Cell, Vol. 88, 561–572, February 21, 1997, Copyright ©1997 by Cell Press

Development of a Novel Polygenic Model of NIDDM in Mice Heterozygous for *IR* and *IRS-1* Null Alleles

Jens C. Brüning,* Jonathon Winnay,* Susan Bonner-Weir,* Simeon I. Taylor,† Domenico Accili,‡ and C. Ronald Kahn*

*Research Division
Joslin Diabetes Center and
Department of Medicine
Harvard Medical School
Boston, Massachusetts 02215
†Diabetes Branch
National Institutes of Diabetes
and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892
‡Developmental Endocrinology Branch
National Institute of Child Health
and Human Development
National Institutes of Health
Bethesda, Maryland 20892

Summary

NIDDM is a polygenic disease characterized by insulin resistance in muscle, fat, and liver, followed by a failure of pancreatic β cells to adequately compensate for this resistance despite increased insulin secretion. Mice double heterozygous for null alleles in the *insulin receptor* and *insulin receptor substrate-1* genes exhibit the expected ~50% reduction in expression of these two proteins, but a synergism at a level of insulin resistance with 5- to 50-fold elevated plasma insulin levels and comparable levels of β cell hyperplasia. At 4–6 months of age, 40% of these double heterozygotes become overtly diabetic. This NIDDM mouse model in which diabetes arises in an age-dependent manner from the interaction between two genetically determined, subclinical defects in the insulin signaling cascade demonstrates the role of epistatic interactions in the pathogenesis of common diseases with non-Mendelian genetics.

Introduction

Noninsulin-dependent diabetes mellitus (NIDDM) is the most common endocrine disorder, affecting over 5% of the population in western countries, with increasing incidence as the population ages and becomes more sedentary and obese (Warram et al., 1995). A strong genetic component in the etiology of NIDDM is suggested by a very high concordance rate between monozygotic twins (Newman et al., 1987) and an increased prevalence of the disorder in first-degree relatives of affected individuals (Rich, 1990). Thus far, attempts to identify relevant mutations or polymorphisms that account for this genetic predisposition have met very limited success. Some subtypes of NIDDM, such as syndromes of severe insulin resistance or maturity onset diabetes in the young (MODY), are due to mutations in the *insulin receptor* (*IR*) or the *glucokinase* genes (Flier, 1992; Froguel et al., 1992; Taylor, 1992). These subtypes, however, represent less than 5% of the patients with NIDDM, and the patterns of inheritance in the remaining NIDDM patients are consistent with a complex pattern of inheritance. Longitudinal studies indicate that the earliest detectable defect in the pathogenesis of NIDDM is the inability of tissues such as muscle and fat to respond to normal levels of circulating insulin, i.e., insulin resistance (Martin et al., 1992; Kahn, 1994). Pancreatic β cells can compensate for insulin resistance by increasing their mass (Bonner-Weir et al., 1989) or their secretory efficiency (Parsons et al., 1992; Piperleers, 1992). If, however, there is inadequate compensation, hyperglycemia eventually develops.

Over the past decade, many components of the insulin signaling network have been identified, and some have been studied as possible sites of genetically determined insulin resistance (Kahn, 1994). The major intracellular substrates of the insulin receptor tyrosine kinase are insulin receptor substrates IRS-1 and IRS-2 (Sun et al., 1991, 1995). IRS molecules become rapidly phosphorylated on multiple tyrosine residues upon ligand stimulation and bind a variety of signaling proteins via their src-homology-2 (SH2) domains, including the growth factor receptor binding protein Grb-2, the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase and the tyrosine protein phosphatase Syp/SHPTP 2 (Backer et al., 1992; Baltensperger et al., 1993; Skolnik et al., 1993; Sun et al., 1993). These signaling molecules bind to IRSs and are activated, thus mediating a variety of biological signals such as mitogenesis, glucose transport, and gene transcription (Waters et al., 1993; Cheatham et al., 1994; Rose et al., 1994; Cheatham and Kahn, 1995). Compared to other signaling molecules, the *IRS-1* gene is highly polymorphic, exhibiting coding sequence variations in about 5% of normal individuals and between 10% and 20% of people with NIDDM (Almind et al., 1993; Laakso et al., 1994; Ura et al., 1996). Moreover, alterations in the level of expression of both the insulin receptor and IRS-1 are observed in humans and rodents with NIDDM owing to down-regulation at the protein level (Saad et al., 1992; Bisbis et al., 1993; Goodyear et al., 1995).

Naturally occurring animal models, such as the *ob/ob* and *db/db* mouse and the Zucker *fatty* rat, that have been commonly employed for the analysis of NIDDM do not appear to accurately mimic the human disease, but are models in which diabetes occurs secondarily to massive obesity resulting from mutations of the gene for leptin or its receptor (Zhang et al., 1994; Chen et al., 1996; Chua, Jr., et al., 1996). We and others have attempted to create new models of NIDDM by eliminating expression of single proteins in the insulin signaling cascade, such as the insulin receptor and IRS-1. However, mice deficient for the *IR* gene exhibit severe insulin resistance and die from ketoacidosis within 3–7 days after birth, and animals lacking IRS-1 are severely growth retarded (due to IGF-1 resistance) as well as insulin resistant. Such severe deficiencies of insulin signaling proteins are very rare in humans (Wertheimer et al.,

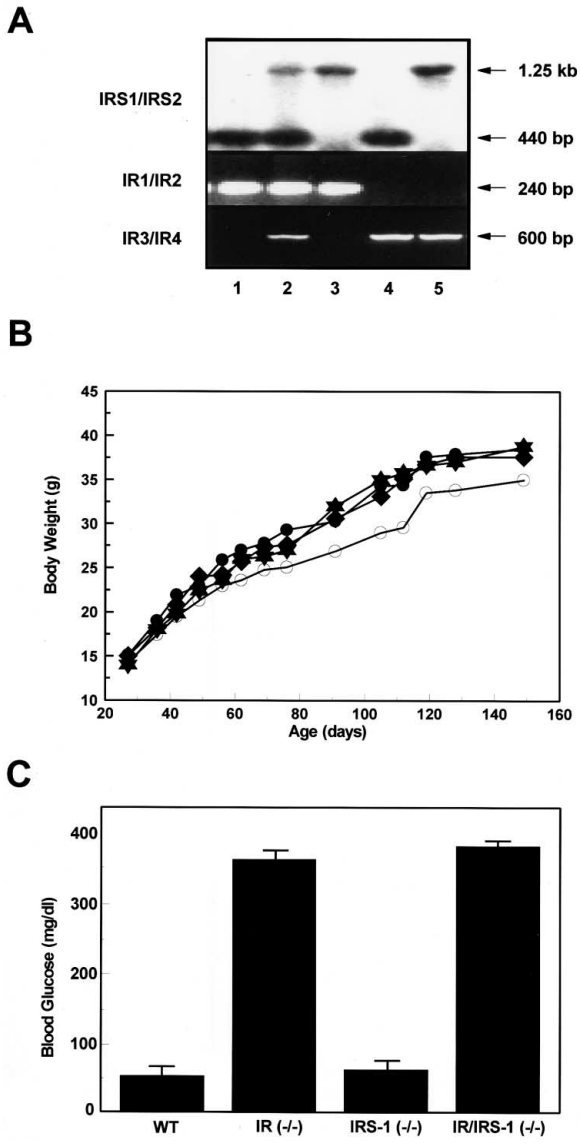

Figure 1. Creation of *IR/IRS-1* Mutant Mice

(A) Mice were bred as described in the text. DNA extracted from tail biopsies was subjected to PCR analysis using primers flanking the insertion site of the neomycin resistance cassette (IRS1/IRS2) in the *IRS-1* gene, primers in intron 3 (IR1) and exon 4 (IR2) of the *IR* gene, and primers in exon 4 (IR3) of the *IR* gene and the neomycin cassette (IR4), which replaced part of exon 4 in the targeted *IR* locus. Presence of the wild-type allele in the *IRS-1* gene results in a 440 bp product, whereas insertion of the neomycin cassette in the targeted allele results in a band of 1.25 kb (upper panel). Primer set IR1 and IR2 detect only the wild-type *IR* allele, since the recognition site of IR2 is replaced in the targeted allele (middle panel). Primer set IR3 and IR4 yield only a product in the presence of the mutant *IR* allele, since primer 4 is anchored in the neomycin resistance cassette (bottom panel). Exemplary results are shown for wild-type (lane 1), *IR/IRS-1* (+/−) (lane 2), *IRS-1* (−/−)/*IR* (+/+) (lane 3), *IRS-1* (+/+)/*IR* (−/−) (lane 4), and *IR/IRS-1* (−/−) (lane 5) mice.

(B) Body weights of wild-type (closed circle), *IRS-1* (+/−) (closed diamond), *IR* (+/−) (asterisk), and *IR/IRS-1* (+/−) (open circle) mice were determined at the indicated age. Data are presented from at least eight animals of each genotype. Values represent the mean body weight; the standard error at each point was <15% within each group.

1993), and heterozygous animals for both knockouts did not exhibit an obvious clinical phenotype (Araki et al., 1994; Tamemoto et al., 1994; Accili et al., 1996; Joshi et al., 1996).

Therefore, to create a polygenic mouse model for NIDDM that might more closely mimic the human disease, we have generated mice with combined heterozygous deficiencies in the *IR* and *IRS-1* genes. These animals are lean but highly insulin resistant, and 40% develop NIDDM between the ages of 4 and 6 months, despite massive hyperinsulinemia and pancreatic β cell hyperplasia. These animals provide a new understanding of the genetic and molecular mechanisms underlying NIDDM in which the combination of two mild defects in the insulin signaling cascade give rise to insulin resistance and subsequent progression to a diabetic phenotype.

Results

IR/IRS-1 (+/−) Mice Develop Normally, While *IR/IRS-1* (−/−) Mice Die from Diabetic Ketoacidosis

Mice double heterozygous for *IR* and *IRS-1* null alleles were obtained by breeding mice heterozygous for either mutation alone and identified according to the scheme outlined in Figure 1A. These *IR/IRS-1* (+/−) mice were born with the expected Mendelian frequency of 25% and were indistinguishable from wild-type, *IRS-1* (+/−), and *IR* (+/−) mice. Their extrauterine development proceeded at normal rates, with a slight, nonsignificant decrease in body weight (Figure 1B). Simultaneously, mice homozygous for null mutations in both genes were created by mating male and female *IR/IRS-1* double heterozygote mice or *IRS-1* (−/−) *IR* (+/−) male mice with double heterozygote *IR/IRS-1* females. Mice homozygous for the *IR* null allele, either alone or in combination with the null allele in the *IRS-1* locus, were also born with the expected frequency. As previously described for the *IR* (−/−) mice, animals of these genotypes died within 72 hr of birth due to uncontrolled diabetes. Blood glucose concentrations, determined 24 hr after birth, revealed severe hyperglycemia, independent of the presence of the *IRS-1* mutation (Figure 1C). Likewise, mice homozygous for the *IRS-1* null allele, whether alone or in combination with the *IR* null alleles, exhibited intrauterine growth retardation.

Heterozygosity for Null Alleles in the *IR* and *IRS-1* Gene Results in Synergistic Impairment of Insulin Signaling

To assess the effect of heterozygosity for a null allele in the *IR* and *IRS-1* genes alone or in combination on protein expression, we performed immunoprecipitations on liver and muscle extracts from wild-type, *IRS-1* (+/−), *IR* (+/−), and *IR/IRS-1* (+/−) mice using anti-IR,

(C) Blood glucose concentrations were determined in wild-type (WT), homozygous insulin receptor–deficient (*IR* [−/−]), homozygous IRS-1-deficient (*IRS-1* [−/−]), and double homozygous mice (*IR/IRS-1* [−/−]) mice 16–24 hours after birth. Data shown represent the mean +/− SEM for at least four animals of each genotype.

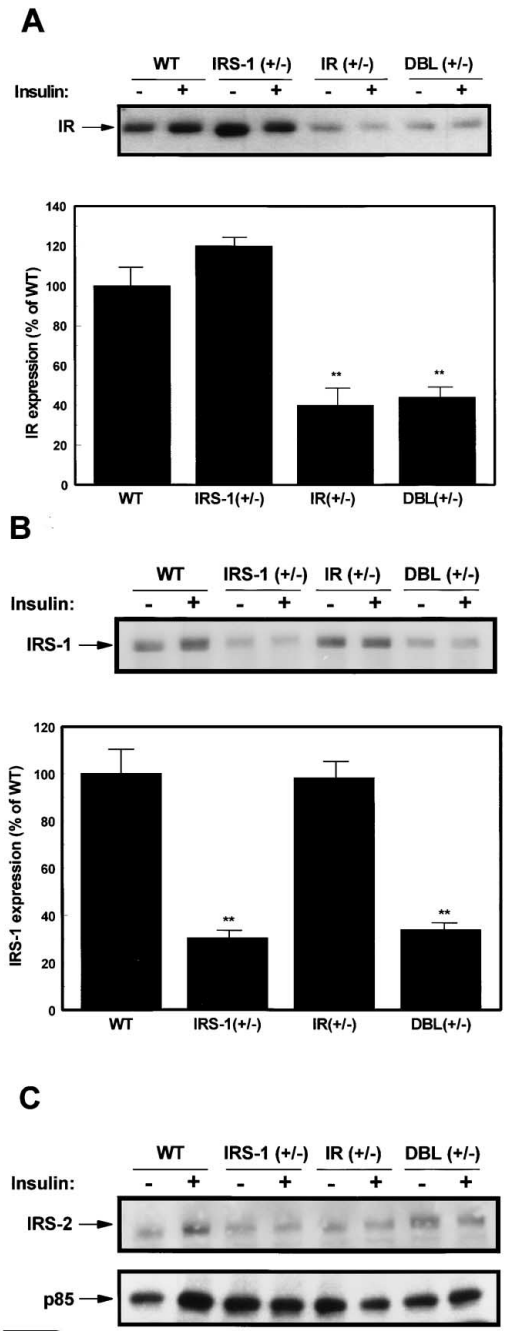

Figure 2. Expression of Insulin Receptor and IRS-1 in Liver of Different Mutant Mice

(A) Liver extracts prepared from wild-type (WT), heterozygous *IRS-1* (*IRS-1* [+/−]), heterozygous *IR* (*IR* [+/−]), and double heterozygous (*IR*/*IRS-1* [+/−]) mice that had been injected with either saline (−) or insulin (+) in the inferior vena cava after an overnight starvation period were subjected to immunoprecipitation with an anti-insulin receptor antibody, followed by Western blotting with the same antibody. The upper panel shows a representative experiment; the lower panel summarizes the quantitation of the results obtained from 4–6 animals of each genotype. The data are expressed as the percentage of insulin receptor expression in the wild-type animals. The data are the mean +/− SEM.

(B) The experiment was performed as described in (A), with the exception that immunoprecipitation and Western blotting were performed with an antibody against IRS-1.

anti-IRS-1, anti-IRS-2, and anti-p85 antibodies, followed by Western blot analysis with the respective antibodies. If a null allele for *IR* was present either alone or in combination with an *IRS-1* null allele, expression of IR was reduced in approximate proportion to gene dosage. Quantification of multiple experiments showed an ~60% reduction in IR expression in liver (Figure 2A) and muscle (data not shown) of heterozygote *IR* (+/−) animals. Similarly, inactivation of one allele of the *IRS-1* gene resulted in an ~60% reduction in IRS-1 protein expression (Figure 2B). As expected, the expression of the alternative substrate of the insulin receptor kinase, IRS-2, and of the p85 regulatory subunit of PI 3-kinase was unaltered by the presence of either the *IR* or *IRS-1* null alleles (Figure 2C). Short-term administration of insulin had no effect on the expression of the insulin signaling molecules examined.

To determine the consequence of reduced insulin receptor and IRS-1 expression on insulin-stimulated signaling, we investigated the early signaling events in both liver and muscle. In wild-type animals, insulin rapidly stimulated the tyrosine autophosphorylation of the insulin receptor, and this was unaltered in *IRS-1* (+/−) mice (Figure 3A). In *IR* (+/−) and *IR*/*IRS-1* (+/−) mice, the 60% reduction of insulin receptor expression led to a similar reduction in receptor autophosphorylation (Figure 3A). As with insulin receptor phosphorylation, wild-type animals also exhibited a robust stimulation of IRS-1 phosphorylation (Figure 3B). Reduction of IRS-1 expression alone in *IRS-1* (+/−) mice or reduction of insulin receptor alone in *IR* (+/−) mice led to a 40%–50% reduced tyrosine phosphorylation of IRS-1 (Figure 3B). In double heterozygous animals, combined deficiency of one copy of the *IR* and one copy of the *IRS-1* gene led to a 70% reduction of insulin-stimulated tyrosine phosphorylation of IRS-1 (Figure 3B). Thus, the combined deficiency for the insulin receptor and IRS-1 resulted in an additive effect on impairment of this step in insulin signaling.

IRS-2 is an alternative substrate of the insulin receptor kinase and provides a partial compensation for insulin and IGF-1 (insulin-like growth factor-1) signaling in homozygous IRS-1-deficient mice (Araki et al., 1994; Patti et al., 1995). In livers of wild-type mice, IRS-2, like IRS-1, becomes rapidly tyrosine phosphorylated following insulin administration (Figure 3C). Reduced expression of IRS-1 in the *IRS-1* (+/−) mice led to an ~20% increase in IRS-2 phosphorylation (Figure 3C). Since this occurs without an increase in IRS-2 expression, this suggests that IRS-1 and IRS-2 act as competitive substrates for the insulin receptor kinase. In contrast, reduced expression of the insulin receptor resulted in a 40% reduction of IRS-2 phosphorylation, in both the *IR* (+/−) and *IR*/*IRS-1* (+/−) mice (Figure 3C). Thus, the double heterozygote mice exhibited a 40% impairment of both insulin receptor and IRS-2 phosphorylation due to reduced insulin receptor expression and a 70% reduction in IRS-1

(C) Shown is a representative experiment for the expression of IRS-2 (upper panel) and p85, the regulatory subunit of PI 3-kinase (lower panel), in liver of the different mutant mice. Immunoprecipitation and Western blotting were performed as described in Experimental Procedures.

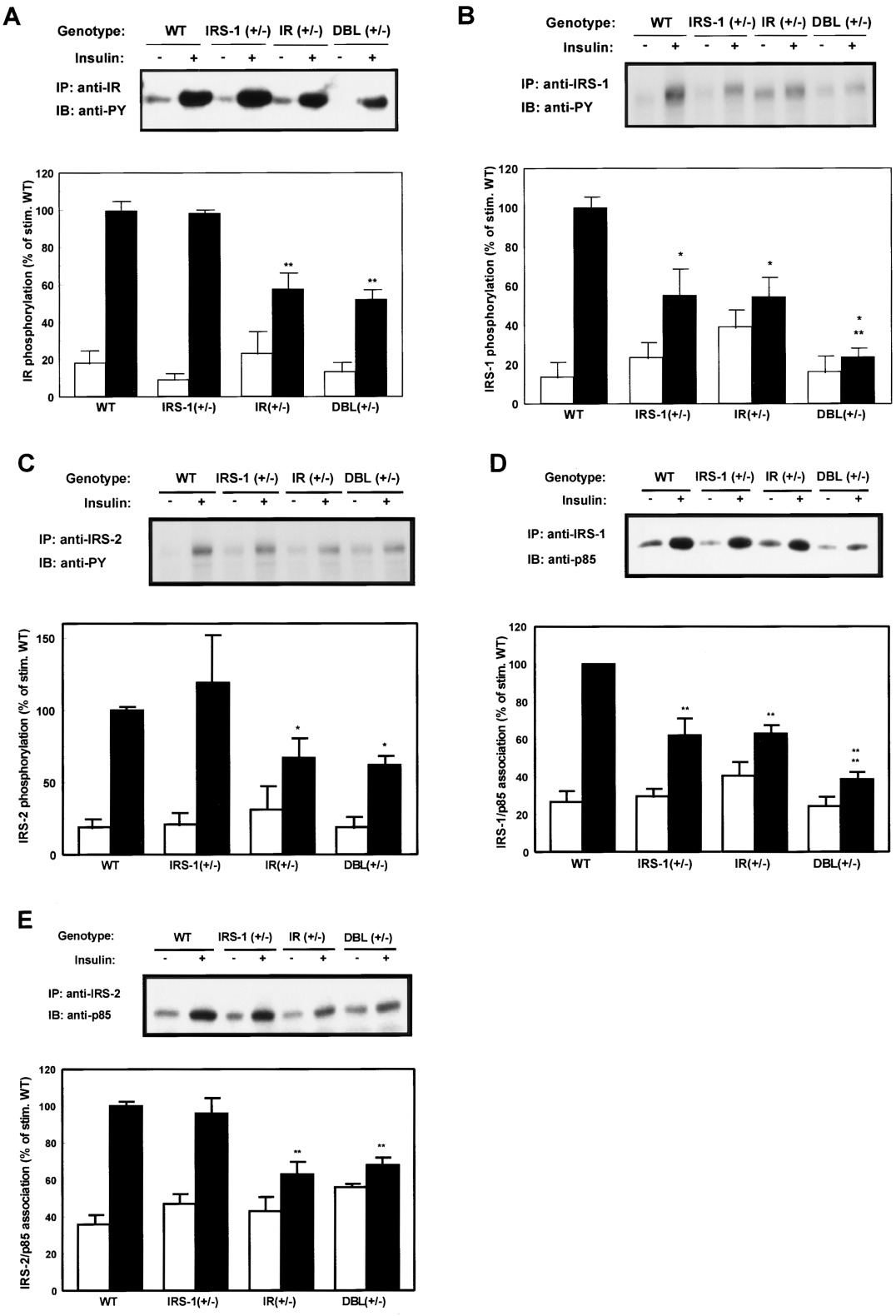

Figure 3. Proximal Steps of Insulin Signaling in Liver of Different Mutant Mice

(A) Insulin-stimulated tyrosine phosphorylation of the β subunit of the insulin receptor was assayed by performing immunoprecipitations using an anti-insulin receptor antibody on liver extracts from mice that had been injected with either saline (−) or insulin (+) in the inferior vena cava after an overnight starvation period. Western blot analysis was performed using an anti-phosphotyrosine antibody. The upper panel shows a representative experiment; the lower panel, the quantitation of results for at least four animals under each experimental condition. Results are expressed as percentage of the signal intensity detected in the insulin-stimulated wild-type (WT) animal. Open bars indicate values

phosphorylation due to combined deficiency in both genes.

IRS-1 and IRS-2 mediate further downstream signaling events by binding to SH2 domain–containing proteins via their tyrosine-phosphorylated motifs. One of the SH2 proteins implicated in mediating insulin-stimulated glucose transport and other metabolic signals is the p85 regulatory subunit of PI 3-kinase that binds to phosphorylated IRS-1, resulting in activation of the p110 catalytical subunit. Association of p85 with IRS-1 was reduced by 40% in liver of *IRS-1* (+/−) mice and *IR* (+/−) mice and by 60% in *IR*/*IRS-1* (+/−) mice, paralleling the decreased IRS-1 phosphorylation in these mice (Figure 3D). In contrast, IRS-2-associated p85 was unaltered in the *IRS-1* (+/−) mice, despite the modest increase in insulin-stimulated IRS-2 phosphorylation (Figure 3E). Reduction of insulin receptor expression, on the other hand, resulted in a 40% reduction in insulin-stimulated association of IRS-2 with p85, whether the level of IRS-1 expression was normal or reduced (Figure 3E).

Insulin Signaling in Muscle versus Insulin Signaling in Liver

Changes in insulin-stimulated signaling in skeletal muscle tissue for the insulin receptor and IRS-1 were similar to those observed in liver of these different mutant mice. Thus, insulin receptor expression and insulin-stimulated tyrosine phosphorylation of the insulin receptor β subunit was reduced by 50% in the muscle of *IR* (+/−) and *IR*/*IRS-1* (+/−) mice (Figure 4, upper panel). Insulin-stimulated tyrosine phosphorylation of IRS-1 and association of p85 to IRS-1 was reduced by ~50% in *IRS-1* (+/−) and *IR* (+/−) mice and by ~80% in *IR*/*IRS-1* (+/−) mice (Figure 4, middle panels).

Examination of IRS-2 expression and its tyrosine phosphorylation upon insulin stimulation, however, revealed an important difference in insulin signaling between liver and muscle. While there was a significant amount of IRS-2 detectable in liver extracts from mice of the different genotypes, immunoprecipitation from muscle extracts using IRS-2-specific antisera revealed a very weak signal, suggesting that IRS-2 expression in muscle is low as compared to its expression in liver.

To address the question as to what extent insulin stimulated association of p85 to any cellular tyrosine phosphoprotein in muscle, we prepared anti-p85 immunoprecipitates from muscle extracts and subjected them to Western blotting with an anti-phosphotyrosine antibody. As previously described, in the wild-type mice the only signal detectable in this assay was a broad band in the molecular weight range of ~185 kDa, which represents both tyrosine-phosphorylated IRS-1 and IRS-2 (Figure 4, lower panel). In *IR* (+/−) or *IRS-1* (+/−) mice, this signal was reduced by ~50%, paralleling the decrease in IRS-1 phosphorylation in these animals. In the double heterozygote, the pp185 band was almost undetectable, indicating that IRS-1 is the major insulin signaling component in muscle mediating insulin stimulation of PI 3-kinase and that very little tyrosine-phosphorylated IRS-2 is available to bind p85.

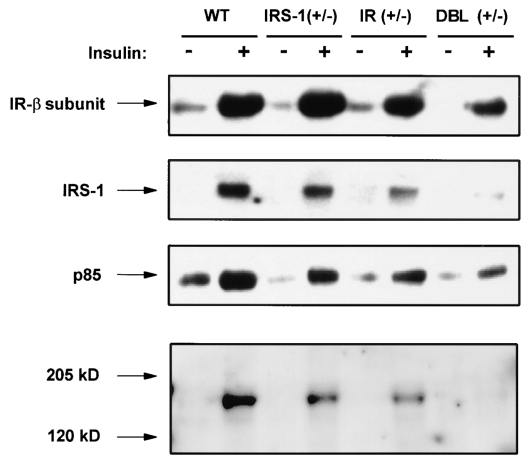

Figure 4. Insulin-Stimulated Signaling Events in Muscle of Different Mutant Mice

Insulin-stimulated tyrosine phosphorylation of the insulin receptor (upper panel) and IRS-1 (second panel from the top), as well as insulin-stimulated association of p85 with IRS-1 (second panel from the bottom) were assayed on muscle extracts from saline-injected (−) or insulin-injected (+) animals of the indicated genotypes as described in Figures 3A, 3B, and 3D. Detection of insulin-stimulated association of cellular tyrosine phosphoproteins with p85 (bottom panel) was assessed by performing immunoprecipitation with an anti-p85-specific antiserum followed by Western blot with an anti-phosphotyrosine antibody. Results shown are representative for 2–4 independent experiments that gave similar findings.

Consequence of Impaired Insulin Signaling in *IR*/*IRS-1* (+/−) Mice on Glucose Homeostasis

Despite the reductions in insulin receptor and IRS-1 protein, there was no detectable difference in blood

for untreated animals; closed bars, values for insulin-treated animals.

(B) Insulin-stimulated tyrosine phosphorylation of IRS-1 was performed as described in (A), with the exception that immunoprecipitations were performed using an anti-IRS-1-specific antiserum. The upper panel shows results of a typical experiment; the lower panel, the quantitation of results obtained for at least four animals under each condition, expressed as in (A).

(C) Insulin-stimulated tyrosine phosphorylation of IRS-2 was assessed in a manner similar to that described for IRS-1 in part (B), except that immunoprecipitations were performed with an anti-IRS-2-specific antiserum. The top panel shows a representative experiment; the bottom panel, the quantitation of 4–6 animals for each experimental condition.

(D) Insulin-stimulated association of p85, the regulatory subunit of PI 3-kinase, with IRS-1 was assayed by performing immunoprecipitations using an anti-IRS-1-specific antiserum, followed by Western blot analysis using an anti-p85-specific antiserum. The upper panel shows results of a typical experiment; the lower panel, quantitation of four independent determinations for each experimental condition. Data are expressed as percentage of the signal intensity obtained for the sample of the insulin-stimulated wild-type animal.

(E) Insulin-stimulated association of p85 with IRS-2 was assayed as described in part (D), with the exception that IRS-2-specific antisera were used for the immunoprecipitations. The upper panel shows the result of an exemplary experiment; the lower panel, the quantitation of five experiments presented as percentage of the intensity found in the insulin-stimulated wild-type animals.

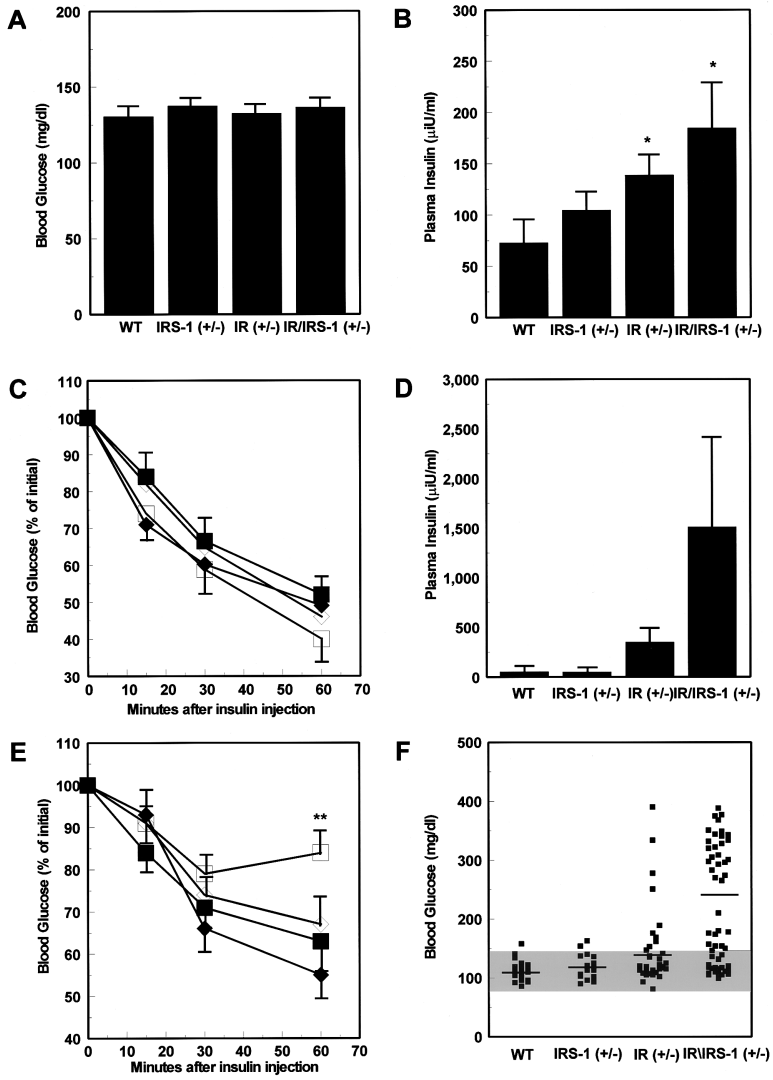

Figure 5. Effect of Heterozygosity for Insulin Receptor and/or IRS-1 Deficiency on Glucose Homeostasis

(A) Blood glucose concentrations were determined in random fed mice of the indicated genotype using samples obtained from tail biopsies at the age of 2 months. Data represent the mean blood glucose concentration +/− SEM for at least ten animals of each genotype.

(B) Plasma insulin concentrations were determined for random fed animals at the age of 2 months. Blood was sampled by orbital puncture from mice that had been anaesthetized by an inhalation anaesthesia using methoxyflurane. Insulin concentrations were determined by RIA using rat insulin as a standard. Data shown are the mean +/− SEM of 8–12 animals for each genotype.

(C) Insulin tolerance tests were performed on conscious 2-month-old animals. 0.75 U/kg body weight of crystalline insulin were injected in the peritoneal cavity. Blood glucose concentrations were determined on blood samples obtained by tail biopsy immediately before and 15, 30, and 60 min after injection. Results are expressed as percentage of blood glucose concentration before insulin injection and are the mean +/− SEM of 6–8 animals of each genotype: wild-type, closed diamond; *IRS-1* (+/−), closed square; *IR* (+/−), open diamond; *IR/IRS-1* (+/−), open square.

(D) Plasma insulin concentrations were determined on random fed animals at the age of 4–6 months as described in (B). Data shown are the mean +/− SEM of 10–12 animals for each genotype.

(E) Insulin tolerance tests were performed on conscious 4- to 6-month-old animals as described in (C). Tests were performed on animals, which were normoglycemic over the period before the test and exhibited blood glucose concentrations in a range of the mean +/− 2 SEM of wild-type animals at the day of the test. Results are the mean +/− SEM of 8–12 animals of each genotype: wild-type, closed diamond; *IRS-1* (+/−), closed square; *IR* (+/−), open diamond; *IR/IRS-1* (+/−), open square.

(F) Blood glucose concentrations were determined in random fed mice at the age of 4–6 months. Data are shown for individual animals of the indicated genotype and represent the mean blood glucose concentration obtained in at least two independent determinations. The shaded area indicates the mean blood glucose concentration of wild-type animals +/− 2 SD. The bar indicates the mean blood glucose concentration for the indicated genotype.

glucose concentrations of *IRS-1* (+/−), *IR* (+/−), and *IR/IRS-1* (+/−) mice when examined shortly after birth (data not shown) or at the age of 2 months (Figure 5A). At 2 months, however, insulin levels already suggested an incremental effect of the two mutations on insulin resistance. Thus, insulin levels in the fed state were increased ~30% in *IRS-1* (+/−) mice (n.s.), 1.9-fold in *IR* (+/−) mice ($p < 0.05$), and 2.6-fold ($p < 0.05$) in the double heterozygotes as compared to wild-type animals (Figure 5B). Intraperitoneal insulin tolerance tests performed with 0.75 U/kg body weight, however, were not sensitive enough to detect a significant difference in the blood glucose–lowering effect of exogenously administered insulin at this age (Figure 5C).

When the animals were reexamined between the ages of 4 and 6 months, insulin resistance had increased dramatically. Thus, by this age, the *IR* (+/−) mice exhibited 5-fold elevated plasma insulin concentrations, and those in *IR/IRS-1* (+/−) mice were 13-fold elevated (Figure 5D). This insulin resistance was confirmed in the insulin tolerance tests. Blood glucose levels in wild-type mice fell by 45% over a 1 hr period following an intraperitoneal injection of insulin, but dropped only by 30%–35% in *IRS-1* (+/−) and *IR* (+/−) heterozygotes (Figure 5E). Even more dramatic results were obtained when *IR/IRS-1* (+/−) mice were examined. In these mice, blood glucose concentrations dropped by ~20% at 30 min after insulin injection and began to rise again by 1 hr. Thus, the double heterozygous mice were the only group examined reaching statistical significance, as compared to wild-type animals with regard to insulin resistance in intraperitoneal tolerance testing ($p < 0.01$)

(Figure 5E). These data, together with the markedly elevated plasma insulin concentrations in *IR/IRS-1* (+/−) mice, indicate that two mild defects in the insulin signaling cascade can act synergistically in producing insulin resistance.

Also, over time, the insulin resistance produced by two 50% defects in insulin signaling was sufficient to cause an increased rate of diabetes, even in the absence of obesity or overt β cell injury. Analysis of blood glucose concentrations revealed a tight range in which random fed glucose concentrations were regulated in wild-type animals (Figure 5F). Blood glucose concentrations in *IRS-1* (+/−) were indistinguishable from those found in wild-type mice (Figure 5F). These data are consistent with our previous findings that no wild-type or *IRS-1* (+/−) animal monitored in our laboratory over the past three years has developed diabetes. Moderate insulin resistance in the *IR* (+/−) mice resulted in the occurrence of hyperglycemia in some of these mice. While most of them exhibited blood glucose concentrations within the range of control animals, some mice exhibited mild hyperglycemia in the fed state (Figure 5F). Out of 40 animals characterized during the course of these studies, only four (10%) developed severe hyperglycemia of more than 250 mg/dl on multiple individual determinations. This rate of diabetes is consistent with an incidence rate of diabetes of ~10% when 150 *IR* (+/−) mice were studied in an independent animal facility (data not shown). In contrast, *IR/IRS-1* (+/−) mice showed a markedly increased incidence of disease. Thus, only ~40% were able to maintain glucose concentrations in a normal range, several exhibited moderate hyperglycemia, and 24 out of 61 *IR/IRS-1* (+/−) mice (39.3%) became overtly diabetic by the age of 6 months with blood glucose concentrations of more than 250 mg/dl (Figure 5F). Although *IR/IRS-1* (+/−) mice of both genders were highly insulin resistant and developed diabetes, the male mice were more strongly affected than their female litter mates (75% of the diabetic animals versus 25%). In summary, although a 50% deficiency in IRS-1 or in insulin receptor alone causes diabetes in only a few mice, these two defects act synergistically to cause a significantly increased incidence of disease.

Increasing Degrees of Insulin Resistance Cause Pancreatic β Cell Hyperplasia and Hyperinsulinemia

In the multistep model for the pathogenesis of NIDDM, insulin resistance precedes the appearance of hyperglycemia and is accompanied by increased pancreatic insulin production. This situation is reflected in the progressively elevated insulin levels found in *IR/IRS-1* (+/−) mice. To assess the impact of different degrees of insulin resistance on pancreatic compensation in these different mutant mice, we examined the pancreas histologically after immunostaining for insulin (Figure 6A) and determined pancreatic β cell and non-β cell masses by point-counting morphometry. In 6-month-old male mice, even though there were no differences in body weight or pancreatic weight, the β cell mass was increased 2- to 3-fold in *IRS-1* (+/−) and *IR* (+/−) mice and 2- to 30-fold (mean 10-fold) in the *IR/IRS-1* (+/−) mice as compared to wild-type animals (Figure 6B, left panel). The β cell mass increased in parallel to the insulin resistance, but in no group did non-β cell mass differ from wild-type mice (Figure 6B, right panel). Thus, there was a selective increase in β cells in these insulin resistant mice. The enormous increase in β cells in the double heterozygote animals resulted in distorted islet morphology. In the wild-type mice, the non-β cells (20%–30% of the islet) form a discontinuous mantle 1–2 cells thick around a core of β cells (70%–80% of the islet) (Figure 6C). However, in the *IR/IRS-1* (+/−) mice, the non-β cells, which represent 8% of the islet, are scattered irregularly throughout the islet periphery (Figure 6C).

According to the multistep model of NIDDM, the occurrence of diabetes can result from a relative or absolute failure of pancreatic β cells to secrete enough insulin to overcome insulin resistance and maintain euglycemia. When we compared additional *IR/IRS-1* (+/−) mice of known phenotype (diabetic or nondiabetic), the most extensive β cell hyperplasia was found in the diabetic mice. Indeed, the diabetic *IR/IRS-1* (+/−) animals had a mean random fed blood glucose value of 300 mg/dl (Figure 7A), a 15-fold increase in β cell mass (Figure 7B), and 21-fold elevated plasma insulin levels (Figure 7C) as compared to wild-type animals. In contrast, the euglycemic double heterozygote mice had mean blood glucose values of 120 mg/dl (Figure 7A), a 3-fold increase in β cell mass (Figure 7B), and a 4-fold elevation in plasma insulin levels (Figure 7C). Even so, the occurrence of diabetes indicates that the β cells were unable to completely compensate for the insulin resistance caused by heterozygosity of *IR* and *IRS-1* null alleles. Thus, there is a state of "relative" insulin deficiency in which these double heterozygote animals were not able to maintain euglycemia. Whether absolute insulin deficiency would develop over time remains to be determined.

Discussion

Insulin and IGF-1 mediate a variety of essential signals for vertebrate growth and metabolism. The most prominent function of insulin is the maintenance of euglycemia, which is achieved by stimulation of glucose disposal in muscle and fat and inhibition of hepatic glucose production. Longitudinal studies have shown that the first step in the pathogenesis of NIDDM is resistance to insulin-stimulated glucose uptake in skeletal muscle (Martin et al., 1992; Lillioja et al., 1993). Insulin resistance can initially be overcome by elevated plasma insulin concentrations, but after a period of time this mechanism is no longer able to compensate sufficiently and hyperglycemia occurs. The underlying molecular and genetic mechanisms for insulin resistance and the factors responsible for progression to frank diabetes are still unknown. The animal model presented here indicates that the combination of minor defects in the insulin signaling cascade can act synergistically to cause insulin resistance and NIDDM.

The double heterozygote *IR/IRS-1* (+/−) mice model fulfills three criteria that are not present in other rodent models but closely mimic human NIDDM. First, this

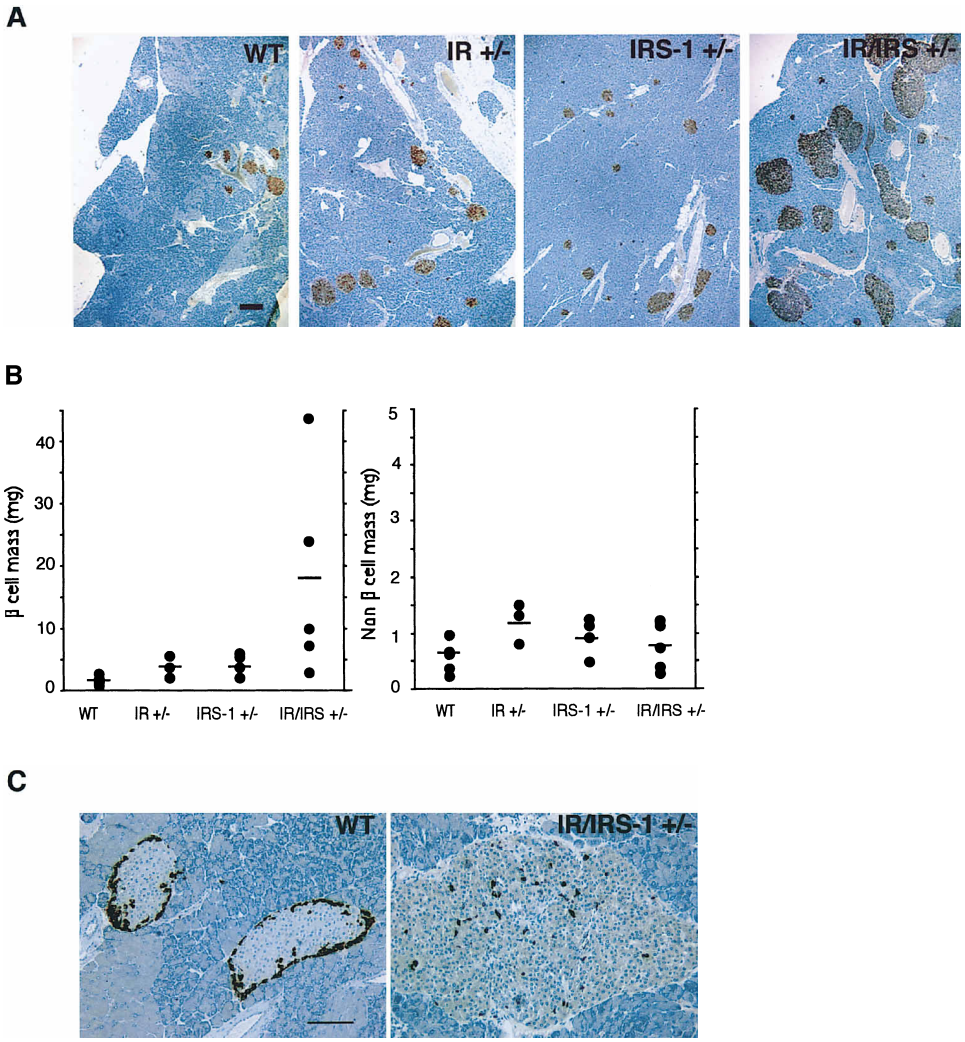

Figure 6. Combined Deficiency for the Insulin Receptor and IRS-1 Has a Synergistic Effect on Pancreatic β Cell Hyperplasia

(A) Sections of pancreas of 6-month-old male mice of the indicated genotype were immunostained for insulin. Representative fields are shown of animals exhibiting pancreatic β cell mass corresponding to the mean found for the respective genotype (bar = 200 mm).

(B) Pancreatic β and non-β cell mass were quantitated by point-counting morphometrics on sections from 6-month-old male animals of the indicated genotype. Data represent determinations for individual animals: wild-type, n = 5; *IRS-1* (+/−), n = 4; *IR* (+/−), n = 3; *IR/IRS-1* (+/−), n = 5. Animals at the appropriate age were randomly chosen for this experiment, blind for the diabetic phenotype. Pancreatic β cell mass was significantly increased in *IRS-1* (+/−) and *IR* (+/−) versus wild-type ($p < 0.05$).

(C) Immunostaining for non-β cell hormones of pancreas sections from a wild-type animal (left panel) and an *IR/IRS-1* (+/−) animal (right panel) (bar = 100 mm).

model is polygenic in nature and is the result of rather modest genetic defects. NIDDM clearly has a genetic component, and several studies suggest a polygenic nature of the disease. Metabolic defects in NIDDM, whether genetic or acquired, are usually relatively mild; these include the defects in signaling that occur with mutant IRS-1 molecules or the decrease in signaling that results from insulin receptor down-regulation (Prince et al., 1981; Olefsky et al., 1982; Caro et al., 1987; Almind et al., 1996). Second, the first detectable defect in our model is resistance to the blood glucose–lowering effect of insulin, and this can occur even in the absence of obesity. Since skeletal muscle is responsible for 80% of insulin-stimulated glucose transport, and since skeletal muscle has a relatively low level of IRS-2 to compensate for the IRS-1 deficiency, *IR/IRS-1* (+/−) mice exhibit a disproportionately severe insulin resistance in skeletal muscle as compared to liver, mimicking the defect seen in most individuals prior to development of NIDDM. Third, the onset of diabetes is age-dependent. This also reflects the typical course of human NIDDM, which is rare before the age of 20 and gradually increases with aging. In humans, this period can be accelerated by obesity and/or sedentary life style, factors that might also be superimposed in the NIDDM mouse. Therefore, the animals created reflect thus far the only rodent model that represents the clinical picture of human NIDDM.

These characteristics of the NIDDM mouse provide clues that may redirect research to find the underlying

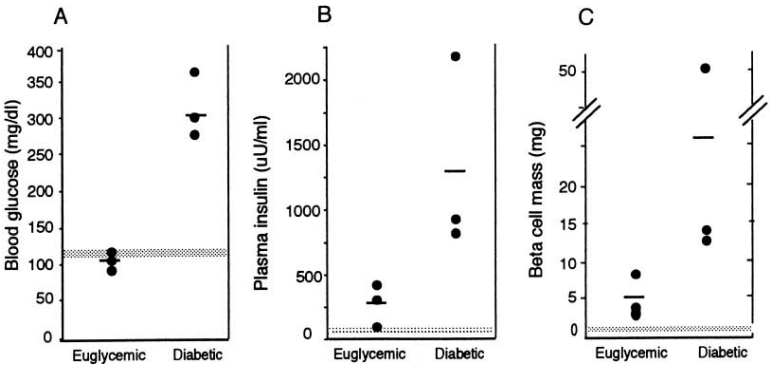

Figure 7. Pancreatic Hyperplasia and Hyperinsulinemia Are Insufficient to Compensate for Insulin Resistance in Diabetic Double Heterozygous Animals

Six-month-old male double heterozygote mice of known phenotype were bled and then sacrificed for morphometric analysis of pancreatic β cell mass. Mean fed blood glucose concentrations for three individual nondiabetic and three diabetic mice (A), plasma insulin concentrations (B), and pancreatic β cell mass (C). The shaded bar in each graph represents the mean +/− SEM detected in wild-type animals in independent experiments.

defects in NIDDM humans. Research thus far has focused on the identification of major gene effects caused by mutations of the insulin receptor signaling network. The animal model presented here suggests that minor defects acting in concert with each other can cause significant insulin resistance and overt NIDDM. Reduced expression of the insulin receptor comparable to what is seen in these mice (~50%) is found in tissues of obese insulin-resistant people (Bar et al., 1976), diabetic patients (Kolterman et al., 1981; Prince et al., 1981; Olefsky et al., 1982; Sinha et al., 1987), and most of the obesity-linked rodent models of NIDDM (Kahn et al., 1973). Likewise, previous studies have shown a reduction of IRS-1 expression in both NIDDM and obesity in humans and rodents (Saad et al., 1993; Goodyear et al., 1995), and this is of similar magnitude to that seen in heterozygous IRS-1 deficiency. Furthermore, as noted above, IRS-1 sequence variations have been found with an increased frequency in many diabetic populations (Almind et al., 1993; Laakso et al., 1994; Clausen et al., 1995). When expressed in cultured cells, the most common of these mutant IRS-1s exhibits an ~30% reduction of insulin-stimulated PI 3-kinase activation (Almind et al., 1996). While the relatively modest magnitude of this defect might be considered of questionable biological significance, it is comparable to the defect seen in *IRS-1* (+/−) mice. Indeed, we have shown here that this defect can act in concert with a second mild defect in the insulin signaling cascade to cause progression toward NIDDM. It is likely that the combination of various other defects in the insulin signaling cascade can cause a similar phenotype.

Another important implication from this mouse model for the genetics of NIDDM is the ability to use these mice of a well-defined phenotype as an indicator for identification of other genetic factors causing insulin resistance. Since environmental factors within our animal colony are similar for all the mice, it seems likely that genetic factors other than the knockout mutation influence the phenotype. The genetics of NIDDM in humans also follows a complex pattern of inheritance, indicating that it is unlikely that NIDDM is caused by a single mutation in a major gene. While different explanations have been set forth to account for this complex pattern of inheritance, the notion that NIDDM may develop as a result of different mutations at different loci acting simultaneously and in a synergistic manner (epistasis) provides a unifying hypothesis for the complex genetics of NIDDM. However, it is particularly difficult to test this hypothesis in human populations, owing to the complexity of the gene pool and the possibility that even within relatively homogenous populations, different genes may interact in the pathogenesis of different individuals to produce the insulin resistance and insulin secretory defects.

The NIDDM mouse provides a practical demonstration of epistatic interactions among different genes in the same pathway. The somewhat bimodal distribution of glucose concentrations into diabetic and nondiabetic animals suggests that only one or two additional genes may be required to give rise to a "clinically apparent" phenotype. The phenotypic variation within each subpopulation may in part be explained by the mixed genetic background (C57Bl/6J and 129 sv) used to derive the *IR/IRS-1* (+/−) mice. It is likely that additional diabetes susceptibility alleles are present in these two strains. Indeed, our use of mixed strains of mice was designed to mimic as closely as possible the genetics of diabetes in an outbred population. The identification of additional susceptibility loci using quantitative trait linkage analysis on backcrosses of the NIDDM mouse should provide information on the number and location of such loci and suggest important syntenic regions for investigators pursuing genome scans of NIDDM in human populations (Vaxillaire et al., 1995).

Another area of extensive research is the search for pancreatic β cell growth and survival factors. We have demonstrated that *IR/IRS-1* (+/−) mice exhibit extensive β cell hyperplasia that allows for partial or complete compensation of the severe insulin resistance by elevated plasma insulin levels. Since even the euglycemic, nondiabetic animals exhibit up to 5-fold elevated β cell mass, this model may provide important clues for islet growth and survival. As has been shown previously, β cell mass is dynamic and can compensate for increased demand (Bonner-Weir, 1994). In double heterozygous animals at 1 month of age, little hyperinsulinemia is detectable, and there is no β cell hyperplasia (data not shown). Therefore, there must exist a β cell growth factor that is able to stimulate the massive β cell proliferation in the young animals. Although glucose has been shown to stimulate β cell growth (Bonner-Weir et al., 1989), this factor is probably something in addition to glucose, since we can already detect a significant degree of islet cell hyperplasia in normoglycemic *IR/IRS-1* (+/−) mice. The mouse model presented here may help to identify

such β cell growth factors. On the other hand, there are mouse strains, such as the C57Bl/*ks*, that carry genes encoding a susceptibility for β cell failure. Breeding the NIDDM mouse onto these background strains will greatly facilitate the identification of such genes, since it is clear that the *IR/IRS-1* (+/−) rely on massive β cell hyperplasia to maintain euglycemia for a period of time. Thus, there should be an increased rate or earlier onset of NIDDM when the *IR/IRS-1* (+/−) genotype is bred onto such a background, and analysis of these animals will serve as a sensitive indicator for the presence of β cell failure susceptibility genes.

In conclusion, we have created a novel polygenic model for the most common endocrine disease, NIDDM. In many ways, NIDDM is similar to other common diseases such as atherosclerosis, hyperlipidemia, and obesity, in which longitudinal studies suggest complex patterns of inheritance and superimposed environmental effects. The *IR/IRS-1* (+/−) mouse allows new insights on the nature of NIDDM, and perhaps of these other common disorders, in which two subclinical defects in the insulin receptor signaling cascade can synergize to cause NIDDM in the absence of pancreatic β cell failure and obesity. These animals also provide a unique tool to identify and characterize genes modulating a diabetic phenotype by independently causing a more severe degree of insulin resistance or pancreatic β cell failure, thereby helping to solve the puzzle of the pathogenesis of human NIDDM and pointing the way to the development of new therapeutic approaches.

Experimental Procedures

Creation and Genotyping of Mutant Mice

Creation of mice deficient for the insulin receptor and IRS-1 has been described previously (Araki et al., 1994; Accili et al., 1996). Mice used in this study were kept on a C57Bl/129sv hybrid background. Genotyping for the *IRS-1* locus was performed by PCR as previously described (Araki et al., 1994), except that the 1.25 kb PCR product resulting from the mutant allele was subcloned into a PCR direct vector and used as a probe to hybridize with PCR products of genotyping reactions, thus enhancing the detectability of the mutant allele in *IRS-1* (+/−) mice. Genotyping for the *IR* locus was performed by PCR using two reactions: one primer set amplified only the wild-type allele, and another primer set amplified only the mutant allele.

Analytical Procedures

Blood glucose values were determined from whole venous blood using an automatic glucose monitor (One Touch II, Lifescan). Insulin levels were measured by radioimmunoassay using rat insulin (Linco) as a standard. Insulin tolerance tests were performed on random fed animals between 2:00 and 5:00 PM. Animals were injected with 0.75 U/kg body weight of human crystalline insulin (Lilly) into the peritoneal cavity. Blood glucose values were measured immediately before and 15, 30, and 60 min after the injection. Results were expressed as percentage of initial blood glucose concentration.

Morphological and Morphometric Analysis

Animals were sacrificed by administering an overdose of sodium amino barbital (Amytal Sodium, Eli Lilly, 200 mg/kg body weight). Pancreases were removed, cleared of fat and lymph nodes, weighed, fixed in Bouin's solution, and embedded in paraffin. Sections (5 μm) were immunostained either for the endocrine non-β cells of the islets, using a cocktail of antibodies rabbit anti-bovine glucagon (final dilution 1:3000, gift of Dr. M. Appel), rabbit anti-synthetic somatostatin (final dilution 1:300), and rabbit anti-bovine pancreatic polypeptide (final dilution 1:3000, gift of Dr. R. Chance, Eli Lilly, Indianapolis, IN) (Montana et al., 1993), or for β cells, using a guinea pig anti-porcine insulin antiserum (Linco, St. Louis, MO). β cell mass was measured by point-counting morphometry as previously described (Bonner-Weir et al., 1989).

Statistical Analyses

Each variable was analyzed using the unpaired Student's t test. For all analyses, a p value of less than 0.05 was considered significant. Results are given as means +/− SEM.

Immunoprecipitations and Western Blot Analyses of Insulin Signaling Proteins

Immunoprecipitations and Western blot analyses of insulin signaling proteins were performed on muscle and liver homogenates as previously described (Araki et al., 1994). Results are expressed as percentage of signal intensity seen in samples from insulin-stimulated wild-type animals.

Acknowledgments

Correspondence regarding this paper should be addressed to C. R. K. We thank T. L. Bellman for excellent secretarial assistance, M. Ginsberg for determining the plasma insulin determinations, Dr. M. Hattori and M. Petruzelli for their help in performing the orbital punctures, and M. Taneja for her contributions to our experiments. The authors wish to thank Dr. M. F. White for the generous gift of numerous antisera used in these studies.

This work was supported by NIH grants DK 31036 and DK 33201 (C. R. K.), DK 44523 (S. B. W.), DERC grant P30DK36836, and the Markey Charitable Trust. J. C. B. was supported by a postdoctoral fellowship of the Deutsche Forschungsgemeinschaft (DFG) and is a Mary K. Iacocca Fellow.

Received November 27, 1996; revised January 20, 1997.

References

Accili, D., Drago, J., Lee, E.J., Johnson, M.D., Cool, M.H., Salvatore, P., Asico, L.D., Jose, P.A., Taylor, S.I., and Westphal, H. (1996). Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nat. Genet. *12*, 106–109.

Almind, K., Biorbaek, C., Vestergaard, H., Hansen, T., Echwald, S.M., and Pedersen, O. (1993). Amino acid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus. Lancet *342*, 828–832.

Almind, K., Inoue, G., Pedersen, O., and Kahn, C.R. (1996). A common amino acid polymorphism in insulin receptor substrate-1 causes impaired insulin signaling. Evidence from transfection studies. J. Clin. Invest. *97*, 2569–2575.

Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B.L., III, Johnson, R.S., and Kahn, C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature *372*, 186–190.

Backer, J.M., Myers, M.G., Jr., Shoelson, S.E., Chin, D.J., Sun, X.J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J., and White, M.F. (1992). Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. *11*, 3469–3479.

Baltensperger, K., Kozma, L.M., Cherniack, A.D., Klarlund, J.K., Chawla, A., Banerjee, U., and Czech, M.P. (1993). Binding of the Ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science *260*, 1950–1952.

Bar, R.S., Gorden, P., Roth, J., Kahn, C.R., and De Meyts, P. (1976). Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients: effects of starvation, refeeding and dieting. J. Clin. Invest. *58*, 1123–1135.

Bisbis, S., Bailbe, D., Tormo, M.A., Picarel-Blanchot, F., Derouet, M., Simon, J., and Portha, B. (1993). Insulin resistance in the GK rat: decreased receptor number but normal kinase activity in liver. Am. J. Physiol. *265*, E807–E813.

Bonner-Weir, S. (1994). Regulation of pancreatic β-cell mass in vivo. Recent Prog. Horm. Res. *49*, 91–104.

Bonner-Weir, S., Deery, D., Leahy, J.L., and Weir, G.C. (1989). Compensatory growth of pancreatic β-cells in adult rats after short-term glucose infusion. Diabetes *38*, 49–53.

Caro, J.F., Sinha, M.K., Raju, S.M., Ittoop, O., Pories, W.J., Flickinger, E.G., Meelheim, D., and Dohm, G.L. (1987). Insulin receptor kinase in human skeletal muscle from obese subjects with and without non-insulin-dependent diabetes. J. Clin. Invest. *79*, 1330–1337.

Cheatham, B., Vlahos, C.J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C.R. (1994). Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase DNA synthesis and glucose transporter translocation. Mol. Cell. Biol. *14*, 4902–4911.

Cheatham, B., and Kahn, C.R. (1995). Insulin action and the insulin signaling network. Endocr. Rev. *16*, 117–142.

Chen, H., Charlat, O., Tartaglia, L.A., Woolf, E.A., Weng, X., Ellis, S.J., Lakey, N.D., Culpepper, J., Moore, K.J., Breitbart, R.E., et al. (1996). Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db*/*db* mice. Cell *84*, 491–495.

Chua, S.C., Jr., Chung, W.K., Wu-Peng, X.S., Zhang, Y., Liu, S.M., Tartaglia, L.A., and Leibel, R.L. (1996). Phenotypes of mouse *diabetes* and rat *fatty* due to mutations in the OB (Leptin) receptor. Science *271*, 994–996.

Clausen, J.O., Hansen, T., Bjorbaek, C., Echwald, S.M., Urhammer, S.A., Rasmussen, S., Andersen, C.B., Hansen, L., Almind, K., Winther, K., et al. (1995). Insulin resistance: interactions between obesity and a common variant of insulin receptor substrate-1. Lancet *346*, 397–402.

Flier, J.S. (1992). Syndromes of insulin resistance. From patient to gene and back again (Lilly Lecture). Diabetes *41*, 1207–1219.

Froguel, P., Vaxillaire, M., Sun, F., Velho, G., Zouali, H., Butal, M.O., Lesage, S., Vionnet, N., Clement, K., Fougerousse, F., et al. (1992). Close linkage of glucokinase locus on chromosome 7p to early-onset non-insulin-dependent diabetes mellitus. Nature *356*, 162–164.

Goodyear, L.J., Giorgino, F., Sherman, L.A., Carey, J., Smith, R.J., and Dohm, G.L. (1995). Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. J. Clin. Invest. *95*, 2195–2204.

Joshi, R.L., Lamothe, B., Cordonnier, N., Mesbah, K., Monthioux, E., Jami, J., and Bucchini, D. (1996). Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. EMBO J. *15*, 1542–1547.

Kahn, C.R., Neville, D.M., Jr., and Roth, J. (1973). Insulin receptor interaction in the obese hyperglycemic mouse. A model of insulin resistance. J. Biol. Chem. *248*, 244–250.

Kahn, C.R. (1994). Insulin action, diabetogenes, and the cause of type II diabetes (Banting Lecture). Diabetes *43*, 1066–1084.

Kolterman, O.G., Gray, R.S., Griffin, J., Burstein, P., Insel, J., Scarlett, J.A., and Olefsky, J.M. (1981). Receptor and postreceptor defects contribute to the insulin resistance in noninsulin-dependent diabetes mellitus. J. Clin. Invest. *68*, 957–969.

Laakso, M., Malkki, M., Kekalainen, P., Kuusisto, J., and Deeb, S.S. (1994). Insulin receptor substrate-1 variants in non-insulin-dependent diabetes. J. Clin. Invest. *94*, 1141–1146.

Lillioja, S., Mott, D.M., Spraul, M., Ferraro, R., Foley, J.E., Ravussin, E., Knowler, W.C., Bennett, P.H., and Bogardus, C. (1993). Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. N. Engl. J. Med. *329*, 1988–1992.

Martin, B.C., Warram, J.H., Krolewski, A.S., Bergman, R.N., Soeldner, J.S., and Kahn, C.R. (1992). Role of glucose and insulin resistance in development of type II diabetes mellitus: results of a 25-year follow-up study. Lancet *340*, 925–929.

Montana, E., Bonner-Weir, S., and Weir, G.C. (1993). Beta cell mass and growth after syngeneic islet cell transplantation in normal and streptozotocin diabetic C57BL/6 mice. J. Clin. Invest. *91*, 780–787.

Newman, B., Selby, J.V., King, M.C., Slemenda, C., Fabsitz, R., and Friedman, G.D. (1987). Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins. Diabetologia *30*, 763–768.

Olefsky, J.M., Kolterman, O.G., and Scarlett, J.A. (1982). Insulin action and resistance in obesity and noninsulin-dependent type II diabetes mellitus. Am. J. Physiol. *243*, E15–E30.

Parsons, J.A., Brelje, T.C., and Sorenson, R.L. (1992). Adaptation of the islets to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology *130*, 1459–1466.

Patti, M.E., Sun, X.J., Bruening, J.C., Araki, E., Lipes, M.A., White, M.F., and Kahn, C.R. (1995). 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.

Piperleers, D.G. (1992). Heterogeneity in pancreatic β-cell population. Diabetes *41*, 777–781.

Prince, M.J., Tsai, P., and Olefsky, J.M. (1981). Insulin binding, internalization and insulin receptor regulation in fibroblasts from type II (noninsulin-dependent) diabetic subjects. Diabetes *30*, 596–600.

Rich, S.S. (1990). Mapping genes in diabetes: genetic epidemiological perspective. Diabetes *39*, 1315–1319.

Rose, D.W., Saltiel, A.R., Majumdar, M., Decker, S.J., and Olefsky, J.M. (1994). Insulin receptor substrate 1 is required for insulin-mediated mitogenic signal transduction. Proc. Natl. Acad. Sci. USA *91*, 797–801.

Saad, M.J.A., Araki, E., Miralpeix, M., Rothenberg, P.L., White, M.F., and Kahn, C.R. (1992). Regulation of insulin receptor substrate 1 in liver and muscle of animal models of insulin resistance. J. Clin. Invest. *90*, 1839–1849.

Saad, M.J.A., Folli, F., Kahn, J.A., and Kahn, C.R. (1993). Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. J. Clin. Invest. *92*, 2065–2072.

Sinha, M.K., Pories, W.J., Flickinger, E.G., Meelheim, D., and Caro, J.F. (1987). Insulin-receptor kinase activity of adipose tissue from morbidly obese humans with and without NIDDM. Diabetes *36*, 620–625.

Skolnik, E.Y., Lee, C.H., Batzer, A.G., Vicentini, L.M., Zhou, M., Daly, R.J., Myers, M.G., Jr., Backer, J.M., Ullrich, A., White, M.F., and Schlessinger, J. (1993). The SH2/SH3 domain–containing protein GRB2 interacts with tyrosine-phosphorylated IRS-1 and Shc: implications for insulin control of ras signalling. EMBO J. *12*, 1929–1936.

Sun, X.J., Rothenberg, P.L., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., and White, M.F. (1991). The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature *352*, 73–77.

Sun, X.J., Crimmins, D.L., Myers, M.G., Jr., Miralpeix, M., and White, M.F. (1993). Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol. Cell. Biol. *13*, 7418–7428.

Sun, X.J., Wang, L.M., Zhang, Y., Yenush, L., Myers, M.G., Jr., Glasheen, E.M., Lane, W.S., Pierce, J.H., and White, M.F. (1995). Role of IRS-2 in insulin and cytokine signalling. Nature *377*, 173–177.

Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., et al. (1994). Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature *372*, 182–186.

Taylor, S.I. (1992). Molecular mechanisms of insulin resistance—lessons from patients with mutations in the insulin receptor gene (Lilly Lecture). Diabetes *41*, 1473–1490.

Ura, S., Araki, E., Kishikawa, H., Shirotani, T., Todaka, M., Isami, S., Shimoda, S., Yoshimura, R., Matsuda, K., Motoyoshi, S., et al. (1996). Molecular scanning of the IRS-1 gene in Japanese patients with non-insulin-dependent diabetes mellitus: identification of five novel mutations in IRS-1 gene. Diabetologia *39*, 600–608.

Vaxillaire, M., Boccio, V., Philippi, A., Vigouroux, C., Terwilliger, J., Passa, P., Beckmann, J.S., Velho, G., Lathrop, G.M., and Froguel, P. (1995). A gene for maturity onset diabetes of the young maps to chromosome 12q. Nat. Genet. *9*, 418–423.

Warram, J.H., Rich, S.S., and Krolewski, A.S. (1995). Epidemiology

and genetics of diabetes mellitus. In Joslin's Diabetes Mellitus. C. R. Kahn and G. C. Weir, eds. (Philadelphia: Lea and Febiger), pp. 201–216.

Waters, S.B., Yamauchi, K., and Pessin, J.E. (1993). Functional expression of insulin receptor substrate-1 is required for insulin-stimulated mitogenic signaling. J. Biol. Chem. *238*, 22231–22234.

Wertheimer, E., Lu, S.P., Backeljauw, P.F., Davenport, M.L., and Taylor, S.I. (1993). Homozygous deletion of the human insulin receptor gene results in leprechaunism. Nat. Genet. *5*, 71–73.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature *372*, 425–432.

Note Added in Proof

While this paper was in press, two additional MODY genes were identified as *hepatic nuclear factors 1*α and *4*α:

Yamagata, K., Oda, N., Kaisaki, P.J., Menzel, S., Furuta, H., Vaxillaire, M., Southam, L., Cox, R.D., Lathrop, G.M., Boriraj, V.V., et al. (1996). Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). Nature *384*, 455–458.

Yamagata, K., Furuta, H., Oda, N., Kaisaki, P.J., Menzel, S., Cox, N.J., Fajans, S.S., Signorini, S., Stoffel, M., and Bell, G.I. (1996). Mutations in the hepatocyte nuclear factor-4α gene in maturity-onset diabetes of the young (MODY1). Nature *384*, 458–462.