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Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality

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Targeted disruption of the insulin receptor gene (*Insr*) in the mouse was achieved using the homologous recombination approach. *Insr*$^{+/-}$ mice were normal as shown by glucose tolerance tests. Normal *Insr*$^{-/-}$ pups were born at expected rates, indicating that *Insr* can be dispensable for intrauterine development, growth and metabolism. However, they rapidly developed diabetic ketoacidosis accompanied by a marked post-natal growth retardation (up to 30–40% of littermate size), skeletal muscle hypotrophy and fatty infiltration of the liver and they died within 7 days after birth. Total absence of the insulin receptor (IR), demonstrated in the homozygous mutant mice, also resulted in other metabolic disorders: plasma triglyceride level could increase 6-fold and hepatic glycogen content could be five times less as compared with normal littermates. The very pronounced hyperglycemia in *Insr*$^{-/-}$ mice could result in an increased plasma insulin level of up to ~300 μU/ml, as compared with ~25 μU/ml for normal littermates. However, this plasma insulin level was still unexpectedly low when compared with human infants with leprechaunism, who lack IR but who could have extremely high insulinemia (up to >4000 μU/ml). The pathogenesis resulting from a null mutation in *Insr* is discussed.

Keywords: animal model/diabetes/gene targeting/insulin receptor/insulin resistance

Introduction

The vital role of insulin in controlling energy metabolism is mediated by the insulin receptor (IR). Although IR is widely distributed in the body, the three major tissues for this action of insulin are muscle, fat tissue and liver. A large body of data is available on the structure and function of IR, a member of the family of membrane receptors with tyrosine kinase activity (for a review see Ullrich and Schlessinger, 1990). It is composed of two types of subunits, designated α and β, that assemble into a heterodimeric $\alpha_2\beta_2$ structure. The α chain bearing the hormone binding site is extracytoplasmic and is attached to the β chain by disulfide bonds. The β chain possesses a short extracellular, a transmembrane and an intracytoplasmic region encompassing a tyrosine kinase domain that is activated upon binding of insulin to IR (for reviews see Olefsky, 1990; Taylor *et al.*, 1991). One of the mechanisms of signal transduction by IR consists of the phosphorylation, by the activated receptor tyrosine kinase, of intermediate adaptor proteins, such as insulin receptor substrate-1 (IRS-1). Such docking proteins subsequently bind various effector proteins containing Src homology 2 domains that, in turn, activate the different signalling pathways for metabolic, as well as other less well-documented, effects of insulin on cell growth and differentiation (for reviews see Moses and Tsuzaki, 1991; White and Kahn, 1994).

Since IR is present in most cells from even the early morula stages of embryogenesis (for a review see Heyner *et al.*, 1989), its complete absence was generally expected to lead to embryonic lethality. Several mutations that alter IR life cycle (synthesis, transport to the plasma membrane, insulin binding, transmembrane signalling, endocytosis, recycling and degradation) have been identified in the insulin receptor gene (*Insr*) in non-insulin-dependent diabetes mellitus (NIDDM) patients with extreme insulin resistance and certain associated syndromes, such as leprechaunism (for reviews see Bell, 1991; Moller and Flier, 1991; Taylor, 1992). However, only recently have infants with leprechaunism who were homozygous for a null mutation in *Insr* been reported, indicating that total absence of IR could be compatible with intrauterine development and even with post-natal life (Jospe *et al.*, 1993; Krook *et al.*, 1993; Psiachou *et al.*, 1993; Wertheimer *et al.*, 1993; Hone *et al.*, 1995). The question, however, remains as to the contribution of mutations in *Insr* in the pathologies observed in syndromes associated with extreme insulin resistance (for reviews see De Meyts, 1993; Kahn, 1994).

To investigate further the effects on growth and metabolism specifically due to the total lack of IR we have introduced a null mutation in *Insr* in the mouse using the homologous recombination approach (Bradley *et al.*, 1984; Mansour *et al.*, 1988). The phenotype of such mutant mice completely devoid of IR is presented here.

Results and discussion

Introduction of a null mutation in mouse Insr

Human *Insr* spans >120 kb and comprises 22 exons (Seino *et al.*, 1989). The gene product is a polypeptide precursor that generates, upon proteolytic cleavage, the signal peptide and the α and β subunits of the receptor. The subunits are glycosylated, assembled and translocated to the plasma membrane. Partial cloning of mouse *Insr* suggested that the gene organization in humans is conserved in the mouse (Sibley *et al.*, 1989).

Upon screening of a mouse genomic library a portion of *Insr* surrounding exon 2 which encodes the insulin

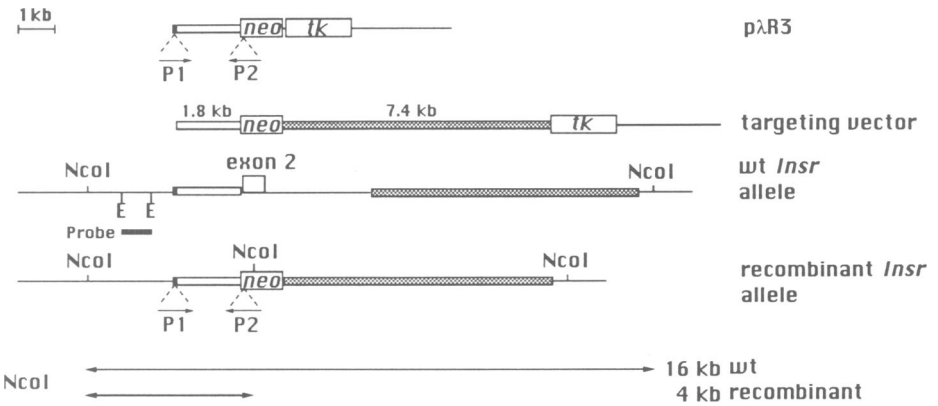

Fig. 1 Structure of the targeting vector, the wild-type (wt) and the recombinant *Insr* alleles. The targeting vector contains the neomycin phosphotransferase gene (*neo*) flanked by 1.8 and 7.4 kb fragments of 5′ and 3′ homology regions around exon 2. It also includes the thymidine kinase gene (*tk*) from herpes simplex virus type 1. The recombinant *Insr* allele could be identified by both PCR and Southern blot analyses. For PCR screening the external primer P1 was derived from the sequence of the 5′ extension (■) in pλR3 as compared with the region of 5′ homology in the targeting vector. The primer P2 was chosen in *neo*. The recombinant *Insr* allele gives rise to a PCR product of ~1.8 kb. Southern blot analysis upon digestion of DNA with *Nco*I and hybridization with the probe synthesized using the genomic *Eco*RI fragment allows the wild-type and recombinant *Insr* alleles to be distinguished.

binding site was cloned (see Materials and methods). The replacement vector we have engineered (Figure 1) contains the selectable marker *neo* flanked by 1.8 and 7.4 kb fragments of the 5′ and 3′ homology regions located in introns on either side of exon 2. It also contains *tk*, allowing negative selection. Homologous recombination between the targeting vector and the *Insr* locus results in replacement by *neo* of a 3.6 kb *Insr* region comprising exon 2. After electroporation of the targeting vector into embryonic stem (ES) cells G418- and Gancyclovir-resistant clones were first screened by PCR to identify recombinant clones (Figure 1; see Materials and methods). Successful targeting at the *Insr* locus in these clones was confirmed by Southern blot analysis (Figures 1 and 2). Upon injection of recombinant ES cells into C57Bl/6 blastocysts and their transfer into foster mothers male germline chimeras were obtained. These were used to produce heterozygous mutant offspring that were normal and fertile. The structure of the recombinant *Insr* allele in DNA from offspring of a heterozygous intercross as analyzed by Southern blot is presented in Figure 2. Homozygous offspring were born from heterozygous intercrosses near the expected 25% frequency (75 $Insr^{-/-}$ animals among 351 pups born).

Western immunoblot analysis of protein extracts from liver of $Insr^{-/-}$ mice confirmed that the targeted deletion of *Insr* around exon 2 resulted in a null mutation, since no IR protein was detected (Figure 3).

Normal glucose tolerance in $Insr^{+/-}$ mice

As mentioned above, $Ins^{+/-}$ mice were fertile and had a normal phenotype. Heterozygous animals did not show any glucose intolerance (Figure 4). This is at variance with the observation that parents of an infant with leprechaunism who lacked IR were near normal with the exception that they showed glucose intolerance (Psiachou *et al.*, 1993). The possible relationships between insulin resistance, fasting glucose levels, glucose tolerance and mutations in *Insr* are not clearly understood. All patients with leprechaunism studied to date have had mutations in both alleles of *Insr* and showed glucose intolerance. In many patients with type A insulin resistance (characterized

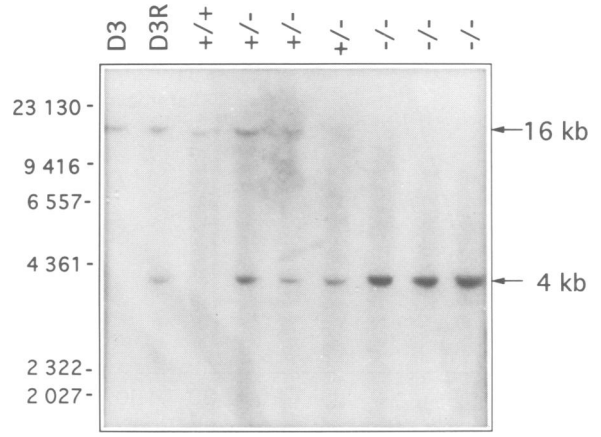

Fig. 2. Autoradiogram of a Southern blot analysis using DNA from the wild-type ES cell clone (D3), one recombined ES cell clone (D3R) and tails of the offspring of a heterozygous $Insr^{+/-}$ intercross. The DNA from tails of newborn mice was extracted, digested with *Nco*I, subjected to electrophoresis on an agarose gel, blotted onto a nylon membrane, hybridized to a ^{32}P-labeled probe prepared using the *Eco*RI genomic fragment (see Figure 1) and exposed to X-ray film. The sizes (in base pairs) of phage λ DNA digested with *Hin*dIII are indicated on the left. The wild-type and recombinant *Insr* alleles generate fragments of 13 and 4 kb respectively that hybridize to the probe.

by the triad of insulin resistance, acanthosis nigricans and hyperandrogenism) only one allele of *Insr* was mutated. They could present with normal levels of fasting glucose and show glucose intolerance. However, patients with type A syndrome who had mutations in both alleles of *Insr* tended to have fasting hyperglycemia (for a review see Taylor, 1992).

$Insr^{-/-}$ mice, normal at birth, display early neonatal lethality

$Insr^{-/-}$ mice were almost indistinguishable at birth from their littermates. This is very unexpected, since infants with leprechaunism completely lacking IR showed marked intrauterine growth retardation and several morphological abnormalities (for a review see Taylor, 1992). The question therefore arises as to whether, in addition to mutations in *Insr*, other genetic defects are responsible for the

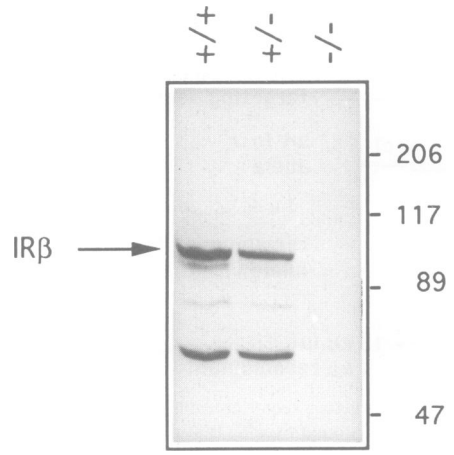

Fig. 3. Western immunoblot analysis of protein extracts from the liver of mice carrying wild-type or mutated *Insr* alleles. The primary antibodies were directed against the cytoplasmic β subunit of IR. The size markers (in kDa) were from Bio-Rad. For details see Materials and methods.

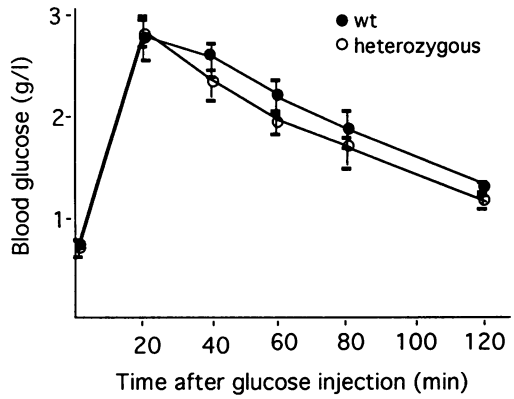

Fig. 4. Intraperitoneal glucose tolerance tests on mice carrying wild-type or $Insr^{+/-}$ alleles. The mean values of experiments on five wild-type and nine heterozygous animals are presented with error bars to indicate standard deviations. For details see Materials and methods.

syndromes associated in humans with severe insulin resistance (for a review see De Meyts, 1993). Gene targeting now allows the effects of defined mutations in single genes in the mouse to be examined. From the targeted disruption of *Insr* in the mouse that we have achieved it appears that IR can be dispensable for intrauterine development, growth and metabolism.

In the days following birth, in spite of normal suckling activity, $Insr^{-/-}$ mice showed increasing post-natal growth retardation (they weighted 30–40% less than their littermates) and a marked skeletal muscle hypotrophy became apparent. Anatomical observation of these animals further revealed a marked hepatomegaly with liver steatosis that could be seen in histological analysis (Figure 5). The cells in the liver of $Insr^{-/-}$ mice were vacuolated, resulting in weak staining of the cytoplasm (Figure 5). Fatty infiltration of the liver could be demonstrated upon further histochemical staining of the sections with Sudan black B (Figure 5). The total absence of IR finally resulted in early neonatal lethality, since all $Insr^{-/-}$ mice died within 1 week after birth.

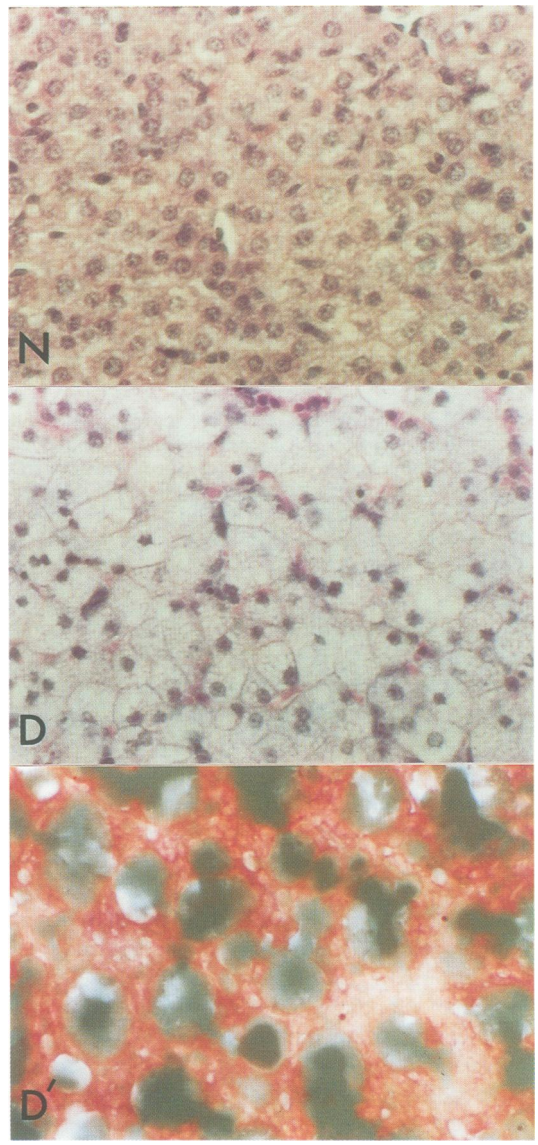

Fig. 5. Histological analysis of liver from normal (N) and $Insr^{-/-}$ diabetic (D, D′) animals. Tissue sections were stained with hematoxylin and eosin (N, D) or Sudan black B and neutral red (D′). Magnifications are ×125 (N, D) and ×200 (D′). For details see text and Materials and methods.

Metabolic disorders in $Insr^{-/-}$ mice leading to liver steatosis and diabetic ketoacidosis

$Insr^{-/-}$ mice developed diabetic ketoacidosis. Within 3 days after birth first glucose and then ketone bodies appeared in the urine and soon reached very high levels (>20 and >1.6 g/l respectively). Hyperglycemia in $Insr^{-/-}$ mice, as expected, led to hyperinsulinemia (Table I). The plasma insulin levels in mice devoid of IR were raised ~2- to 10-fold as compared with normal littermates. The total absence of IR resulted in other metabolic disorders. The fatty acid levels in the plasma of $Insr^{-/-}$ mice were visibly elevated, as judged by the slightly turbid aspect of the plasma. The plasma triglyceride levels were measured and were indeed found to be ~6-fold higher as compared with normal littermates (Table I). Finally, the glycogen content in the liver of $Insr^{-/-}$ mice could decrease up to about five times as compared with normal littermates (Table I).

Table I. Metabolic parameters in normal and diabetic mice

	Normal[a]	Diabetic
Glucose[b] (g/l)	1.4 ± 0.3	18.1 ± 2.4
Insulin[c] (μU/ml)	22–30	60–300
Triglycerides[d] (g/l)	2.2 ± 0.1	14.6 ± 4.5
Hepatic glycogen[e] (mg/g tissue)	15.7 ± 0.5	3.4 ± 0.3

[a]Normal mice include wild-type and heterozygous animals.
[b]Average of two measurements on different plasma pools from six mice (3 days old).
[c]Values ranged between these levels using different plasma pools from five mice (2–4 days old).
[d]Average of three determinations on different plasma pools from three to four mice (2–4 days old).
[e]Average of two independent determinations (6-day-old mice). For experimental details see Materials and methods.

Energy metabolism, controlled by several hormones that play counter-regulatory roles, has been the subject of extensive investigation. It is well established that insulin has primary anabolic effects, while other hormones, such as glucagon, subserve catabolic functions. Insulin stimulates glucose uptake by muscle and fat tissue and the incorporation of glucose essentially into glycogen in muscle and liver and into lipids in fat tissue. The effect of insulin in fat tissue for fatty acid mobilization is achieved by inhibition of hormone-sensitive lipase. Most fatty acids taken up by the liver are esterified to form triglycerides and re-exported to the plasma. A small fraction of fatty acids captured by the liver can, however, be used for oxidative degradation leading to production of CO_2 and ketone bodies, which are secreted into the plasma and are poorly utilized by peripheral tissues. On the other hand, glucagon action in the liver consists of activation of glycogen breakdown and gluconeogenesis and derepression of the fatty acid oxidizing ketogenic machinery. It is known that a small change in the insulin:glucagon ratio can result in large effects (see Foster, 1983, and references therein).

Histological analysis of the skin and pericapsular kidney region of *Insr*$^{-/-}$ mice revealed that fat tissue was present (not shown), indicating that signalling by IR is not absolutely required for the development and differentiation of fat tissue (for a review see Smas and Sul, 1995). However, storage of fatty acids must be impaired in *Insr*$^{-/-}$ mice by derepression of hormone-sensitive lipase, leading to an imbalance between storage and degradation of lipids. As a consequence, the levels of free fatty acids in the plasma could increase, as we have observed. At the same time, lack of signalling by IR in the liver could result in increased effects of glucagon and particularly in activation of the fatty acid oxidizing ketogenic cycle. The rate of ketogenesis is determined by the concentration of fatty acids present in the liver according to first order kinetics and the hepatic uptake of fatty acids is the passive consequence of plasma concentration. Thus in *Insr*$^{-/-}$ mice the high plasma concentrations of fatty acids could lead to hepatic steatosis and ketoacidosis. In insulin-dependent diabetes (IDDM) an increase in fatty acid levels in the plasma as a consequence of insulin deficiency results in severely increased ketogenesis and the appearance of ketoacidosis is a major risk factor that can lead to death of the patient. In contrast, this is not observed in NIDDM. The mechanisms for the resistance to ketogenesis in NIDDM are not yet elucidated (see Foster, 1983, and references therein). Moreover, the glycemia of a diabetic *Insr*$^{-/-}$ animal after overnight fasting remained very high (>8 g/l). This contrasts with patients having insulin resistance, who can present with fasting hypoglycemia. A possible resistance to glucagon effects in the liver in NIDDM patients might explain some of these differences from the phenotype of *Insr*$^{-/-}$ mice.

Alternative signalling pathways for insulin?

The insulin-like growth factor-1 receptor (IGF-1R) is structurally very homologous to IR. Moreover, the signal transduction pathways activated by these two receptors are common, although the kinetics and the magnitude of activation could be different (for a review see De Meyts *et al.*, 1994; Lamothe *et al.*, 1995, and references therein). Activation of IR and IGF-1R thus leads to certain specific, as well as some common, biological effects. It is known that insulin can bind to IGF-1R and insulin-like growth factors to IR, albeit with lower affinities. The existence of hybrid receptors has also been reported (for a review see Werner *et al.*, 1991). Therefore, study of the biological effects specifically mediated by each of these receptors remains somewhat difficult. In this respect IR-deficient mice should prove to be a useful animal model for studying some of these issues. In any event, activation of IGF-1R could therefore represent an alternative signalling pathway for insulin. Interestingly, human *Insr*$^{-/-}$ infants with leprechaunism could have extremely high insulinemia (up to >4000 μU/ml) and it is conceivable that this could lead to activation of IGF-1R. In contrast, insulin levels in *Insr*$^{-/-}$ mice were raised only up to ~300 μU/ml and this level might not be enough to allow efficient binding to IGF-1R. One can wonder whether this difference in activation of insulin gene expression reveals the existence of a major difference in glucose regulation of human and mouse insulin genes that has not yet been uncovered. Immunohistochemical staining of pancreatic sections from diabetic and normal mice using anti-insulin and anti-glucagon antibodies were comparable (not shown) and did not reveal any disorganization of the islets in the pancreas. Alternatively, insulin secretion could somehow be limited in the state of ketoacidosis in *Insr*$^{-/-}$ mice.

Targeted inactivation of the IGF-1R gene (*Igf-1r*) has already been achieved and resulted in embryonic growth retardation and immediate post-natal lethality (Liu *et al.*, 1993). It therefore appears that, at least post-natally, IR and IGF-1R cannot substitute for each other. The question, however, remains as to whether this is also the case during embryonic development and it would be interesting to examine the phenotype of *Igf-1r*$^{-/-}$ and *Insr*$^{-/-}$ double mutants.

Transgenic mice have been used for a decade to study altered expression of genes potentially important in NIDDM (for a review see Moller, 1994). Gene targeting now allows the effects of defined mutations in a single gene or a set of genes to be examined in the mouse. Genes encoding IRS-1 and the glucose transporter GLUT4, two proteins that are considered crucial in the action of insulin downstream of IR, have already been inactivated and have led to very unexpected phenotypes (Araki *et al.*, 1994; Tamemoto *et al.*, 1994; Katz *et al.*, 1995). The

application of this approach to other genes that play a role in insulin action might help in understanding the molecular mechanisms of insulin resistance and the pathogenesis of NIDDM.

Materials and methods

Engineering of the replacement vector

All bacterial, phage and plasmid DNA manipulations were performed as described (Sambrook *et al.*, 1989). Enzymes used for cloning were from Biolabs. Amplified plasmid DNAs were prepared using a Qiagen kit (Promega).

A mouse strain 129 genomic DNA library (obtained from P.Chambon) constructed by partial *Mbo*I digestion and cloning at the *Bam*HI site in the λGEM12 phage vector (Stratagene) was screened with a probe synthesized using full-length human cDNA encoding IR (Ebina *et al.*, 1985; Ullrich *et al.*, 1985; provided by A.Ullrich). Characterization of the DNA inserts in recombinant phages by hybridization using different fragments of IR cDNA as probes, PCR amplification of exons (Flores-Riveros *et al.*, 1989) and restriction mapping identified five recombinant phages containing exon 2. The restriction map of the genomic region of ~30 kb around exon 2 was established and several genomic fragments were subcloned. Particularly, two fragments of 7.4 and 14.8 kb located respectively on the 3′- and 5′-sides of exon 2 were obtained by digestion of independent phage DNA with *Not*I/*Spe*I and *Not*I/*Sma*I. They were cloned into pSK+ (Stratagene) using *Not*I/*Spe*I and *Not*I/*Sma*I sites, leading to pλR2 and pλR7 respectively. In recombinant phage DNA the *Not*I site is present in the polylinker.

To engineer the targeting vector pJorg (obtained from J.Hamm) a pSK+ derivative containing *tk* cloned at *Xho*I/*Hin*dIII sites and *neo* at the *Bam*HI site was first modified by inserting a *Sal*I linker at the *Hin*dIII site, generating pKO. A 1.8 kb fragment from the 5′-side of exon 2, obtained by digestion of a recombinant phage DNA with *Not*I/*Xba*I, was directly cloned into pKO using *Not*I/*Xba*I sites, leading to pλR4. To obtain the final targeting vector (pIRKO) a fragment of 7.4 kb located on the 3′-side of exon 2, obtained by digestion of pλR2 with *Not*I/*Spe*I, was cloned in pλR4 at the *Sal*I site using *Sal*I linkers. The final construct was linearized with *Not*I prior to electroporation into ES cells.

For PCR screening of recombinant ES clones a fragment of 2 kb having an extension on the 5′-side as compared with the region of 5′ homology in the targeting vector, obtained by digestion of a recombinant phage DNA with *Not*I/*Xba*I, was cloned into pKO using *Not*I/*Spe*I sites, generating pλR3. The nucleotide sequence of this 5′ extension was determined using the Sequenase kit (United States Biochemical Corp.). The external primer chosen in this region was: 5′-GTGCAGG-ACTATGGGAAGCC-3′ (R1). The internal primer was chosen in *neo*: 5′-CGCAGCGCATCGCCTTCTAT-3′ (R2).

Generation of recombinant ES cell clones and mutant mice

All ES cell cultures and mouse embryo manipulations were performed as described (see references in Robertson, 1987; Joyner, 1993). Cell culture media (Dulbecco's modified Eagle's medium) and fetal calf serum were from Techgen and ESGRO-LIF was from Gibco BRL.

After electroporation of *Not*I-linearized pIRKO DNA (25 μg) into 2×10^7 ES cells (D3 line) 60 colonies that were resistant to G418 (250 μM; Gibco) and Gancyclovir (2 μM; Syntex) were picked and amplified. Screening of recombinant clones was first performed by PCR (see below) on pools of DNA from five resistant clones and then on individual DNA in positive pools. Three positive ES cell clones were identified and were further confirmed by Southern blot analysis (see below). A total of 30 C57Bl/6 blastocysts were each injected with 10–15 cells from two recombinant ES cell clones, re-implanted into pseudopregnant B6CBAF1 females and allowed to develop to term. Eight male chimeras were born, as judged by the coat color, among which four proved to be germline chimeras. Heterozygous mutant offspring were generated by crossing the male germline chimeras with B6D2F1 and their intercrosses produced homozygous mutant mice. Genotyping of offspring was performed by Southern blot analysis on mouse tail DNA (see below).

DNA extraction

DNA from ES cell clones or from tails of newborn mice was extracted by proteinase K (Boehringer) digestion at 56°C overnight, followed by 2 h incubation with RNase A (Boehringer) as described (see references in Joyner, 1993). The cellular DNA was precipitated with ethanol and the tail DNA with isopropanol. After centrifugation the pellets were washed with 70% ethanol, air dried and resuspended in TE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA). DNA concentrations were determined using a spectrophotometer.

PCR screening

DNA from ES cell clones (in pools or individually) was subjected to PCR with primers P1 and P2 (Genosys) and Taq DNA polymerase (Perkin-Elmer Cetus) under the following conditions. After an initial denaturing step at 95°C for 5 min 40 cycles were performed each consisting of 1 min at 95°C, 1 min at 62°C and 2 min at 72°C. The final cycle was followed by a further 5 min incubation at 72°C. The products were run on agarose gels and the amplified band of ~1.8 kb was visualized by ethidium bromide staining. The conditions for PCR were optimized using pλR3 (see above) in the presence of various amounts of ES cell DNA.

Southern blot analysis

DNA (20 μg) prepared from ES cell clones or from mouse tails was digested with *Nco*I, subjected to electrophoresis on 0.8% agarose gels, transferred onto Hybond N+ membranes (Amersham) and hybridized to the probe synthesized using the *Eco*RI fragment of pλR7 (see Figure 1) with a random priming kit (Amersham) and [α-^{32}P]dCTP (3000 Ci/mmol; Amersham). Filters were hybridized and washed at 65°C and exposed to Kodak X-ray film with an intensifying screen for 1–2 days at –70°C.

Western immunoblot analysis

Protein extracts were prepared by homogenizing ~100 mg liver in 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM $Na_4P_2O_2$, 2 mM Na_3VO_4, 0.1 M NaF, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 0.5 μg/ml pepstatin). Upon centrifugation the supernatant was collected, diluted with an equal volume of Laemmli sample buffer, boiled and 10 μl subjected to SDS–PAGE (Laemmli, 1970) and the proteins blotted onto nitrocellulose (Towbin *et al.*, 1979). Immunodetection was performed using rabbit polyclonal antibodies against the β chain (Transduction Laboratories), peroxidase-conjugated goat anti-rabbit antibodies (Immunotech) and the ECL detection system (Amersham).

Glucose tolerance tests

Glucose tolerance tests were performed on wild-type and heterozygous mutant animals (1–2 months old) after overnight fasting by intraperitoneal injection of 2 g/kg D-glucose at time zero. Blood samples were taken from the orbital sinus at the indicated time points and glucose levels were determined as described below.

Assays to determine metabolic parameters

Blood glucose was determined using a Glucometer II and Glucostix strips (Ames). Plasma glucose concentration was measured using the Peridochrom Glucose kit (Boehringer) based on a colorimetric enzymatic procedure that uses glucose oxidase and peroxidase (Trinder, 1969). Urinary glucose and ketone bodies were determined using Keto-Diastix strips (Bayer Diagnostics). Plasma insulin levels were measured with an Insulin-CT kit (CIS Biointernational) based on a radioimmunoassay using pork insulin as standard. Plasma triglyceride concentrations were determined using the Triglyceride (GPO-Trinder) kit (Sigma) based on a modified colorimetric enzymatic procedure described by McGrown *et al.* (1983) that uses lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase. The amount of hepatic glycogen was measured in perchloric acid extracts adjusted to pH 5 with K_2CO_3 using the α-amyloglucosidase (Boehringer) method (Keppler and Decker, 1981).

Histological analysis

For histopathology tissue samples were fixed in 4% paraformaldehyde and paraffin embedded. Sections were cut at 5 μm, mounted on poly-L-lysine-coated slides and stained with hematoxylin and eosin. For staining of lipids frozen tissue samples were fixed in 4% paraformaldehyde, cut at 10 μm with a cryostat, mounted as above, stained with Sudan black B and counterstained with neutral red. Slides were photographed using a photomicroscope.

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Accili *et al.* recently also reported the obtention of insulin receptor-deficient mice using another gene targeting strategy [Accili,D., Drago,J., Lee,E.J., Johnson,M.D., Cool,M.H., Salvatore,P., Asico,D., José,P.A., Taylor,S.I. and Westphal,H. (1996) Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nature Genet.*, **12**, 106–109].