REVIEW ARTICLE

Genetic engineering in mice: impact on insulin signalling and action¹

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The expression of a number of genes encoding key players in insulin signalling and action, including insulin, insulin receptor (IR), downstream signalling molecules such as insulin receptor substrate-1 (IRS-1) and IRS-2, glucose transporters (GLUT4, GLUT2) and important metabolic enzymes such as glucokinase, has now been altered in transgenic or knockout mice. Such mice presented with phenotypes ranging from mild defects, revealing complementarity between key molecules or pathways, to severe diabetes with ketoacidosis and early postnatal death. Insulin action could also be improved by overproduction of proteins acting at regulatory steps. The development of diabetes by combining mutations, which alone do not lead to major metabolic

alterations, validated the ‘diabetogenes’ concept of non-insulin-dependent diabetes mellitus. Genes encoding insulin-like growth factors (IGF-I and IGF-II) and their type I receptor (IGF-IR) have also been disrupted. It appears that although IR and IGF-IR are both capable of metabolic and mitogenic signalling, they are not fully redundant. However, IR could replace IGF-IR if efficiently activated by IGF-II. Studies with cell lines lacking IR or IGF-IR lend support to such conclusions. Concerning the issues of specificity and redundancy, studies with cell lines derived from IRS-1-deficient mice showed that IRS-1 and IRS-2 are also not completely interchangeable.

INTRODUCTION

Insulin is a peptide hormone that plays a vital role in glucose homeostasis in vertebrates by regulating carbohydrate, lipid and protein metabolism. Insulin is synthesized, stored and secreted by the β -cells of the pancreatic islets in a highly regulated manner. Muscle, liver and adipose tissue represent the three major targets for the metabolic effects of insulin. Insulin action is mediated by the insulin receptor (IR), a receptor tyrosine kinase present on the surface of most cells. IR is composed of two pairs of subunits, designated α and β , assembled into a heterotetrameric $\alpha_2\beta_2$ structure that binds one molecule of insulin with high affinity [1]. Insulin binding to the extracellular α -subunits leads to autophosphorylation of specific tyrosine residues of the intracellular part of the β -subunits through a transphosphorylation mechanism [2] that results in the activation of their tyrosine kinase domain. This enables the receptor kinase to phosphorylate intermediate docking proteins such as insulin-receptor substrate (IRS)-1, -2, -3 or -4, Src homology/ α -collagen protein (Shc) and Grb2-associated binder-1 (Gab1), which subsequently recruit various intracellular Src homology 2 (SH2)-domain-containing proteins. As presented in Scheme 1, this association, in turn, results in the activation of downstream signalling cascades, most prominently the Ras/Raf/mitogen-activated protein (MAP) kinase/MAP kinase kinase (MEK) and

the phosphatidylinositol 3-kinase (PI 3-kinase) pathways, leading to a variety of consequences and/or biological effects (Table 1) at the cellular level [3–7].

More detailed knowledge of the signalling network depicted in Scheme 1 has revealed that, far from being highly specific for insulin signalling, it is shared by a number of other extracellular signals. Among those are the insulin-like growth factors (IGF-I and IGF-II), which are primarily growth factors involved in foetal and/or postnatal development. IGFs exert their effects through the type I IGF receptor (IGF-IR), a receptor tyrosine kinase that is very similar to IR. Insulin and the IGFs cross-react with the non-cognate receptor with weak affinity [8] and therefore can activate it at high ligand concentration. One major debate centres around whether the two receptors share completely the same pluripotential signalling ability, but lead to differential biological effects (IR being primarily metabolic and IGF-IR primarily mitogenic), because either their biological effects are controlled by certain elements of the cellular context in which they operate or, alternatively, differences in the structure of the two receptors types or other aspects of their activation by the cognate ligand (i.e. kinetics, signal duration) generate truly distinct choices in the array of signalling molecules that are engaged by each receptor [8].

The question of signalling specificity is compounded by the fact that still other unrelated ligands, including those which do

Abbreviations used: Gab1, Grb2-associated binder-1; GK, glucokinase; GLUT, glucose transporter; Grb2, growth-factor-receptor-bound protein 2; IGF, insulin-like growth factor; IGF-IR, type I IGF receptor; IR, insulin receptor; IRS-1, -2, -3, -4, insulin receptor substrate-1, -2, -3, -4; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; *neo*, neomycin phosphotransferase gene; NIDDM, non-insulin-dependent diabetes mellitus; p70 S6 kinase, the 70 kDa ribosomal protein S6 kinase; p90 S6 kinase, the 90 kDa ribosomal protein S6 kinase; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-kinase, phosphatidylinositol 3-kinase; pY, phosphotyrosine; Ras, p21^{ras}; SH2, Src homology 2; Shc, Src homology/ α -collagen protein; SH-PTP2 (Syp), protein tyrosine phosphatase; Sos, Son-of-Sevenless; LDM, low-density membrane; PM, plasma membrane; MODY, maturity-onset diabetes of the young.

¹ As an expression of our admiration and gratitude we dedicate this review to Dr. Jacques Jami, whose wisdom of Science and Society is of great inspiration, on the occasion of his retirement from the Directorship of INSERM U257.

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Scheme 1 Insulin and IGFs signalling network

Table 1 Consequences and/or biological effects of insulin signalling

Modification of subcellular localization of proteins
Activation or inhibition of certain enzymic activities
Stimulation or repression of the transcription of certain genes
Translocation of GLUT4 and stimulation of glucose uptake and metabolism (muscle/adipocyte)
Stimulation of glucose incorporation into glycogen (muscle/liver)
Inhibition of glucose output (liver)
Stimulation lipogenesis (adipocyte/liver)
Inhibition of lipolysis (adipocyte)
Activation of the Na ⁺ /K ⁺ pump (adipocyte/muscle)
Stimulation of amino acid uptake (all cells)
Stimulation of protein synthesis (all cells)
Stimulation of DNA synthesis and cell growth (all cells)
Inhibition of apoptosis (all cells)

not normally act through receptor tyrosine kinases, such as interleukin-4, growth hormone and angiotensin II, engage elements of the IRS signalling cascade [4].

TRANSGENIC/KNOCKOUT MICE AS NEW INVESTIGATIVE TOOLS

Genetic engineering in mice has proven to be a powerful tool for studying the specificity, complementarity and redundancy in the signalling cascades by altering the structure or expression of a single gene or a set of genes, from the ligands themselves to the specific receptors to the downstream signalling players.

The general strategy for overexpressing the product of a gene of interest or knocking it out by homologous recombination has been reviewed elsewhere ([9–13] and references cited therein). In transgenic mice, a transgene construct with its own promoter will normally be expressed in all tissues where the gene is normally expressed, while it can be targeted to selected tissues by the choice of an appropriate promoter. The normal function of a given gene can be altered by overexpressing a mutated gene encoding a dominant negative protein in transgenic mice. Knockout mice carrying a homozygous null mutation can be used to examine the effects of total lack of a specific gene product, whereas the effects of gene dosage can be studied in

heterozygous null mutants. Breeding of various heterozygous and/or homozygous transgenic/knockout animals can be used for instance to combine alterations in the structure or expression of multiple genes. Tissue-specific knockouts can also be achieved using the Cre/loxP system [14].

The present review will focus on applications of these techniques to answer questions of physiological relevance by examining the effects of increasing or decreasing the levels of insulin, its receptor or key elements in signalling such as glucose transporters, signalling molecules or regulatory metabolic enzymes. We will also discuss the implications of the findings regarding sensitivity, specificity and redundancy in signalling by insulin and IGFs, and their interchangeability and ability to rescue each other in case of defective function.

GENETIC MANIPULATION OF INSULIN SIGNALLING AND ACTION

IR down-regulation concept of insulin resistance

A major physiological determinant of insulin sensitivity *in vivo* is the reciprocal relationship which exists between circulating ligand concentrations and cellular receptor levels. Chronically elevated insulin levels are associated with decreased concentration of IR on target cells, as shown *in vitro* by varying the insulin level using cultured cells [15], in animal models of insulin resistance such as the *ob/ob* mouse [16] or in obese patients with or without non-insulin-dependent diabetes mellitus (NIDDM) [17,18]. These observations have led to the concept of receptor down-regulation by excess ligand. An inverse linear relationship between fasting insulin levels and receptor concentrations on monocytes or adipocytes has been demonstrated in insulin-resistant patients. The following scenario has been proposed to explain the generation of insulin resistance. Hyperphagia in animals or men predisposed to obesity raises blood sugar and therefore increases insulin secretion. The resulting hyperinsulinaemia 'down-regulates' IR on target tissues, which generates insulin resistance and leads to further compensatory hyperinsulinaemia. As a corollary, chronic hyperinsulinaemia has been proposed to be a major pathophysiological factor in insulin resistance, but this issue has been controversial [18]. The problem is that, in animal models or human patients with insulin resistance where the vicious circle is already in place, it is impossible to distinguish primary alterations from their consequences. The transgenic/knockout approach has brought a number of novel insights which directly or indirectly pertain to this question by altering in a primary fashion either insulin production, circulating insulin/glucose levels or peripheral IR concentrations.

Insulin

Several transgenic mice were generated using the human insulin gene under the control of its own promoter with the aim of creating primary hyperinsulinaemia. The first transgenic mouse lines that were obtained presented with normal insulin levels and glucose homeostasis [19,20]. Although total insulin mRNA levels were increased nearly twofold, the levels of serum and pancreatic insulin remained normal, indicating post-transcriptional controls of insulin synthesis [21]. Two transgenic mouse lines were later reported which showed 2–4.6-fold elevated fasting insulin levels (depending on transgene copy number). Fasting glucose remained normal, but glucose intolerance appeared upon a glucose load [22]. This was apparently due to a lack of further insulin increase after the glucose challenge. Therefore, these mice validate the notion that chronic hyperinsulinaemia results in insulin resistance in the fasting state, but

its mechanism remains unclear, since no attempts were made to measure IR levels.

Transgenic mice carrying genes encoding mutant insulins were also created. The expression of a transgene encoding proinsulin with His^{B10} changed to Asp [23], as in a human kindred with familial proinsulinaemia, resulted in high levels of the pro-hormone in islets of transgenic mice carrying 100 copies of the transgene. The animals were normoglycaemic, which is not surprising, since the endogenous proinsulin was normally processed, as well as a fraction of the mutant proinsulin resulting in mutant insulin for which enhanced potency was shown *in vitro*. This suggests that the diabetes observed in the patients may originate from other predisposing factors rather than the mutant proinsulin. The most striking feature was that 15% of the mutant proinsulin was rapidly secreted via an unregulated constitutive pathway, probably due to impaired aggregation of this proinsulin.

Interestingly, Asp^{B10} insulin, which was being tested as a fast-acting analogue for use in diabetic patients, was found to have increased mitogenic potency *in vitro*, owing to a lower dissociation rate from IR, resulting in more sustained signalling [1,24], and to induce mammary tumours after long-term treatment in rats [25]. No tumours have been reported so far in either patients or transgenic mice expressing the Asp^{B10} proinsulin gene, but the β -cells in such transgenic mice were found to be twice as abundant and to proliferate at threefold higher rates than in control mice [26].

Transgenic mice expressing another mutant proinsulin gene encoding a monomeric insulin with Ser^{B9} changed to Asp and Thr^{B27} to Glu [27] also showed secretion of the mutant proinsulin at high levels via the unregulated constitutive pathway.

In order to explore the effect of a decreased availability of circulating free insulin which could result in chronic hyperglycaemia, an original approach consisted of creating transgenic mice expressing a mutated human IR cDNA encoding only the extracellular domain of the receptor using the mouse transferrin-gene promoter. The truncated form of human IR was secreted in a soluble form in such mice. This in effect creates a circulating high-affinity binding protein similar to that which normally exist for growth hormone, IGFs and other cytokines (unrelated to the receptor in these cases) and which effectively decrease the bioavailability of these hormones. The creation of such a circulating binding protein for insulin in transgenic mice resulted in altered glucose homeostasis, including postabsorptive hyperglycaemia concomitant with increased hepatic glucose production, and hyperinsulinaemia [28].

IR at the cell surface

A number of attempts have been made to manipulate insulin action in mice by expressing wild-type or mutated forms of the IR selectively in muscle, liver or fat tissue.

First, in order to increase the concentration of functional IR in muscle, transgenic mice were produced using the human IR cDNA under the control of the promoter/enhancer of rat myosin light-chain gene promoter [29]. The transgene was expressed specifically in skeletal muscle, but only a moderate increase in the total IR number (70% in homozygotes) was detected. The basal levels of blood glucose and insulin in the fasting state were not significantly different in transgenic mice as compared with control animals. However, the increase in blood glucose and insulin levels after intraperitoneal glucose injection was 25% lower in transgenic mice as compared with control animals. Also, after intraperitoneal insulin injection, the decrease in blood glucose

levels of transgenic mice was more pronounced. Thus a modest primary increase of IR in muscle significantly increased insulin-sensitivity.

Attempts were also made to decrease the concentration of functional IR in target tissues. Transgenic mice were produced using a human IR cDNA carrying a Lys¹⁰³⁰-to-Met mutation in the ATP-binding site, which codes for a tyrosine kinase-deficient form of IR, under the control of the IR gene promoter [30]. This mutated IR should have a dominant negative effect through the formation of hybrid receptors with the endogenous receptors. Although the transgene was expressed in all tissues, no glucose intolerance was observed in hemizygous mice after intraperitoneal glucose injection, even after sucrose feeding for 55 weeks. It is remarkable that the transgene was expressed in muscle and adipose tissue at higher levels than the endogenous receptor, but at a much lower level in liver, possibly suggesting a key role of the liver in maintaining glucose homeostasis even in the presence of insulin resistance in the other two major target tissues. The serum insulin levels in homozygous transgenic animals were significantly higher before and after glucose loading than in heterozygous or in wild-type mice, but insulin tolerance as well as glucose tolerance were normal.

Subsequently, tissue-specific transgenic mice were generated using a different dominant negative human IR cDNA carrying a Thr¹¹³⁴-to-Ala mutation (found in a human patient with severe insulin resistance), under the control of the rat muscle creatine kinase gene promoter [31,32]. Young hemizygous mice were first studied. These mice overexpressed the transgene at a 5–12-fold increased level in skeletal and cardiac muscle. These mice presented with IR kinase activity reduced by 80% in gluteal muscle (where glycogen content was also reduced) and 42% in soleus muscle (although 2-deoxyglucose uptake was, surprisingly, unaffected). They had normal levels of fasting blood glucose and insulin. After feeding, blood glucose levels increased only slightly, with insulin levels a little more than doubled, showing a mild degree of insulin resistance that was also demonstrated by a lesser decrease in glucose levels in an insulin-tolerance test. In older homozygous transgenic mice the metabolic defects were more profound, with clearly decreased insulin-stimulated 3-O-methylglucose uptake in muscle as well as total hindlimb glucose uptake, glycogen synthesis and lactate production. These mice also presented with increased fasting glucose and altered glucose tolerance with hyperinsulinaemia. Their serum triacylglycerol and fatty acid levels were modestly increased. The mass of the adipose tissue was increased by 22–38%. This animal model therefore suggests that insulin resistance confined to muscle could contribute to the development of dyslipidaemia and obesity associated in man with the so-called 'syndrome X'.

The consequences of the presence of this tyrosine kinase-deficient form of IR on downstream insulin signalling were further analysed. The levels of autophosphorylation and activation of the tyrosine kinase domain of the endogenous IR were reduced as well as IRS-1 phosphorylation and its association with the p85 regulatory subunit of PI 3-kinase. Insulin stimulation of MAP kinase and the 90 kDa ribosomal protein S6 kinase (p90 S6 kinase) was undetectable and c-Fos induction was impaired. However, activation of the 70 kDa ribosomal protein S6 kinase (p70 S6 kinase) and glycogen synthase were preserved, suggesting that glycogen synthase activation by insulin does not require MAP kinase or p90 S6 kinase activation [33], which was also shown recently by others using a specific MEK inhibitor [34]. It was shown that the majority of IR was present as heterodimeric hybrid receptors, validating the concept of a dominant negative effect of the tyrosine kinase-mutated form of IR on insulin signalling in the muscle of these transgenic animals [35].

The gene-targeting approach was also applied to directly inactivate the IR gene in mice. One strategy consisted of inserting a stop codon in exon 4 [36] and another strategy deleted part of the IR gene around exon 2 [37]. Interestingly, heterozygous null mutants (IR^{+/-}) did not present with any major metabolic abnormalities and had normal glucose tolerance following an intraperitoneal glucose tolerance test. Their insulin levels were not significantly different from those of wild-type mice (1.2 ± 1.1 versus 0.78 ± 0.57 ng/ml) [36]. This is noteworthy, since it had been postulated that the 50% decrease in IR (due to IR down-regulation) seen in *ob/ob* mice was a major pathogenic factor in the severe insulin resistance seen in this animal model [16], as well as in obese humans [17]. The data reported for IR^{+/-} mice suggest that a primary decrease in IR alone is not sufficient to generate severe insulin resistance and that additional genetic factors are required (e.g. those affecting postreceptor signalling), in line with the 'diabetogenes' concept [18,38]. Kido and Accili reported recently [39] that IR^{+/-} mice show increased insulin levels at 24 weeks and that 10% eventually developed NIDDM, depending on the genetic background.

Breeding of IR^{+/-} mice leads to IR-deficient homozygous null mutant pups (IR^{-/-}) appearing among the progenies. At birth, IR^{-/-} pups could not be distinguished from other littermates. Human patients with mutations in IR including null alleles usually have severe intrauterine growth retardation.

However, IR deficiency in IR^{-/-} mice led to a number of major metabolic alterations soon after suckling. The pups developed a severe diabetes mellitus with ketoacidosis. Their hyperglycaemia led to hyperinsulinaemia. The triacylglycerol and non-esterified-fatty-acid levels were raised leading to hepatic steatosis. The absence of insulin signalling in the liver also resulted in reduced hepatic glycogen content. IR-deficient pups develop a marked postnatal growth retardation and skeletal-muscle hypotrophy. White- and brown-fat tissue were present, but in decreased amounts. The number of fat cells appeared to be normal, and all stages of differentiation could be observed, but the cell fat content was markedly decreased [40]. All of these disorders led to the death of IR-deficient pups within 1 week after birth. A brief summary of certain striking features of mice lacking IR is presented in Table 2.

This is very different from the phenotypes observed in humans with mutations in IR, usually compound heterozygotes but including a few double null mutants, which present with either leprechaunism, severe insulin resistance with acanthosis nigricans [41] or, in one kindred, congenital fibre-type disproportion myopathy [42]. Those patients present usually with fasting hypoglycaemia. This again stresses the importance of the genetic background in determining the phenotype obtained by targeting a given gene.

Recently attempts were made to test whether metabolic disorders of IR^{-/-} pups could be corrected with IGF-I [43]. Intraperitoneal administration of IGF-I caused a prompt and sustained decrease of plasma glucose levels in IR^{-/-} mice. Increased phosphorylation of IGF-IR and higher amounts of the PI 3-kinase p85 subunit in anti-phosphotyrosine immunoprecipitates were detected in muscle and liver, where a decrease in phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels was observed, suggesting that IGF-IR could substitute for IR in mediating glucose uptake in muscle and inhibit hepatic gluconeogenesis through the PI 3-kinase pathway. However, non-esterified-fatty-acid levels were unaffected, and the survival of mutant pups could not be prolonged. This may be related to an inability of IGF-I to induce lipogenesis and block lipolysis in adipocytes in the absence of IR. In this respect, Christoffersen et al. have recently shown that IGF-IR present on adult rat

Table 2 Effects of manipulating insulin signalling or action in various knockout mice

Knockout mice lacking: Viable		Diabetes mellitus	Ketoacidosis	Other metabolic disorders	Other striking phenotypic characteristics	References
Insulin	No (death within 2 days)	Yes	Yes	Hepatic steatosis	Intra-uterine growth retardation Hyperplastic islets	[93]
IGF-I	No/Yes (death at birth depending on genetic background)	No	No	—	Dwarfism Reduced reproductive organs Infertility Underdevelopment of muscle tissue Less organized lungs Slower long-bone ossification	[91,92]
IGF-II	Yes	No	No	—	Dwarfism	[90]
IR	No (death within 1 week)	Yes	Yes	Hyperglycaemia Hyperinsulinaemia Hepatic steatosis Reduced hepatic glycogen	Postnatal growth retardation Skeletal muscle hypotrophy	[36,37]
IGF-IR	No (death at birth)	No	No	—	Dwarfism Respiratory failure at birth Muscle hypoplasia Abnormal development of skin, central nervous system and bone	[91]
IRS-1	Yes	No	No	Mild degree of peripheral insulin resistance Hypertriglyceridaemia	Intra-uterine and postnatal growth retardation Hypertension	[47,48,52]
IRS-2	Yes (few months for males)	Yes	No	Hyperglycaemia Hyperinsulinaemia Polydipsia Polyuria	Intra-uterine and postnatal growth retardation β -Cell deficiency	[55]
GLUT 4	Yes (5–7 months)	No	No	Hyperinsulinaemia Decreased levels of lactate, non-esterified fatty acids and β -hydroxybutyrate	Growth retardation Cardiac hypertrophy Reduction in fat tissue deposition	[79]
GLUT 2	No (death within 2–3 weeks)	Yes (mild)	No	Moderate hyperglycaemia Slight hypoinsulinaemia Elevated glucagon, free fatty acid and β -hydroxybutyrate Blood hyperosmolarity	Postnatal growth retardation Altered postnatal development of pancreatic islets	[82]
GK	No (death within 1 week)	Yes	Yes	High cholesterol and triacylglycerol levels Hepatic steatosis Depleted hepatic glycogen	—	[85,87]

adipocytes is incapable of signalling (C. T. Christoffersen, L. Larsø, P. De Meyts, and H. Tornqvist, unpublished work), while it is active in pre-adipocytes, where IGF-I activates adipose differentiation. Also, the low expression level of IGF-I receptors in liver [43] may not be sufficient to replace the normally abundant IR expression in preventing ketogenesis. The observed effect of IGF-I on PEPCK mRNA levels in liver is known to occur normally at infinitesimal insulin concentrations and seems to require the activation of very few receptors [44]. Studies with cells derived from the knockout mice should allow a resolution of these intriguing findings.

More recently, muscle-specific IR gene knockout was achieved using the Cre/loxP system [45]. Mice carrying an altered IR gene with exon 4 flanked by loxP sites were generated and bred with transgenic mice that express the Cre recombinase gene under the control of the muscle creatine kinase gene promoter/enhancer. The resultant muscle-specific IR knockout offspring showed a 90–95% reduction in IR expression specifically in skeletal muscle and a parallel decrease in insulin-stimulated IR and IRS-1 phosphorylation. Despite impaired insulin-stimulated glucose uptake in skeletal muscle, these animals remained physiologically normal. Muscle-specific IR knockout mice were normoglycaemic at birth and showed normal growth and development. Intra-

peritoneal glucose tolerance tests showed no evidence of glucose intolerance or insulin resistance. This shows that isolated insulin resistance in skeletal muscle cannot lead to impaired glucose tolerance in mice and suggests the existence of important, as yet unidentified, compensating mechanisms to maintain normoglycaemia. Tissue-specific knockout of the IR gene in the pancreatic β -cell has also been achieved by breeding the mutant mice mentioned above with transgenic mice expressing the Cre recombinase gene under the control of the rat insulin promoter [46]. The first analysis of such mice has revealed a defect in their early insulin secretory response. Thus IR may play a functionally important role in pancreatic β -cells.

IRS-1/IRS-2

To assess the relative importance of IRS-1 in insulin signalling, the corresponding gene was disrupted in mice by gene targeting. The strategy consisted of inserting a neomycin phosphotransferase gene (*neo*) cassette in the first exon of the IRS-1 gene, which resulted in its inactivation [47,48]. Surprisingly, homozygous null mutants (IRS-1^{-/-}) did not present with any major metabolic disorders. Their fasting glycaemia was normal. Tamemoto et al. reported normal glucose tolerance [47], while

Araki et al. found a significant hyperglycaemia after intra-peritoneal glucose load [48]. A mild degree of insulin (and IGF) resistance were observed. This rather mild phenotype suggested that another signalling intermediate could substitute for IRS-1 in the metabolic action of insulin in muscle and liver. It was soon identified (and cloned) as 4PS/IRS-2 [49–51].

IRS-1 deficiency resulted, however, in intra-uterine and post-natal growth retardation (more severe in the mice studied by Araki et al. than those studied by Tamemoto et al.), indicating that IRS-1 is indispensable at least in part for the growth-promoting function of IGFs. More recently, it was reported that blood pressure and plasma triacylglycerol levels were significantly increased in IRS-1-deficient mice. Endothelium-dependent vascular relaxation was also impaired. Furthermore, lipoprotein lipase activity in such mice was lower, suggesting that impaired lipolysis could lead to increased triacylglycerol levels under insulin-resistant conditions [52]. Table 2 presents a brief summary of the phenotype of mice lacking IRS-1.

Insulin signalling and action were further studied in muscle and liver from IRS-1^{-/-} mice. One study [53] showed that the responses to insulin-induced activation of PI 3-kinase, glucose transport, p70 S6 kinase, MAP kinase, mRNA translation and protein synthesis were markedly impaired in muscle, while insulin-induced activation of PI 3-kinase and MAP kinase were not significantly reduced in liver. This suggested that the insulin resistance of IRS-1^{-/-} mice is mainly due to resistance in the muscle. A second study, however, showed a twofold decrease in insulin-induced stimulation of phosphotyrosine-associated PI 3-kinase activity in both liver and muscle of IRS-1^{-/-} mice versus controls [48]. However, IRS-2-associated PI 3-kinase activation by insulin was 55 % greater in liver and 144 % greater in muscle in IRS-1^{-/-} mice than in controls. It appears somewhat difficult to quantitatively relate the data obtained in immunoprecipitates to the overall signalling economy in order to fully assign the observed degree of resistance, or compensation, to specific pathways.

IGF-I signalling and mitogenic effects were studied in embryonic fibroblasts and 3T3 cell lines from IRS-1^{-/-} embryos [54]. These data revealed that the cells lacking IRS-1 exhibit three classes of IGF-I-stimulated responses: (i) those that do not require IRS-1 (MAP kinase activation); (ii) those that are impaired by IRS-1 deficiency and can be reconstituted by either IRS-1 or IRS-2 (PI 3-kinase activation and early gene induction); and (iii) those that are impaired by IRS-1 deficiency and can be reconstituted by IRS-1, but not fully by IRS-2 (mitogenesis). These data already suggested that IRS-1 and IRS-2 are not fully interchangeable, which was amply confirmed by subsequent targeted disruption of the IRS-2 gene.

The entire IRS-2 gene was inactivated by replacement with a *neo* cassette [55]. The phenotype obtained was strikingly different from the IRS-1^{-/-} mice. Heterozygous mice (IRS-2^{+/-}) had normal birth size and phenotype, survived to adulthood and were bred to homozygosity. Homozygous IRS-2^{-/-} mice were born 10 % smaller than wild-type, a difference which persisted into adult life. Mildly resistant at birth, the animals progressively developed a marked glucose intolerance and had full-blown diabetes at 10 weeks. Males developed polydipsia and polyuria without ketosis and died from dehydration and hyperosmolar coma. Females followed a similar disease progression, but rarely died. The most striking finding was that IRS-2 deficiency not only caused peripheral insulin resistance, as did IRS-1 deficiency, but also prevented adequate β -cell compensation. Morphometric analysis showed that, as early as 4 weeks, a more-than-twofold reduction in β -cell mass compared with wild-type. Moreover, insulin secretion in response to glucose loading decreased as

hyperglycaemia progressed, possibly due to glucose toxicity. Table 2 presents a brief summary of the phenotype of IRS-2-deficient mice.

Thus a single gene mutation in this animal model was capable of inducing both the peripheral insulin resistance and the β -cell deficiency seen in typical type 2 diabetes. However, an initial study in human type 2 diabetic patients has shown no linkage between polymorphisms in IRS-2 and NIDDM [56].

In terms of insulin signalling, it was found that the degree of stimulation of PI 3-kinase activity associated with IRS-1 in liver and muscle of IRS-2^{-/-} mice was reduced by more than 50 %, due in part to an increased basal activity, in contrast with the marked enhancement of insulin-stimulated PI 3-kinase associated with IRS-2 seen in the IRS-1^{-/-} mice. This functional defect in PI 3-kinase regulation in the IRS-2^{-/-} mice may be the major factor underlying defective glucose metabolism.

PI 3-kinase

PI 3-kinase is an essential step in insulin signalling and has been shown to be essential for insulin-stimulated GLUT4 translocation and glucose transport. The p85 α regulatory subunit associates with IRS molecules through its SH2 domain, which results in PI 3-kinase activation.

Targeted disruption of the gene encoding the p85 α regulatory subunit of PI 3-kinase was attempted by disrupting the exon containing the initiation codon for p85 α [57]. Paradoxically, the p85 α -deficient mice showed hypoglycaemia due to increased sensitivity and increased basal and insulin-stimulated glucose disposal in peripheral tissues. There was a preferential localization of GLUT4 at the plasma membrane (PM) in both the basal and the insulin-stimulated state. Insulin-stimulated PI 3-kinase activity associated with IRS-1 was normal in the liver or reduced by 30–50 % in skeletal muscle and adipocytes; the association was through the p50 α alternative splicing isoform of the same gene with a lower affinity towards IRS-1. The p50 α PI 3-kinase/IRS-1 complex appears to dissociate more readily than p85 α PI 3-kinase/IRS-1 in the low-density membrane (LDM) fraction, resulting in increased translocation of GLUT4 from the LDM to the PM. These results support an essential role of PI 3-kinase in glucose transport and glucose homeostasis *in vivo* [57].

Glucose transporters and other metabolic enzymes

The transgenic and/or gene targeting approaches have been applied to alter the expression of a number of other genes encoding glucose transporters or other key metabolic enzymes such as glucokinase (GK), glycogen synthase or PEPCK. Studies using transgenic mice have already confirmed a number of important concepts. The pivotal role of glucose transport in glucose homeostasis was demonstrated by overproducing GLUT4 or GLUT1 in all or selected insulin target tissues [58–74]. The key role of glycogen synthase in regulating glycogen synthesis could also be demonstrated [75]. It was shown that primary hyperglycaemia from increased hepatic glucose production due to PEPCK overproduction can cause insulin resistance [76]. Finally, overproduction of GK in the liver could prevent the development of streptozotocin-induced diabetic alterations, thus identifying GK as a potential target for therapeutic interventions [77,78]. In this review we present only the phenotypes of knockout mice which have been recently generated.

GLUT4/GLUT2

Glucose transporters represent a large family of proteins encompassing GLUT1–GLUT5 which facilitate glucose transport into

the cells across the PM. The genes encoding these proteins are differentially expressed and regulated in various tissues, and these transporters have different biochemical properties. For instance, GLUT1 is found in nearly every tissue type. GLUT4 is the major insulin-sensitive transporter in muscle and adipose tissue, the major sites for postprandial glucose disposal, where it is present in intracellular vesicles that are translocated to the PM in response to insulin. GLUT2 is present in liver, kidney, intestine and pancreatic β -cells. GLUT2 has a high K_m for glucose and has been proposed to function together with GK (see below) in glucose sensing by β -cells for regulated insulin secretion.

Application of the gene-targeting approach to the GLUT4 gene recently produced mice carrying a null mutation in this gene [79]. The strategy consisted of inserting a *neo* cassette in exon 10 of the GLUT4 gene which resulted in its inactivation. Surprisingly, homozygous null mutants (GLUT4^{-/-}) did not show a diabetes phenotype as one might have expected. Blood glucose levels in females were not significantly elevated in either the fasted or fed states. Males had 34 % lower fasting glucose and 20 % increase in fed glycaemia. Insulin resistance was present in animals of both sexes, as evidenced by 5–6-fold higher postprandial hyperinsulinaemia and impaired glucose and insulin tolerance tests. GLUT4 deficiency resulted in decreased levels of lactate and non-esterified fatty acids in both the fasting and fed states, and of β -hydroxybutyrate in the fasting state. These changes are the opposite of those seen with GLUT4 overproduction and also the opposite of what is seen in the diabetic phenotype. There was a marked reduction in fat tissue deposition in GLUT4^{-/-} mutants, contrasting with the increased adiposity of mice overproducing GLUT4 in adipocytes. Finally, GLUT4-deficient mice were growth-retarded and showed significant cardiac hypertrophy, which may be related to hyperinsulinaemia and decreased supply of free fatty acids. Possibly as a result of abnormal cardiac function, the GLUT4^{-/-} mice had reduced longevity (5–7 months). Increased expression of other glucose-transporter genes was detected in liver (GLUT2) and heart (GLUT1), but not in skeletal muscle. The phenotype of GLUT4-deficient mice is briefly summarized in Table 2. The unexpected phenotype of these mutant mice has stimulated interest in the possibility of another insulin-responsive glucose transporter in muscle or fat cells. Recently, GLUT4-deficient mice in which GLUT4 expression was reconstituted in skeletal muscle were obtained upon breeding mice carrying a null mutation in the GLUT4 gene with transgenic mice expressing GLUT4 in skeletal muscle. The alterations in glucose metabolism due to GLUT4 deficiency could be corrected by GLUT4 expression in skeletal muscle only, but this did not restore normal adipose-tissue function, since fat-pad weight and non-esterified-fatty-acid levels remained lower in such mice [80].

The heterozygous GLUT4^{+/-} mice were born with normal phenotype and were fertile. Interestingly, the male mice developed, as they aged, hyperinsulinaemia and subsequently hyperglycaemia and hypertension [81]. The hyperinsulinaemia persisted until death, with no evidence of β -cell deficiency. GLUT4 content and glucose uptake in muscle of hyperinsulinaemic mice was markedly reduced. There was a drastic decrease in GLUT4 expression in adipose tissue as well, with no change in total mass, but average cell volume increased by 35 % (the opposite of what was seen in adipocytes of mice selectively over-producing GLUT4 in fat). The mice also showed diabetic cardiomyopathy and liver steatosis.

The role of GLUT2 in the control of glucose-stimulated insulin secretion has been controversial [82]. Recently, targeted disruption of the GLUT2 gene was achieved in mice by replacing exons 5, 6 and 7 by a *neo* cassette [82]. Heterozygous null

mutants did not present with any abnormality and were fertile. Homozygous null mutants (GLUT2^{-/-}) were normal at birth, but developed growth retardation and usually died at the age of 2–3 weeks. Animals 10–15 days old presented with moderate hyperglycaemia, slight hypoinsulinaemia, markedly elevated glucagon, non-esterified fatty acid and β -hydroxybutyrate. They also had extreme glycosuria (probably from lack of GLUT2 in kidney proximal tubules) and resulting blood hyperosmolarity. GLUT2-deficient mice showed a markedly altered glucose tolerance. Isolated islets of Langerhans from 12–15-day-old mice had a complete absence of insulin secretion in response to stimulatory concentrations of glucose, and a lack of rapid onset response in the presence of isobutylmethyl xanthine, but a normal response to non-glucidic secretagogues. Insulin gene transcription was also affected. Therefore, GLUT2 deficiency impaired glucose-stimulated insulin secretion by the β -cells. Unexpectedly, altered postnatal development of pancreatic islets was also observed (inversion of α/β cell ratio similar to that seen in NIDDM). The phenotype could be rescued by insulin given in subcutaneous pellets starting on days 15–17 of life, stressing the importance of the insulin secretory defect. A brief summary of the phenotype of mice lacking GLUT2 is presented in Table 2.

GK

The conversion of glucose into glucose 6-phosphate is the first step in glucose metabolism. This reaction is catalysed by a family of enzymes, hexokinases, with different affinities for glucose, kinetic properties and tissue distribution. These enzymes can be classified into two classes. The first class includes hexokinase I, II and III, and their activity is subjected to feedback inhibition by physiological concentrations of glucose 6-phosphate. The second class is represented by hexokinase IV or GK, which has a low affinity for glucose and is not inhibited at physiological concentrations of glucose 6-phosphate. One liver-specific isoform and one β -cell-specific isoform of GK are produced upon alternative splicing of the GK gene transcript. GK in the β -cells acts in glucose sensing. Heterozygous point mutations in the GK gene resulting in reduced enzymic activity have been found in the majority of patients with maturity-onset diabetes of the young (MODY) [83,84].

The GK gene was inactivated in mice in both β -cells and liver using two strategies that consisted of inserting a *neo* cassette between exons 3 and 5 [85] or in exon 2 [86]. The heterozygous null mutants (GK^{+/-}) had normal size and were fertile. The GK activity was reduced in their islets and liver by 37 and 28 % respectively [86], due to a reduced V_{max} of the enzyme [85]. Although the circulating insulin levels in these mice were comparable with those in control animals, glucose-stimulated insulin secretion during a hyperglycaemic clamp was decreased by 37 %, although it was not significant. Glucose production by the liver persisted even under hyperglycaemic and hyperinsulinaemic conditions. The blood glucose levels after overnight fasting were elevated by 25 %. The lipid metabolism in these mice was also affected, since circulating triacylglycerol levels were raised. Overall, the phenotype was quite similar to that of MODY patients. While one study found that fed GK^{+/-} mice had normal glucose levels [86], another study [85] reported consistently elevated random (non-fasted) glucose values from birth in GK^{+/-} mice. The first group was unable to generate homozygous null mutants due to embryonic lethality around E9.5 [86], but the second group was able to obtain viable GK^{-/-} pups which became severely diabetic with ketoacidosis, had high cholesterol and triacylglycerol levels and died at postnatal days 3–5 [85]. Their livers were steatotic and depleted of glycogen. Upon breeding

GK^{+/-} mutants and transgenic mice expressing the rat GK cDNA in β -cells under the control of the human insulin promoter, homozygous null mutants expressing the transgene in β -cells were recovered which were viable [85]. Thus, reconstitution of GK activity in β -cells in a GK-deficient background was sufficient to normalize the levels of cholesterol and triacylglycerols. However, blood glucose levels in the fed and fasting state were not normalized in 50 % of the animals which developed mild diabetes. These studies pointed to β -cell GK having a greater impact on glucose homeostasis than liver GK and provided strong support for the concept that GK is important for glucose sensing [85]. Table 2 presents a brief summary of some striking features of GK-deficient mice.

What would happen then if the GK gene were selectively inactivated in β -cells? This was done by inserting a *neo* cassette in exon 1b, which resulted in GK gene inactivation specifically in β -cells without affecting the liver isoform [87]. The heterozygous mutants generated in this way had GK and hexokinase activities unaffected in the liver and reduced GK activity in the islets. At the age of 10 weeks these mice developed mild diabetes due to defective insulin secretion in response to glucose. Homozygous null mutants developed the same postnatal metabolic disorders as complete GK-deficient pups described above and died within a week after birth (Table 2). *In vitro*, insulin secretion from their islets in response to 20 mM glucose was abolished, while that to arginine was preserved. All these studies indicate that GK activity in the β -cell plays a vital role as a glucose sensor, and that liver GK could be dispensable.

COMBINING MULTIPLE GENE DEFECTS

IR and IRS-1

As mentioned above, mice heterozygous for a null allele of IR or IRS-1 had no obvious clinical phenotype. Double heterozygous mutant mice carrying one null allele for both IR and IRS-1 were obtained upon breeding the single mutants [88]. Such animals were indistinguishable from other littermates at birth. The expression of IR and IRS-1 in liver and muscle of these mice was diminished by ≈ 60 %. As a consequence, insulin-stimulated autophosphorylation of IR, tyrosine-phosphorylation of IRS-1 and IRS-2 and association of the p85 subunit of PI 3-kinase with IRS-1 were all reduced. Similar results were seen in liver and muscle, with the exception of IRS-2, which was expressed at a much lower level in muscle than in liver. Blood glucose was normal and remained so up to 4–6 months of age, but insulin levels were already more elevated at 2 months in the double heterozygotes than in each single mutant. Interestingly, at the age of 4–6 months, these animals developed a dramatic insulin

resistance, as evidenced by very elevated levels of plasma insulin (13-fold) and blunted response to exogenous insulin. At 6 months, 40 % of double heterozygous null mutants for IR and IRS-1 were overtly diabetic. The development of insulin resistance was accompanied by an increase in β -cell mass, which was most pronounced in the diabetic animals. This contrasts with the lack of compensatory β -cell hyperplasia seen in the IRS-2 knockout mice. A brief summary of the phenotype of these double knockout mice is presented in Table 3. It therefore appears that the combination of minor defects in the insulin signalling cascade can act synergistically to cause insulin resistance and NIDDM [88].

IRS-1 and GK

A different approach was used for combining two genetic defects which did not cause diabetes in isolation, this time a peripheral and a β -cell defect. Double mutant mice that were IRS-deficient (homozygous) and carried only one functional allele of GK were obtained upon breeding individual mutants and analysed [89]. As described above, IRS-1^{-/-} mice showed normal glucose tolerance, despite insulin resistance due to compensatory hyperinsulinaemia. Heterozygous β -cell GK-deficient mice showed non-progressive glucose intolerance due to impaired insulin response to glucose. The double knockout mice were growth-retarded like the IRS-1^{-/-} mice. Interestingly, the double-knockout mice presented with glucose intolerance, fasting hyperinsulinaemia and decreased secretory response to glucose and, with age, became overtly diabetic (30–40 weeks). These results indicate that mild insulin resistance and a slight defect in insulin secretion, when combined, can lead to the development of diabetes. Selective β -cell hyperplasia was observed in such animals as in double heterozygous null mutants for IR and IRS-1. The phenotype of these double-knockout mice is briefly summarized in Table 3.

INTERPLAY BETWEEN INSULIN AND IGF SYSTEMS

The issue of possible functional redundancies in the insulin and IGF systems *in vivo* has been addressed using knockout mice for genes encoding IGF-I, IGF-II or the mouse insulin1 or insulin2 genes [90–93] or their respective receptors [36,37,91]. Targeted disruption of the IGF-I gene was achieved either by inserting a *neo* cassette in exon 3 or by deleting part of exon 4 by insertion of a *neo* cassette. The gene encoding IGF-II was inactivated by inserting a *neo* cassette in exon 2, the first coding exon of the gene. The strategy used to inactivate the IGF-IR gene consisted in deleting part of exon 3 during insertion of the *neo* cassette. Finally, insulin1 and insulin2 genes were inactivated by deleting most of the coding sequences by insertion of *neo* cassettes.

We will not review here in detail the wealth of information gathered by simple or multiple knockouts on the role of the IGFs and insulin and their receptors in fetal development, which is not the focus of this review (see [94] for a brief, but exhaustive, review of the findings). Some relevant features of the phenotypes of these mutant mice are, however, briefly described here. Mice lacking IGF-I either died at birth or were viable dwarfs, depending on the genetic background. Liu et al. reported that progress of long-bone ossification in adult mice proceeded at a reduced rate and that there was a reduction in the sizes of reproductive organs and infertility for both sexes [91]. Powell-Braxton et al. observed an underdevelopment of muscle tissue and a less organized lung in IGF-I-deficient pups [92]. Mice lacking IGF-II were viable dwarfs [90]. IGF-IR deficiency resulted in marked intra-uterine growth retardation and abnormalities in the development and differentiation of central

Table 3 Mice carrying multiple gene defects as new animal models of NIDDM

Double-knockout mice	Phenotype	Reference
IR ^{+/-} IRS-1 ^{+/-}	Hyperinsulinaemia Selective hyperplasia of β -cells Development of diabetes mellitus within 4–6 months	[88]
IRS-1 ^{-/-} GK ^{+/-}	Growth retardation Glucose intolerance Fasting hyperinsulinaemia Selective hyperplasia of β -cells Development of diabetes mellitus within 7–10 months	[89]

Scheme 2 Interplay in the insulin and IGFs system

The black broken, black thin or red thick arrows indicate the efficiency (in the increasing order) of the interactions of different ligands with their cognate or non-cognate receptors. In knockout (KO) mice, certain ligand–receptor interactions are abolished and others are forced to take place due to an increase in the concentration of a particular ligand. + + +, increased ligand concentration. The lessons from these single- or double-knockout mice concerning the issues of specificity and redundancy in the insulin and IGFs system are discussed in the text.

nervous system, muscle, bone and skin, and death of the mutant pups at birth due to respiratory failure [91]. Thus, in single IGF-IR null mutants, IR cannot substitute for the lack of IGF-IR. However, IGF-IR null mutants could be completely rescued by a second mutation in the gene encoding the IGF-II/mannose-6-phosphate receptor (IGF-IIR) [95]. This receptor is thought to act as a scavenger that binds IGF-II and removes it from the circulation, thus playing an important role in controlling serum IGF-II levels. Mutant mice lacking both IGF-IR and IGF-IIR presented with elevated IGF-II levels in the serum. Under such conditions, IGF-II, which binds to IR with a higher affinity than IGF-I [96], could efficiently act via IR, which possibly explains the viable phenotype of the double knockout mice. Most remarkably, this worked also in postnatal growth (normally regulated by IGF-I and growth hormone), since the double-knockout mice exhibited only a brief growth delay. As mentioned above, IR-deficient pups were near normal at birth. It has been shown, however, that double homozygous null mutants for both the IGF-IR and IR are only 30% of normal size at birth, while IGF-IR null mutants are 45% of normal size, indicating that IR also plays a role in mediating some effects of IGF-II during development [97]. The fact that the IR^{-/-} mice have normal size was shown to be due to compensatory up-regulation of the IGF-IR protein [97]. Postnatally, IR-deficient pups developed several metabolic disorders leading to death within 1 week [36,37]. It therefore appears that IGF-IR cannot substitute for the lack of expression of IR in all tissues. In contrast with IR-deficient pups, insulin-deficient knockout pups were growth-retarded ($\approx 22\%$ on average) at birth, indicating that insulin also exerts a growth-

promoting function during fetal life. Postnatally, insulin-deficient pups developed the same metabolic disorders as IR-deficient pups, but more rapidly and died sooner [93]. This suggests that, in IR-deficient mice which become hyperinsulinaemic, some of the biological effects of IR could be obtained from insulin action through IGF-IR. Insulin deficiency also led to hyperplastic islets, suggesting that insulin might function as a negative regulator of islet-cell growth during pancreatic organogenesis. The molecular basis for this action of insulin remains to be elucidated. Table 2 summarizes the phenotypes of these mutant mice. The various ligand–receptor interactions that could take place *in vivo* in the normal situation or in mutant mice lacking IR, IGF-IR or IGF-IR and IGF-IIR are also shown in Scheme 2.

CELLULAR SYSTEMS FROM KNOCKOUT MICE

The derivation of cell lines (fibroblasts, muscle cells, hepatocytes, pre-adipocytes) from various knockout mice can provide unique tools to further investigate *in vitro* the effects of the lack of specific proteins on insulin and IGF signalling and action.

Cell lines lacking IR

A fibroblast cell line was derived from IR-deficient mice to investigate metabolic and mitogenic effects of IGF-I and insulin [98]. It was reported that IGF-I is able to stimulate glucose uptake, glucose incorporation into glycogen and thymidine incorporation in such cells, as well as to activate PI 3-kinase and MAP kinase. Interestingly, all these effects were also achieved

when IR-deficient cells were stimulated with insulin, albeit at higher concentrations. Thus, IGF-IR can indeed represent an alternative receptor through which insulin might exert some of its biological effects in some cell types. Comparison of the dose–response curves obtained with the two ligands for the metabolic and mitogenic effects suggests that there might be differences in the nature of the interaction of these ligands with their cognate versus non-cognate receptors. Indeed, the dose–response curves for the metabolic effects were sigmoidal with both IGF-I and insulin, whereas the dose–response curves for the mitogenic effects were bell-shaped with IGF-I and sigmoidal with insulin. In this respect, it is interesting to note the results obtained using the LB cell line, which completely lacks IGF-IR and is derived from a murine T-cell lymphoma that was insulin-dependent for growth [99]. Insulin stimulated thymidine incorporation in these cells [99,100], and the dose–response curve for this mitogenic effect of insulin through IR was bell-shaped [101], as was that for the mitogenic effect of IGF-I acting through IGF-IR in IR-deficient cells [98]. In the LB cell line, negligible effects were observed with IGF-I, and none at all were observed with IGF-II [99]. In IR-deficient fibroblasts, insulin was clearly able to cause mitogenic effects, but the dose–response curve was sigmoidal. The biological significance of the bell-shaped dose–response curves for the mitogenic effects of insulin acting through IR or IGF-I acting through IGF-IR escapes our understanding, and the underlying molecular basis remains to be elucidated. It can, however, be recalled that a bell-shaped curve was previously reported for the action of growth hormone on lipolysis in adipocytes [102]. It was demonstrated that growth hormone binding to its receptor in solution resulted in the formation of a dimer [103,104], and receptor dimerization was critical for this biological effect. The bell-shaped curve in this case was explained by possible self-antagonism at high ligand concentration [102]. Cell lines lacking IR represent unique tools with which to investigate further possible differences in the interaction of IGF-I, IGF-II and insulin with IGF-IR.

Cell lines lacking IGF-IR

Fibroblast cell lines were derived from IGF-IR-deficient mouse embryos [105,106]. Such cells could grow in media containing 10 % serum, but with a significantly lower growth rate than wild-type cells and with prolonged cell cycles. No cell growth was observed in serum-free medium supplemented with growth factors, owing to arrest before the S-phase of the cell cycle. Thus IGF-IR is required for optimal growth and normal cell cycle. Interestingly, IGF-IR-deficient cells could not be transformed by simian-virus-40 large T antigen or an activated Ha-ras oncogene, revealing a critical role of IGF-IR in the transformation process or growth of transformed cells. This defect in the transformability of these cells was specifically due to the absence of IGF-IR, since cells in which IGF-IR gene expression was reconstituted by transfection with the corresponding cDNA could be transformed. Furthermore, it was shown that IGF-IR-deficient cells overproducing IR upon transfection of the corresponding cDNA became capable of growing in serum-free medium supplemented with insulin or IGF-II, but not IGF-I [107]. In fact, IGF-II had a greater maximal effect than insulin in promoting growth of these cells. In addition, if cells overexpressing IR cDNA were transfected with a plasmid carrying the IGF-II gene for overproduction of IGF-II, they could grow in serum-free medium without supplementation with any growth factors. These studies demonstrate that IGF-II, at high concentrations, can efficiently stimulate cell proliferation by acting through IR, and they fully

support the earlier explanation given for the viable phenotype of mice lacking both IGF-IR and IGF-IIR.

Cell lines lacking IRS-1

As mentioned above, fibroblast cell lines were also derived from homozygous null mutants for IRS-1 [54]. The effects of stimulating such cells with IGF-I were examined. Interestingly, stimulation of MAP kinase activity was unaffected, indicating that IRS-1 can be dispensable for the activation of this pathway. On the other hand, stimulation of PI 3-kinase activity and induction of *c-fos* and *erg-1* gene transcription were altered. If IRS-1 gene expression was reconstituted or the IRS-2 gene was overexpressed in IRS-1-deficient cells by expressing the corresponding cDNAs using retroviral vectors, the association of p85 with IRS-1 or IRS-2 and induction of *c-fos* and *erg-1* genes in response to IGF-I were restored or increased. However, the ability of IRS-1-deficient cells to enter in the S-phase in response to IGF-I was restored upon reconstitution of IRS-1, but not of IRS-2 gene expression, pointing to some interesting differences in these two docking proteins.

CONCLUSIONS AND PERSPECTIVES

Application of the transgenic and gene-targeting approaches to the field of insulin action has already provided a wealth of novel information and a means to examine the relevance, for *in vivo* metabolic physiology and pathophysiology, of key concepts often derived from *in vitro* experiments. It has been established, for instance, that primary hyperinsulinaemia (resulting from increasing the insulin gene copy in the β -cell) or hyperglycaemia secondary to a decrease in free insulin (due to production of a secreted soluble circulating form of IR) can cause insulin resistance.

The mild or absent phenotype obtained after targeted deletion of genes encoding IRS-1 or GLUT4 demonstrated the robustness of glucose homeostasis and validated the ‘diabetogenes’ concept of NIDDM, according to which the accumulation of several gene defects may be required to produce an overt diabetes phenotype. In some cases this was clearly due to β -cell compensation. Its failure to occur in the IRS-2 knockout mice resulted in a monogenic overt diabetes phenotype. Identification of the IRS-2-dependent β -cell trophic factor will be of great interest. Combination of gene deletions such as IR and IRS-1 or IRS-1 and GK further exemplified the synergistic impact of combined sublethal defects. The importance of the genetic background observed in some phenotypes, however, cautions against extrapolating unconditionally findings in mice to pathology in man. The development of animal models with well-specified phenotypes certainly opens the door for selected therapeutic trials.

Gene-targeting studies also showed that, while there is some overlap between the functions of IRS-1 and IRS-2 on one hand, and insulin and the IGFs on the other, they are not functionally redundant. It has become clear, however, that IGF-IR is capable of metabolic signalling, while IR is capable of mitogenic signalling. Interestingly, IR could completely substitute for IGF-IR if efficiently activated by IGF-II.

Gene-targeting studies also demonstrated the key role of PI 3-kinase in glucose transport and homeostasis. The relative contributions of GLUT2 and pancreatic GK to β -cell glucose sensing were also established.

In the future, genetic engineering in mice to perform tissue-specific gene disruptions, to reconstitute expression of a given

gene in selected tissues in a null background using transgenic mice or to replace specific genes with mutated genes should provide new investigative tools for further studies. In addition, cell lines derived from the various animal models should allow more in-depth investigation of insulin signalling and action as well as provide a means to address in more detail the issues of specificity and redundancy between various proteins or pathways at the molecular level.

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