In the hyperglycemic IR-deficient pups, one might have expected to find higher levels of insulin transcripts than in normoglycemic wild-type pups. Because total insulin transcripts detected in IR-deficient and wild-type pups were comparable, this suggests that transcriptional activation of the insulin gene by high glucose might

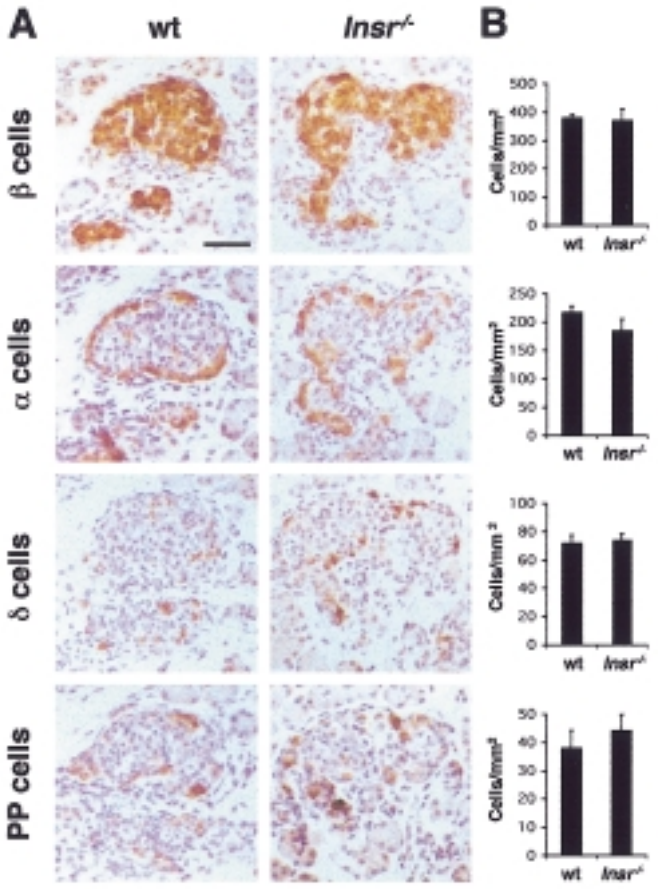

FIG. 1. Immunocytochemical analysis of pancreas. *A*: Pancreatic islets from IR-deficient and wild-type pancreata stained using antibodies against insulin, glucagon, somatostatin, and PP to detect β-, α-, δ-, and PP cells, respectively. *B*: Quantification of β-, α-, δ-, and PP cells (from top to bottom) in pancreata from IR-deficient and wild-type mice. The numbers are expressed in relation to total area of the counted tissue sections. Results are shown as means ± SE for four animals of each genotype. Bar = 40 μm.

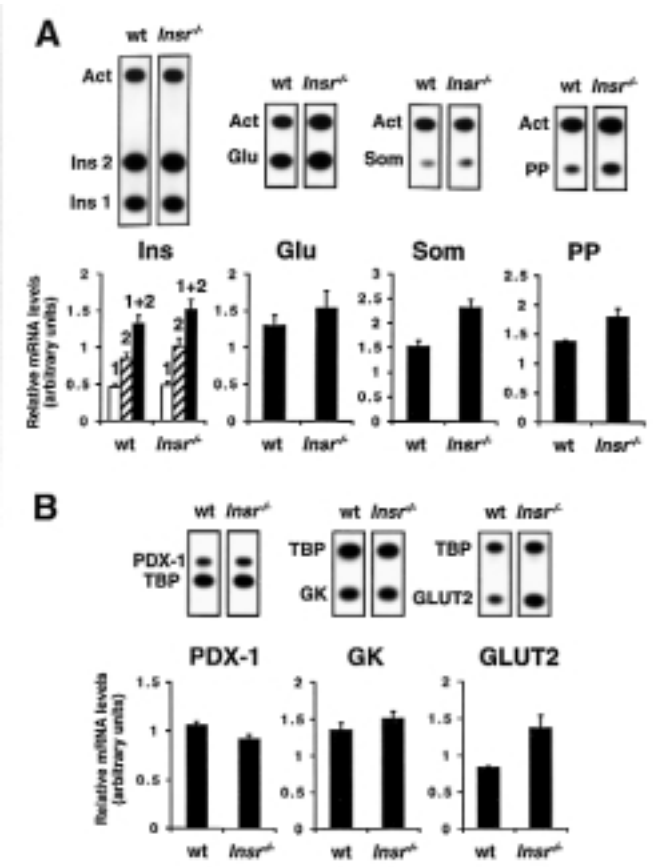

FIG. 2. RT-PCR analysis of pancreatic gene expression. *A*: Analysis of transcripts for insulin 1/insulin 2 (Ins1/Ins2), glucagon (Glu), somatostatin (Som), and PP. β-Actin (Act) mRNA was coamplified as control. Lower panel shows quantitative analysis. *B*: Analysis of the transcripts for PDX-1, GCK, and GLUT2. TBP mRNA was coamplified as control. Lower panel shows quantitative analysis. The quantitative data are presented as the ratio of the band intensities of the specific gene product in relation to internal controls. All RT-PCR reactions shown were performed using total RNA from IR-deficient and wild-type 3- to 4-day-old mice and the products were analyzed by Southern blotting. The ratio found with RNA from wild-type samples (stage P2; not shown) was normalized to 1. The sizes of the various PCR products correspond to 71 bp (insulin 1), 112 bp (insulin 2), 170 bp (glucagon), 165 bp (somatostatin), 150 bp (PP), 243 bp (β-actin), 275 bp (PDX-1), 162 bp (GCK), 150 bp (GLUT2), and 233 bp (TBP). Data are shown as means ± SE from triplicate assays.

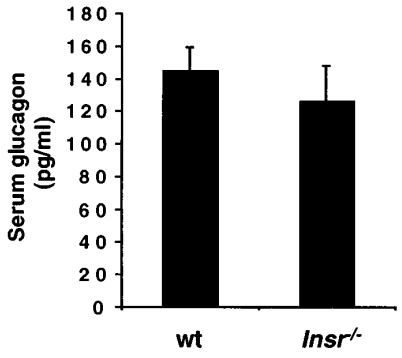

FIG. 3. Serum glucagon levels. Glucagon was measured by RIA in serum pools from 2- to 4-day-old wild-type (n = 9) and IR-deficient (n = 8) pups. Serum from nondiabetic heterozygous IR knockout pups was pooled with that from wild-type mice. Data are given as means ± SE.

require the presence of IR on β-cells. To further explore this issue, we plan to achieve transgenic reconstitution of IR gene expression in β-cells in IR-deficient mice, which we also expect to be hyperglycemic, to see whether and to what extent the level of insulin transcripts can be increased.

We further analyzed the expression of PDX-1, a key regulator of insulin gene transcription and the proposed mediator of the glucose responsiveness of insulin gene expression (20). In parallel with the equivalence of total insulin transcripts found in normal and mutant mice, PDX-1 gene expression was also unchanged in IR-deficient pups (Fig. 2*B*). PDX-1 has also been suggested to regulate the expression of other β-cell-specific genes, like those for the glucose-sensing proteins GCK and GLUT2 (1). RT-PCR revealed normal levels of GCK transcripts in hyperglycemic newborn mice lacking IR (Fig. 2*B*). This result is in agreement with an in vitro study showing no effect of glucose on GCK gene transcription in β-cells (21). In contrast, the RNA levels of GLUT2 were slightly increased in 3- to 4-day-old pups lacking IR (Fig. 2*B*), thereby confirming studies showing that hyperglycemia increases GLUT2 gene expression in β-cells (22).

In line with the similar levels of glucagon transcripts observed in the mutant and wild-type mice (Fig. 2*A*), serum glucagon levels in IR-deficient pups and their nondiabetic littermates were also comparable (Fig. 3). Thus, downregulation of glucagon secretion, which normally takes place under hyperinsulinemic and hyperglycemic conditions, was not seen in IR-deficient mice. This suggests a possible involvement of IR in the regulation of α-cell function. In the absence of counterregulatory insulin signaling, the normal serum glucagon levels must result in sustained glucagon action in the liver, leading to glycogen breakdown and increased ketogenesis.

IR deficiency and hyperinsulinemia. Because IR-deficient mice become both hyperglycemic and hyperinsulinemic, it is interesting to understand the mechanism leading to hyperinsulinemia in these mutants, i.e., lack of clearance versus increased insulin secretion from β-cells. It has been documented that insulin is eliminated from the circulation upon binding to IR in target tissues (23); therefore, it is conceivable

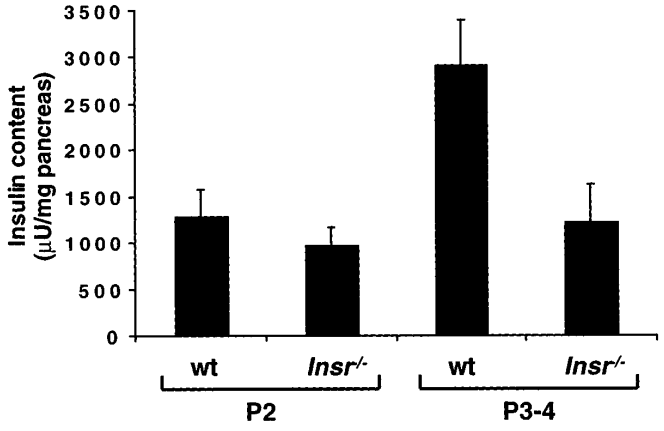

FIG. 4. Pancreatic insulin content. Insulin content was measured by RIA in pancreatic extracts prepared from wild-type and IR-deficient pups at 2 (P2) or 3–4 days (P3–4). Each extract was prepared from a pool of five pancreata. Data are given as means ± SE of four independent extracts for each genotype and age.

that reduced insulin clearance contributes to hyperinsulinemia in IR-deficient mice. However, and more importantly, insulin action through IR was also reported to play an active role in insulin secretion by β-cells (7). This issue was further investigated by generating mice with a β-cell–specific knockout of the IR gene (βIRKO). It was shown that insulin secretion in response to glucose was impaired in these mutants (24). In addition, βIRKO mice become hyperinsulinemic with age. We thus determined the total insulin content in pancreatic extracts from our hyperinsulinemic IR-deficient pups by RIA. Clearly, there exists a significant depletion of pancreatic insulin in mice lacking IR compared with wild-type controls (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day, than in 2-day-old pups, diabetic for less than 1 day. Thus, whereas the amounts of insulin transcripts are similar in *Insr*$^{-/-}$ and wild-type pancreas, sustained insulin secretion persists in the absence of IR and actively contributes to hyperinsulinemia in hyperglycemic IR knockout mice. It will be interesting to see whether insulin depletion would be higher in IR-deficient mice in which IR gene expression has been reconstituted in β-cells.

ACKNOWLEDGMENTS

We thank Dr. L.-I. Larsson providing us with the antiserum to human pancreatic polypeptide. M.J. is the recipient of a fellowship from Carlsbergfondet, A.B. from the Ministère de l'Enseignement Supérieur et de la Recherche, and B.L. from Fondation pour la Recherche Médicale. This work was supported by grants from the Comité de Paris de la Ligue Nationale Contre le Cancer, Association de la Recherche pour le Cancer (ARC), Fondation de France, and Lilly-Alfediam.

REFERENCES

1. Nielsen JH, Serup P: Molecular basis for islet development, growth, and regeneration. *Curr Opin Endocrinol Diabetes* 5:97–107, 1998
2. Lamothe B, Duvillié B, Cordonnier N, Baudry A, Saint-Just S, Bucchini D, Jami J, Joshi RL: Genetic manipulation of insulin action and β-cell function in mice. *Mol Cell Biochem* 182:161–168, 1998
3 White MF, Kahn CR: The insulin signaling system. *J Biol Chem* 269:1–4, 1994
4. Harbeck MC, Louie DC, Howland J, Wolf BA, Rothenberg PL: Expression of insulin receptor mRNA and insulin receptor substrate 1 in pancreatic islet β-cells. *Diabetes* 45:711–714, 1996
5. Leibiger IB, Leibiger B, Moede T, Berggreen PO: Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 S6 kinase and CaM kinase pathway. *Mol Cell* 1:933–938, 1998
6. Xu GG, Rothenberg PL: Insulin receptor signaling in the β-cell influences insulin gene expression and insulin content: evidence for autocrine β-cell regulation. *Diabetes* 47:1243–1252, 1998
7. Aspinwall CA, Lakey JRT, Kennedy RT: Insulin-stimulated insulin secretion in single pancreatic beta cells. *J Biol Chem* 274:6360–6365, 1999
8. Joshi RL, Lamothe B, Cordonnier N, Mesbah K, Monthioux E, Jami J, Bucchini D: Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. *EMBO J* 15:1542–1547, 1996
9. Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H: Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* 12:106–109, 1996
10. Duvillié B, Cordonnier N, Deltour L, Dandoy-Dron F, Itier JM, Monthioux E, Jami J, Joshi RL, Bucchini D: Phenotypic alterations in insulin-deficient mutant mice. *Proc Natl Acad Sci U S A* 94:5137–5140, 1997
11. De Meyts P, Urso B, Christoffersen CT, Shymko RM: Mechanism of insulin and IGF-I receptor activation and signal transduction specificity: receptor dimer cross-linking, bell-shaped curves, and sustained versus transient signaling. *Ann N Y Acad Sci* 766:388–401, 1995
12. Withers DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL, White MF: Irs-2 coordinates Igf-1 receptor-mediated β-cell development and peripheral insulin signaling. *Nat Genet* 23:32–40, 1999
13. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White M: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900–904, 1998
14. Bonner-Weir S, Deery D, Leahy JL, Weir GC: Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. *Diabetes* 38:49–53, 1989
15. Bernard C, Berthault MF, Saulnier C, Ktorza A: Neogenesis vs. apoptosis as main component of pancreatic beta cell mass changes in glucose-infused normal and mildly diabetic adult rats. *FASEB J* 13:1195–1205, 1999
16. Morrione A, Valentinis B, Xu SQ, Yumet G, Louvi A, Efstratiadis A, Baserga R: Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc Natl Acad Sci U S A* 94:3777–3782, 1997
17. Devedjian JC, George M, Casellas A, Pujol A, Visa J, Pelegrin M, Gros L, Bosch F: Transgenic mice overexpressing insulin-like growth factor-II in β cells develop type 2 diabetes. *J Clin Invest* 105:731–740, 2000
18. Pandini G, Vigneri R, Costantino A, Frasca F, Ippolito A, Fujita-Yamaguchi Y, Siddle K, Goldfine ID, Belfiore A: Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. *Clin Cancer Res* 5:1935–1944, 1999
19. Rothenberg PL, Willison D, Simon J, Wolf BA: Glucose-induced insulin receptor tyrosine phosphorylation in insulin-secreting β-cells. *Diabetes* 44:802–809, 1995
20. Marshak S, Totary H, Cerasi E, Melloul D: Purification of the β-cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. *Proc Natl Acad Sci U S A* 93:15057–15062, 1996
21. Liang Y, Najafi H, Smith RM, Zimmerman EC, Magnuson MA, Tal M, Matschinsky FM: Concordant glucose induction of glucokinase, glucose usage, and glucose-stimulated insulin release in pancreatic islets maintained in organ culture. *Diabetes* 41:792–806, 1992
22. Chen L, Alam T, Johnson JH, Hughes S, Newgard CB, Unger RH: Regulation of β-cell glucose transporter gene expression. *Proc Natl Acad Sci U S A* 87:4088–4092, 1990
23. Sato H, Terasaki T, Mizuguchi H, Okumura K, Tsuji A: Receptor-recycling model of clearance and distribution of insulin in the perfused mouse liver. *Diabetologia* 34:613–621, 1991
24. Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn RC: Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329–339, 1999