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Review

Regulation of insulin receptor function

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Abstract. Resistance to the biological actions of insulin contributes to the development of type 2 diabetes and risk of cardiovascular disease. A reduced biological response to insulin by tissues results from an impairment in the cascade of phosphorylation events within cells that regulate the activity of enzymes comprising the insulin signaling pathway. In most models of insulin resistance, there is evidence that this decrement in insulin signaling begins with either the activation or substrate kinase activity of the insulin receptor (IR), which is the only component of the pathway that is unique to insulin action. Activation of the IR can be impaired by post-translational modifications of the protein involving serine phosphorylation, or by binding to inhibiting proteins such as PC-1 or members of the SOCS or Grb protein families. The impact of these processes on the conformational changes and phosphorylation events required for full signaling activity, as well as the role of these mechanisms in human disease, is reviewed in this article.

Keywords. Tyrosine kinase, autophosphorylation, insulin resistance, ectonucleotide pyrophosphatase phosphodiesterase 1, PC-1, suppressor of cytokine signaling, protein kinase C, tyrosine phosphatise.

Overview

The regulation of blood glucose levels requires a coordinated interaction between several tissues that is mediated by the release of and response to the hormone insulin. Secreted by pancreatic β-cells in response to an increase in circulating glucose levels, insulin triggers an increase in glucose uptake in the target tissues muscle and fat, and suppression of hepatic glucose release. The relative capacity of insulin to promote a decrease in blood glucose is referred to as insulin sensitivity. Insulin sensitivity can have a different meaning at the tissue level, referring to the stimulation of glucose uptake in muscle, and the processes of glucose production and release in the liver.

The study of insulin sensitivity in humans typically involves investigation of insulin action in skeletal muscle. This results from the fact that the gold standard for quantifying insulin sensitivity, the hyperinsulinemic, euglycemic clamp procedure, measures muscle insulin action, as this tissue accounts for 75 % of glucose disposal under these conditions [1]. In contrast, hepatic glucose output, although contributing significantly to glucose tolerance, is fully suppressed under the conditions normally employed during the glucose clamp. This review will correspondingly focus on the regulation of IR function in skeletal muscle in both *in vivo* and *in vitro* models of insulin resistance. The fact remains, however, that insulin resistance can occur in any insulin target tissue, and will produce a phenotype distinct for the biological characteristics of that tissue, as has been demonstrated by the study of mice with tissue-specific genetic knockout of the IR (reviewed in [2]).

Insulin resistance

Tissue insulin resistance predisposes an individual to significant health risks in addition to its role in the pathogenesis of type 2 diabetes mellitus [3]. In humans, insulin-stimulated muscle glucose disposal rates vary widely across the normal population, and the insulin-resistant state refers to individuals in the lower end of a normal distribution, rather than a discreet pathological condition. *In vivo*, muscle insulin sensitivity is regulated on a long-term basis by factors such as obesity, and is altered in a more rapid manner by changes in dietary habits and physical activity [4]. Though more difficult to quantify, there is also evidence for genetic or intrinsic differences in muscle insulin sensitivity [3]. In any individual, therefore, the degree of insulin sensitivity is determined by the interaction of numerous factors, both genetic and environmental. Both acute and chronic regulation of tissue insulin sensitivity can occur via multiple avenues through which the cellular mediators of insulin signaling can be altered by protein interactions and other modifications.

Insulin signaling pathway

Following binding of insulin to the extracellular portion of the IR, the second messenger system involved in insulin signaling diverges into separate pathways that regulate distinct biological effects (Fig. 1). These specific second messenger proteins are also employed in mediating the effects of a variety of other hormones. Thus, a specific and coordinated cellular response to insulin stimulation requires the integration of a full network of signaling processes [5]. The substrate tyrosine kinase activity of the IR initiates a cascade of cellular phosphorylation reactions that regulate protein interactions and enzymatic activities. Substrates of the IR include the insulin receptor substrates IRS-1 and IRS-2, as well as Shc (Src homology collagen) and APS (adaptor protein with a PH and SH2 domain) [5]. These phosphorylated substrates then serve as docking molecules that bind to and activate cellular kinases, initiating the divergent signaling pathways that mediate cellular insulin action. The simplified scheme of insulin signaling is that the stimulation of glucose transport and most other metabolic effects of insulin are regulated by activation of the phosphatidylinositol 3-kinase (PI3K) pathway, facilitated by binding of the regulatory subunit of PI3K to phosphotyrosine residues on IRS-1. The ultimate effector system for regulating glucose disposal is the translocation of GLUT4-containing vesicles to the plasma membrane to increase the rate of cellular glucose transport [6]. Cell growth and protein synthesis are more closely associated with the Ras/MAPK pathways which are activated primarily by Shc phosphorylation (reviewed in [7]).

In insulin resistance, the ability of insulin to initiate these phosphorylation cascades that regulate the activity of insulin second messengers is diminished [8, 9]. The enzymatic activity and phosphorylation state of kinases and substrates in these signaling pathways are reduced in muscle biopsies from insulin-resistant subjects [10]. However, the site(s) of the initial perturbation in signal transduction remains unclear. Significant evidence suggests that acute and chronic regulation of IR function contributes to alterations in insulin sensitivity and glucose homeostasis.

The insulin receptor

The IR gene consists of 22 exons and 21 introns spanning 150 kb of chromosome 19 [11]. The IR gene product is synthesized as a prepro-receptor, from which a 30-amino acid signal peptide is cleaved. The pro-receptor is then processed further, undergoing glycosylation, folding, and dimerization. In the Golgi apparatus, the dimerized amino acid chains are then cleaved into α- and β-subunits. These peptides are then linked via disulfide bonds to form a hetero-tetrameric holo receptor consisting of two identical extracellular α-subunits that bind insulin, and two identical transmembrane β-subunits that have intracellular tyrosine kinase activity (Fig. 2). Binding of insulin to the α-subunits of the IR results in a conformational change that induces autophosphorylation of distinct tyrosine residues on the β-subunits. As described below, autophosphorylation produces an additional conformational change that activates the receptor's protein tyrosine kinase activity [6]. While linked, autophosphorylation and tyrosine kinase activity are separate aspects of IR function that can be uncoupled and are subject to different mechanisms of regulation.

Impairments in IR function have been reported in tissues from insulin-resistant humans and animals. Reduced autophosphorylation has been observed for IR stimulated by insulin *in vivo* as well as in IR isolated from tissue biopsies and stimulated *in vitro*. In addition, a reduced capacity for IR substrate tyrosine phosphorylation has been demonstrated in IR isolated from insulin-resistant subjects and animal models. *In vivo*, a reduced phosphorylation of IRS proteins following insulin infusion is a common finding in insulin resistance; although this result does not, by

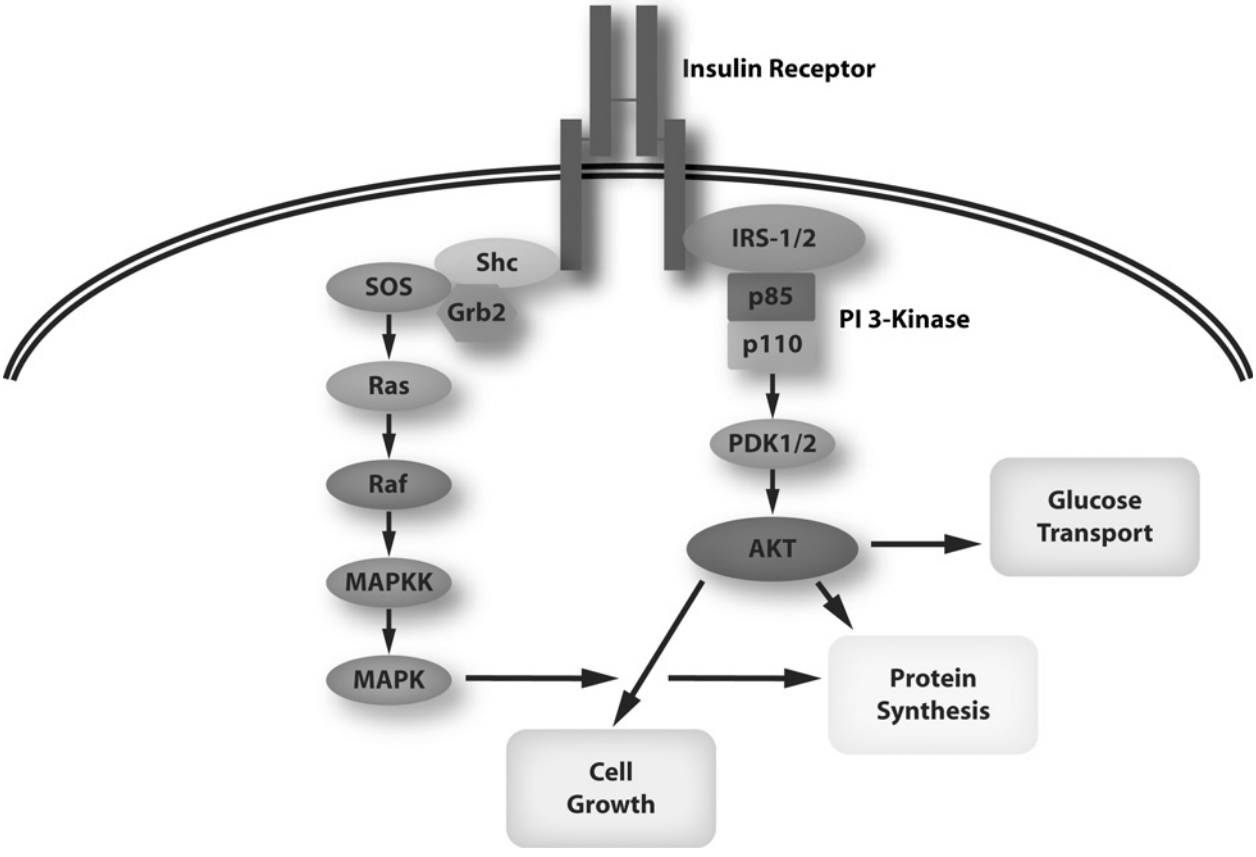

Figure 1. The insulin signaling pathway. The binding of insulin to its receptor leads to autophosphorylation of the β-subunits and the tyrosine phosphorylation of insulin receptor substrates (IRS) and other signaling intermediates such as Shc. Phosphorylated IRS proteins serve as docking proteins for other second messengers. Binding of the SH2 domains of PI 3-kinase (PI3K) to phosphotyrosines on IRS-1 activates this enzyme. PI3K activity produces phosphatidylinositol phospholipids, which activate phosphatidylinositol phosphate-dependent kinase-1 (PDK-1) and subsequently Akt/PKB. The net effect of this pathway is to produce a translocation of the glucose transporter (GLUT4) from cytoplasmic vesicles to the cell membrane to facilitate glucose transport. Signaling through the Ras/MAP kinase pathway primarily stimulates mitogenic and catabolic processes, rather than the metabolic effects of insulin.

itself indicate altered IR function, as the ability of IRS-1 to serve as a substrate for the activated IR can be altered by its phosphorylation state [12].

The physiological impact induced by impairments in IR function can be ascertained from recent studies of transgenic mice. Mice with specific ablation of IR in liver, muscle, white adipose tissue, pancreatic β-cell, vascular endothelium, and neurons all display distinct phenotypes depending on the physiological role of insulin in the respective tissue (reviewed in [2]). Significantly, however, all aspects of the metabolic syndrome can be recreated by a loss of IR expression in these target tissues [2]. The targeted expression of dominant-negative kinase-deficient IR in skeletal and cardiac muscle of transgenic mice [13, 14] provides a better model for the fractional downregulation of IR tyrosine kinase activity typically seen in insulin-resistant states. These dominant-negative IR produce substantial impairments in insulin-stimulated muscle IR tyrosine kinase activity, as well as diminished activation of downstream signaling intermediates IRS-1 and PI3K [13, 14]. These transgenic mice develop obesity, hyperinsulinemia, glucose intolerance, and hypertriglyceridemia [15]. Genetically induced alterations in IR function can, therefore, induce phenotypes very similar to those seen in insulin-resistant humans.

Physiological regulation of the IR

Mutations in the IR gene are rare and unlikely to account for reductions in IR function observed physiologically. However, several mechanisms exist whereby insulin signaling can be modulated at the level of the IR. Generally, these processes involve either the modification of IR content in the cells at the level of transcription or protein degradation, or by modification of the enzymatic activity of individual IR. In fact, IR tyrosine kinase activity is a relatively

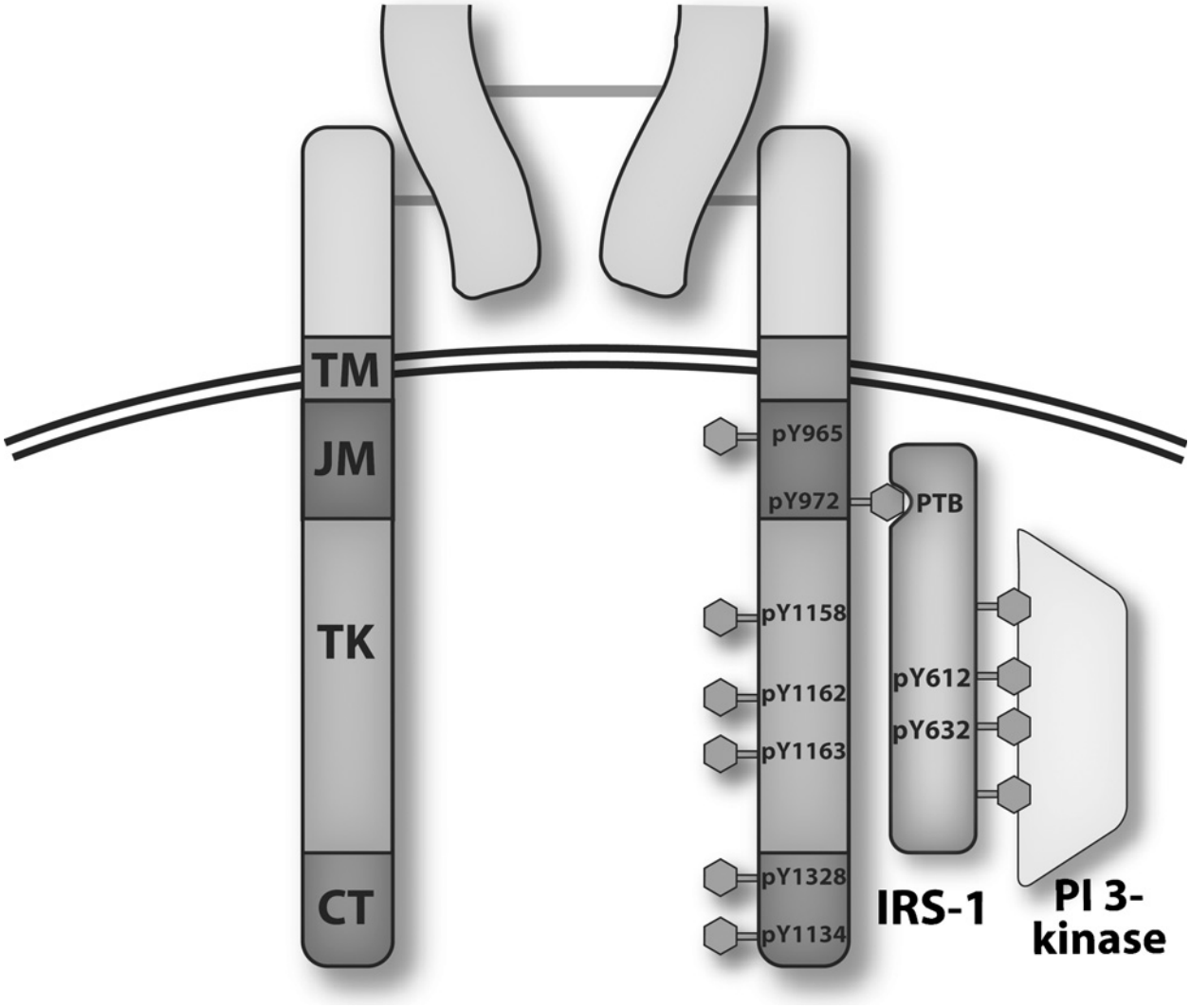

Figure 2. Schematic structure of the insulin receptor. The intracellular β-subunit of the IR contains several functionally distinct domains. The IR is anchored to the plasma membrane by the transmembrane domain (TM). The juxtamembrane domain (JM) contains two tyrosine residues that are autophosphorylated in response to insulin binding. Of these, tyrosine 972 is instrumental in binding the PTB domains of IRS-1 and Shc. The phosphorylated IRS-1 can then serve as a docking site and activator of PI3K. The tyrosine kinase domain (TK) contains the enzymatic active site of the molecule, as well as the ATP-binding site and three key tyrosines (1158, 1162, and 1163) which must be phosphorylated to produce full kinase activity of the IR. Tyrosines in the C-terminus (CT) region are not critical for receptor activation, but are thought to bind IRS-2 and to participate in the mitogenic effects of IR signaling.

labile parameter that can be modulated acutely in cells and *in vivo*, and it is the regulation of this enzymatic activity, rather than regulation of IR content, that is the focus of this review.

Still, the content of IR in cells can impact insulin signal transduction and the biological response to insulin. Previously, the recognition that the maximal response to insulin was elicited under conditions of less than complete IR binding (occupancy) led to a "spare receptor theory" [16]. While some investigators have postulated that with spare IR, moderate changes in IR number or even function might not impact insulin action, the studies of IR knockout mice described above demonstrate that a reduction in tissue IR content has a significant physiological impact. IR knockout heterozygotes present, as expected, half the number of IR as their control littermates, and downstream activation of the insulin pathway, as well as insulin sensitivity and glucose tolerance are reduced [17].

Transcriptional factors that regulate IR expression *in vivo* are not well understood. However, Puig and Tijan [18] have demonstrated that the FOXO1 transcription factor mediates a downregulation of IR transcription in response to insulin stimulation of IR signaling. In the absence of insulin, as during fasting, FOXO1 binds

to the IR promoter and stimulates IR gene transcription. Insulin stimulation of IR signaling results in a phosphorylation of FOXO1 which disrupts the interaction with the IR promoter, and reduces transcription [18]. Insulin also reduces the effective concentration of cellular IR by increasing internalization and degradation of the protein as well [19]. The mechanism underlying the insulin-mediated degradation involves activation of the APS activation protein, which recruits c-CBL to the IR, targeting it for ubiquitination and internalization [20]. Activation of this system produces a downregulation of multiple components of the insulin signaling system [21].

While the downregulation of IR number by insulin has been accepted for several decades, less appreciated are the mechanisms that modulate the function of individual IR at the level of autophosphorylation, substrate tyrosine kinase activity, or both. Alterations in autophosphorylation and tyrosine kinase activity can result from the interaction with directly inhibiting compounds, or by post-translational modification of the IR protein.

Mechanisms of IR autophosphorylation

The process of receptor activation involves a sequence of structural changes to the IR following ligand binding. Although the crystal structures have not been generated for either the full IR or the homologous insulin-like growth factor-1 receptor (IGF-1R), models of IR structure have been produced from single-molecule electron-microscopic imaging studies and from crystal structures of receptor peptide fragments (Reviewed in [22]). Mutated IR have been employed in biochemical and structural studies to further develop the models elucidating the structural aspects of IR activation.

Full substrate kinase activity requires the phosphorylation of several key tyrosine residues on the β-subunit. There are seven tyrosine residues in the cytoplasmic domain of the β-subunit that have been identified as autophosphorylation sites (Fig. 2). Tyrosines 965 and 972 are located in the juxtamembrane region. Tyrosine residues 1158, 1162, and 1163 are located in the activation loop of the kinase domain, and tyrosines 1328 and 1334 are in the C-terminus [23]. As described below, phosphorylation of tyrosines in these different regions regulate separate aspects of IR function.

The autophosphorylation of β-subunit tyrosine residues occurs in both a cis- and trans- manner, meaning that certain tyrosines are phosphorylated by the phosphotransferase activity of the same subunit, while others are substrates for the kinase activity of the opposite subunit [24]. Tyrosines in the activation loop are trans-phosphorylated by the opposing β-subunit following ligand binding. The structural model of Ottensmeyer and co-workers [25] offers a theory on the mechanism whereby ligand binding leads to trans-autophosphorylation. Their studies employing scanning-transmission electron microscopy of the ligand-bound and unbound IR indicate that occupancy of the insulin-binding site induces the movement of opposing subunits towards each other [25]. This close opposition would then allow for tyrosine phosphorylation of the opposing subunits. There is some discrepancy between opposing models for the basic structure of the IR [22], and a definitive model for the structural changes induced by ligand binding has not been developed. Still, it is likely that the functional consequence of binding is that the β-subunits are brought into close opposition to facilitate trans-phosphorylation.

Crystallographic studies of the IR kinase domain in the unphosphorylated state and in the fully active state indicate that autophosphorylation activates the IR tyrosine kinase activity due to a series of alterations in the β-subunit conformation that facilitate ATP binding, β-subunit phosphorylation, the binding of protein substrates and their phosphorylation. In the basal state, the activation loop of the IR is situated within the kinase active site, effectively limiting access of ATP and protein substrates to their respective binding sites (Fig. 3a) [26]. Phosphorylation of tyrosines 1158, 1162 and 1163 within the activation loop destabilize this conformation, resulting in a shift of the activation loop that allows access to the ATP- and substrate-binding sites (Fig. 3b) [26]. The fully phosphorylated activation loop is stabilized in a position that allows full ATP and substrate access and full expression of IR enzymatic activity. Biochemical studies of the purified IR kinase support this structural model. Phosphorylation of the kinase domain increases the affinity for ATP and substrate [27], and studies of the highly homologous IGF-1R kinase domain demonstrate a dose-dependent change in substrate affinity in going from the unphosphorylated to the mono-, bis- and tris-phosphorylated state [27].

Mechanisms of IR substrate tyrosine phosphorylation

The essential requirement of IR tyrosine kinase activity for the cellular insulin response has been demonstrated in a variety of IR mutant cell lines with varying capacities of IR tyrosine kinase activity [28]. In contrast to tyrosines in the activation loop, mutation of tyrosine 972 has a more direct effect on IR substrate phosphorylation. Tyrosine 972 serves as a

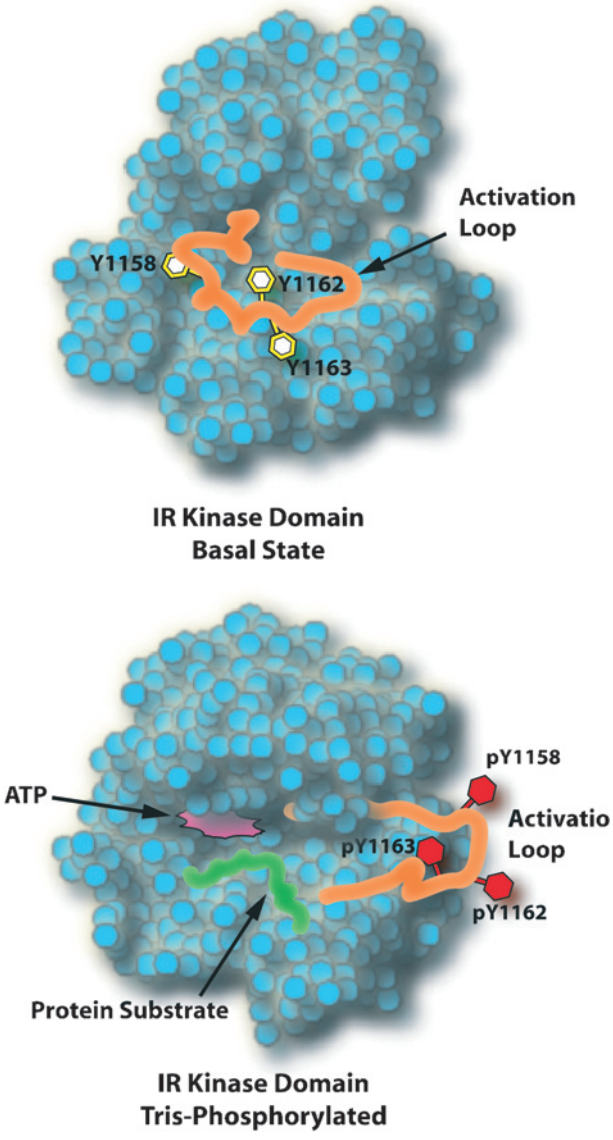

Figure 3. Structural component of IR kinase activation. X-ray crystallography studies of the IR kinase domain indicate that the natural conformation of this peptide is a globular structure, rather than the linear representation typically employed in schematic diagrams. In the basal state (*a*), the activation loop of the kinase domain overlies the critical sites for enzymatic activity. In this conformation, tyrosine 1162 sits in the active site, blocking access to protein substrates, and the proximal end of the activation loop interferes with ATP binding. Autophosphorylation of tyrosines 1158, 1162, and 1163 (*b*) stabilizes the kinase domain in an active state in which the activation loop is shifted out of the enzyme active site, allowing access to substrate- and ATP-binding sites. (Adapted from [154]).

binding site for the phosphotyrosine-binding (PTB) domains of IRS-1, as well as IR substrates Shc and STAT5B, facilitating the phosphorylation of these substrates on numerous tyrosine moieties (Fig. 2) [29–32]. The other primary IRS protein contributing to insulin signaling, IRS-2, is not dependent on tyrosine 972 for phosphorylation by the IR [29] and likely interacts with the phosphotyrosines of the activation loop. This suggests that specific blockade of tyrosine 972 phosphorylation could impact only on IRS-1-mediated signaling. Other modifications of the IR or interactions with separate proteins that interfere with interactions between IR and its substrates can modify IR signal transmission despite normal autophosphorylation (see below).

IR function in insulin resistance

Insulin target tissues such as muscle and fat display a decreased capacity for activation of the insulin signaling pathway in human and animal models of insulin resistance (for review see [10]). Reduced insulin-stimulated IRS-1 phosphorylation has been reported in the majority of insulin-resistant models [10]. However, tyrosine phosphorylation of IRS-1 can be affected by post-translational modification of IRS-1 distinct from alterations in IR function, making this a poor readout of IR tyrosine kinase activity. Insulin stimulation of IR autophosphorylation is therefore often employed as a readout of IR signaling capacity within the cell. When tissues from insulin-resistant humans or animals have shown decreased IRS-1 tyrosine phosphorylation without concurrent reductions in IR autophosphorylation, investigators have concluded that IR function is normal in these models, and that impaired insulin signaling can be attributed to the well documented alterations in IRS-1 substrate potential that can be induced by, for example, serine phosphorylation [33]. However, since IR kinase activity can be uncoupled from the autophosphorylation response via mechanisms detailed below, the IR may still be the primary site of impaired insulin signal transduction even when IR tyrosine phosphorylation appears normal. In fact, impaired activation of muscle IR has been reported in nearly every model of human and animal insulin resistance. While there are often discrepant findings on IR function by different investigators studying similar models of insulin resistance, this review will focus on research that supports the role of the IR as a regulator of insulin sensitivity.

Type 2 diabetes is the primary disease state associated with insulin resistance. In patients with type 2 diabetes IR autophosphorylation is reduced in muscle biopsies obtained following *in vivo* insulin infusion [34–37]. The tyrosine kinase activity of isolated muscle IR stimulated with insulin *in vitro* is also reduced in these subjects [38–42]. The tyrosine kinase activity of isolated muscle IR is also reduced with gestational diabetes [43]. Altered insulin signaling in type 2 diabetes is difficult to interpret, however, as it does not represent a specific form of insulin resistance. Insulin

resistance in this disease results from a variety of factors, typically obesity, but can also be exacerbated by the detrimental effects of hyperglycemia (see below).

Obesity in the absence of diabetes can also be accompanied by impaired IR autophosphorylation and tyrosine kinase activity in muscle. The tyrosine kinase activity of IR isolated from muscle and simulated *in vitro* is reduced in obese versus controls [38, 39] and in obese insulin-resistant patients with polycystic ovary syndrome (PCOS) [44]. In obese subjects, IR autophosphorylation is reduced in isolated muscle strips incubated with insulin [45, 46]. We observed that the autophosphorylation capacity of immunopurified muscle IR is negatively correlated with percent body fat [47]. This result suggests both that there is a dose effect of adiposity on muscle IR function, and that the effect of obesity on the IR autophosphorylation is maintained in isolated IR and therefore likely involves a modification of the IR protein.

Several dietary interventions known to induce insulin resistance in rodents have been shown to modulate IR function. A reduced insulin stimulation of IR autophosphorylation *in vivo* has been reported following administration of diets high in fat [48, 49]. Diets high in fructose also have been shown to impair IR autophosphorylation [50]. We demonstrated that a diet high in fat and refined sugar (HFS) diminished *in vitro* stimulation of IR autophosphorylation, [51], again suggestive of a post-translational modification of the IR. That the HFS diet induced IR alterations prior to an increase in total body fat stores indicates that insulin resistance in diet-induced obesity is due at least in part to effects of the diet alone.

Physical activity improves insulin sensitivity, and a cessation of regular exercise habits leads to a rapid decline in muscle insulin action [4, 52]. Chronic physical activity increases the signaling capacity of the IR in muscle of humans and rodents, and IR function rapidly declines with a reduction of physical activity or with muscle disuse. We observed that participation in a moderately intense exercise training program for seven days enhanced insulin stimulation of muscle IR autophosphorylation *in vitro* [53]. In rodents, there are reports that IR function is increased by standard exercise training regimens [54, 55], and through spontaneous physical activity induced by providing access to a running wheel [56]. In the latter case, when physical activity is curtailed by locking the running wheel, muscle IR autophosphorylation returns to control levels within approximately 2 days [56]. In more severe models of reduced muscle activity, IR function is dramatically impaired following limb immobilization [57] or muscle denervation [58, 59].

Despite the fact that the insulin resistance underlying the development of diabetes is often due to these lifestyle factors rather than genetic influences, taken as a group, the offspring of patients with type 2 diabetes are insulin-resistant compared to the average population even if lean and normoglycemic [60]. Muscle IR autophosphorylation and tyrosine kinase activity is reduced in the lean offspring of diabetic parents [61, 62]. In addition, we have observed that in some insulin-resistant subjects, impaired IR autophosphorylation may be an intrinsic component of muscle tissue that persists in muscle cells in culture [63].

Together, the studies demonstrating impaired IR autophosphorylation in insulin-resistant subjects suggest that impairments in IR signaling capacity can be both acquired and inherited, and that IR function is therefore a labile and regulated parameter. This idea is well supported by studies *in vitro* that demonstrate alterations in IR function induced by activating specific biochemical pathways or by modifying the expression of proteins that interact with the IR.

Mechanisms of Inhibition of IR autophosphorylation

Several mechanisms have been described that could account for the reduction in insulin-stimulated IR autophosphorylation observed in tissues from the various models of insulin resistance described above. Within the cell, two proteins have been demonstrated to produce a reduced level of tyrosine phosphorylation by inhibiting activation of the IR or by dephosphorylating the activated IR.

ENPP1/PC-1

Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1 also referred to as Plasma cell membrane glycoprotein 1 or PC-1) has been identified as a direct inhibitor of IR function. Although the full mechanisms whereby this plasma membrane protein inhibits the IR remain to be elucidated, the available evidence suggests that a direct interaction between PC-1 and the IR blocks autophosphorylation. PC-1 has been demonstrated to bind to amino acids 485–599 of the IR connecting domain [64], a region required for the conformational change in the IR β-subunits in response to ligand binding that permits autophosphorylation. Binding of PC-1 to the connecting domain presumably interferes with the movement of the IR that allows close opposition of the two β-subunits and trans-phosphorylation as described by Ottensmeyer and co-workers [25] (Fig. 4a). Overexpression of PC-1

in cells and tissues results in impaired IR autophosphorylation and insulin resistance [64–68].

Two lines of evidence suggest a role for PC-1 in inherited or genetic forms of insulin resistance. First, PC-1 mRNA transcription and protein levels are dramatically upregulated in cells from some individuals. The initial identification of PC-1 as an IR inhibitor came from studies of fibroblasts derived from a patient with type 2 diabetes whose cells expressed this protein at levels at least ten-fold higher than fibroblasts from controls [67], and from the identification of other insulin-resistant individuals whose cells overexpress PC-1 in culture. Second, analysis of PC-1 gene polymorphisms in a variety of individuals and family cohorts suggests that alterations in this gene are associated with the risk for development of childhood and adult obesity as well as type 2 diabetes [69]. A polymorphism in exon 4 of the human PC-1 gene that produces an amino acid change in codon 121 from lysine to glutamine (K121Q) has received the majority of attention to date.

Studies on allele frequencies suggest that the expression of the Q allele of the PC-1 gene significantly contributes to genetic insulin resistance in some populations. Carriers of the Q allele of the PC-1 gene demonstrate a 25% increase in the risk for developing type 2 diabetes with an earlier onset of disease as well [70, 71]. When expressed in cells, the Q allele variant of PC-1 displays a stronger association with the IR, and an increased capacity for IR inhibition [65], although it is not yet clear what the full physiological impact of expressing the Q allele might be. In addition, a three-allele "at risk" haplotype has been identified involving the Q allele and two other polymorphisms in the PC-1 gene [69]. While each polymorphism is associated with obesity risk, only the Q allele independently confers risk for type 2 diabetes. The mechanisms whereby the presence of the additional polymorphisms further increase risk for diabetes is not clear, as the functional consequences of these polymorphisms that map to untranslated regions of the PC-1 gene have not been studied.

In addition to genetic transmission of insulin resistance, PC-1 overexpression may also contribute to insulin resistance acquired secondarily to obesity. Through mechanisms that have not been elucidated, tissue content of PC-1 increases with the development of obesity in humans and rhesus monkeys [72, 73]. However, muscle content of PC-1 does not appear to be a highly labile parameter, and is not altered in many models of short-term regulation of insulin action. Improved insulin sensitivity resulting from dramatic weight loss induced by bariatric surgery is not associated with altered PC-1 content in muscle [74]. Similarly, there is no change in muscle PC-1 content

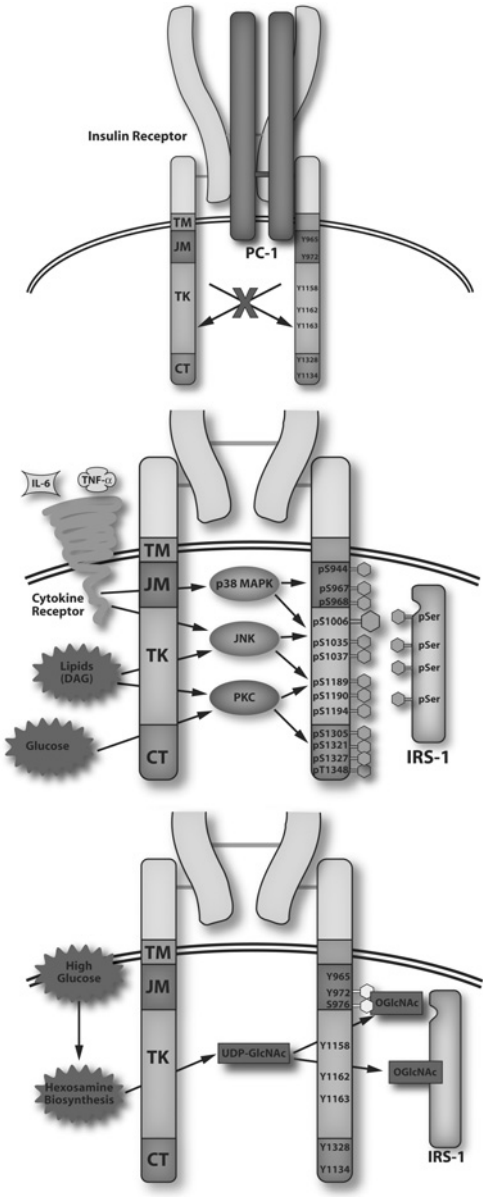

Figure 4. Models for inhibitors of insulin receptor autophosphorylation. (*a*) Membrane glycoprotein PC-1 has been shown to directly bind to the connecting domain of the IR α-subunit. With the model of IR activation proposed by Ottensmeyer and Yip [25], binding of PC-1 to this region could block the conformational change induced by ligand binding that brings the opposing β-subunits together to allow for trans-phosphorylation and activation. (*b*) Multiple serine and threonine phosphorylation sites have been demonstrated or hypothesized on the IR β-subunit, as well as IRS-1. Serine and threonine phosphorylation of the IR results from activation of PKC enzymes due to accumulation of lipid intermediates or high levels of glucose. JNK and p38 MAPK are also activated by lipid accumulation and can interfere with insulin signaling, although direct effects on the IR have not been demonstrated. It is likely that structural alterations resulting from these phosphorylations interfere with conformational changes and/or tyrosine phosphorylation of the IR. (*c*) Serine 976 is a putative site for addition of an O-linked glycosylation with the substrate UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc levels are elevated by increased flux through the hexosamine pathway resulting from hyperglycemia. Glycosylation on this site may interfere with IRS-1 binding to IR tyrosine 972 and subsequent phosphorylation.

when insulin sensitivity is reduced by high fat feeding [51] or improved by short-term exercise training [53], despite the alterations in IR function that accompany these interventions. Factors responsible for regulating expression of the PC-1 gene have not yet been identified.

Tyrosine phosphatases

Because the active conformation of the IR is maintained by the phosphorylation of the key regulatory tyrosines, dephosphorylation of the IR by protein tyrosine phosphatases (PTPs) is responsible for deactivation of IR kinase activity. Leukocyte common antigen-related phosphatases (LAR) and PTP-1B are specific PTPs that contribute to IR dephosphorylation in skeletal muscle and that have been hypothesized to produce the reduced levels of IR tyrosine phosphorylation observed in muscle from insulin-resistant subjects [75]. Evidence for the role of LAR and PTP-1B in downregulating IR function comes the demonstration that altered expression of these enzymes can alter insulin signal transduction [76].

PTP-1B directly associates with the IR following insulin stimulation in a process that is dependent on phosphotyrosines 1158, 1162 and 1163 of the IR [77]. Overexpression of PTP-1B decreases IR autophosphorylation in cultured muscle cells and adipocytes [78, 79]. However, only in muscle cells is PTP-1B overexpression associated with reduced cellular insulin action [78, 79]. *In vivo*, tissue insulin sensitivity and IR autophosphorylation are enhanced in mice heterozygous or homozygous for a knockout of PTP-1B [80]. Correspondingly, overexpression of PTP-1B in muscle induces tissue insulin resistance in mice concurrently with a decrement in the capacity for IR autophosphorylation [81].

However, the data supporting an altered expression or activity of PTP-1B in insulin-resistant states is scant. PTP-1B expression is increased in muscle from the obese diabetic Goto-Kakizaki rats [82]. In humans, associations between insulin resistance and PTP-1B content have been demonstrated in adipose tissue [83]. PTP-1B is increased in muscle from obese subjects but reduced in obese, diabetic subjects [84]. Expression of PTP-1B is not altered by exercise protocols that impact IR signaling function [56]. The role of the membrane-bound PTP LAR on IR function is less clear. LAR directly interacts with the IR [85], and alteration in LAR expression in cells modifies IR signaling [86, 87]. However, there is no discernable defect in IR autophosphorylation in transgenic animals overexpressing LAR in skeletal muscle. The majority of evidence suggests a role for LAR in insulin signaling distinct from the IR, perhaps regulating IRS-2 phosphorylation [88, 89].

Overall, the cellular content of PTPs can undoubtedly regulate IR function, and there is evidence for increased PTP activity in muscle from insulin-resistant and diabetic subjects [90, 91]. However, definitive evidence of altered expression of IR-specific PTPs in muscle corresponding to changes in IR function is lacking. The contribution of altered PTP expression to human insulin resistance is unclear.

Inhibition of IR autophosphorylation by post-translational modification

While the IR-inhibiting proteins described above have been shown to modulate IR phosphorylation in cells and tissues, in many insulin-resistant states the capacity for autophosphorylation is reduced in IR isolated from the intracellular milieu and stimulated with insulin *in vitro*. Barring protein-protein interactions sufficiently robust to be maintained through the purification process, this suggests that some post-translational modification of the IR protein itself must regulate IR activity. Evidence exists for modulation of IR autophosphorylation by serine/threonine phosphorylation and by O-linked glycosylation.

Serine/threonine phosphorylation

IRS-1 serves as a model for the ability of serine/threonine phosphorylation to modulate cellular insulin signal transduction. Serine phosphorylation sites have been mapped on IRS-1 that inhibit the ability of IRS-1 to serve as a substrate of IR tyrosine kinase activity [12]. The modulation of IRS-1 function by serine/threonine phosphorylation results from the activation of multiple serine kinase pathways, such as the c-jun N-terminal kinase (JNK) [92]. Similarly, there is evidence that serine phosphorylation of the IR impairs the autophosphorylation response to insulin binding, and likely contributes to impaired insulin signaling related to obesity as well as that resulting from glucose toxicity.

In studies of cells or with isolated proteins, protein kinase C (PKC) -mediated phosphorylation of the IR has been demonstrated on serine residues 967 and 968 of the juxtamembrane region [93], serines 1006, 1035 and 1037 in the catalytic domain [94, 95], and serines 1288, 1305, 1306, 1321, 1327 and threonine 1348 in the C-terminus [93, 96–100] (Fig. 4b). It is not clear that each of these phosphorylation sites is involved in the regulation of IR autophosphorylation or substrate kinase activity [95, 101]. The down-

regulation of IR activity by PKCβ1, β2 or θ requires serines 1006, 1035 and 1037, as mutations of the IR at these residues abolishes the PKC effect [102, 103]. Several of these putative phosphorylation sites are in close proximity to autophosphorylation sites on the IR, or are within the catalytic domain, and could therefore affect IR conformation or access to key tyrosine residues.

An increased PKC activity is thought to play a significant role in several models of human insulin resistance (for reviews see [101, 104]). PKCβ activity is increased in muscle from obese insulin-resistant subjects [105]. Pharmacological inhibition of PKC activity can reverse the impaired insulin stimulation of glucose transport in muscle strips obtained from obese subjects [45]. Serine phosphorylation of the IR likely contributes to this effect as increased phosphorylation of the IR on serine 944 has been demonstrated in muscle and liver from obese insulin-resistant rodents [94, 106]. In humans, serine phosphorylation of muscle IR has been observed in muscle from PCOS patients [44]. In fibroblasts from PCOS patients, treatment of cells with either a general protein kinase inhibitor or an inhibitor of protein kinase A restored IR autophosphorylation capacity, although a PKC inhibitor was without effect [107]. Serine phosphorylation of the IR has been difficult to demonstrate in IR from human muscle in other models of insulin resistance. However, this may be due largely to methodological difficulties. In an indirect technique for demonstrating the contribution of serine phosphorylation to altered IR function, IR purified from muscle of obese insulin-resistant subjects and from patients with gestational diabetes have been treated with phosphatase enzymes to strip away any existing phosphorylation [43, 105]. This approach normalizes receptor tyrosine kinase activity in these models of IR dysfunction [43, 105]. The causative link between obesity and serine phosphorylation of the IR may involve more than one mechanism. Obesity could lead to activation of PKC due to an oversupply of fuel substrates (either an accumulation of lipid intermediates within the cell or elevated glucose levels), or by activation of stress kinase pathways by adipose-secreted cytokines. Obesity and other insulin-resistant states are associated with an accumulation of intramyocellular lipids (IMCL), and this lipid deposition increases the cellular levels of diacylglycerols (DAG), which are known to activate PKC enzymes [101, 108]. Lipid infusion in humans and rodents increases muscle DAG content and PKC activity coincident with the induction of tissue insulin resistance [109–111]. Lipid infusion in rats increases IR serine phosphorylation and reduces insulin-stimulated IR autophosphorylation in PKC-dependent manner [109]. JNK and MAP kinases are also activated by lipid accumulation and are associated with impaired insulin signaling (reviewed in [101]), although neither have been shown to directly inhibit IR activation. However, the decrement in IR autophosphorylation induced by lipid incubation in 3T3-L1 adipocytes is prevented by blocking JNK expression [112].

Serine phosphorylation of muscle IR in obesity may also occur independently of PKC due to circulating, rather than local factors. Circulating levels of the adipose-secreted cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are increased in obese subjects [113]. TNF-α produces cellular insulin resistance in part by increasing phosphorylation of IRS-1 on the key regulatory site serine 307 via activation of the serine kinases IKK-β, JNK, p38 MAPK and/or mTOR [12, 114]. In addition, in muscle cells, TNF-α incubation induces serine phosphorylation of the IR on undisclosed residues coincident with inhibition of insulin-stimulated IR autophosphorylation. This effect is mediated by p38 MAPK [114]. In cultured muscle cells, incubation with IL-6 results in phosphorylation of serine 318 on IRS-1 and a decrease in IR autophosphorylation [115], although it is not known whether the IR is directly serine-phosphorylated. JAK/STAT activation of MAPK pathways likely mediates the phosphorylation of IRS-1 on serine 318 by IL-6 incubation [116]. Hyperglycemia can also induce insulin resistance (a phenomenon referred to as glucotoxicity) in a manner that may be related to PKC-mediated IR serine phosphorylation [117]. Incubation of cells with high glucose levels produces a PKC-mediated inhibition of IR autophosphorylation [118]. The impaired IR function observed in tissues from patients with type 2 diabetes, and related animal models, could therefore result from the chronically elevated glucose levels. However, these effects would likely be layered upon any perturbations in IR signaling that contributed to the initial state of insulin resistance and preceded the progression to hyperglycemia.

O-linked glycosylation

Glucose toxicity may also lead to decreased IR autophosphorylation via another form of post-translational modification. It has long been recognized that flux of a surplus of glucose through the hexosamine biosynthetic pathway produces insulin resistance [8]. The end product of this pathway is UDP-N-acetylglucosamine (UDP-GlcNAc) that serves as a substrate for the enzyme O-linked N-acetylglucosamine transferase (OGT). OGT O-glycosylates proteins by adding a GlcNAc moiety to the hydroxyl groups of serine/

threonine residues. O-linked N-acetylglucosamine represents a post-translational modification known to modify the function of numerous proteins in a manner more similar to phosphorylation than simple glycosylation [119].

In pancreatic RIN β-cells, glucosamine induces O-glycosylation of both the IR and IRS-1 with a coinciding decrease in insulin-stimulated IR autophosphorylation [120]. Treatment of cells with an inhibitor of OGT reverses the decrement in IR function. Although the exact site of glycosylation has not been determined, one potential O-glycosylation site on the IR is at serine 976 adjacent to the key autophosphorylation site, tyrosine 972, on the juxtamembrane region. It is possible that O-glycosylation at this site would sterically inhibit phosphorylation of tyrosine 972 on the IR following insulin biding and thereby interfere with binding and phosphorylation of IR substrates (Fig. 4c). In support of this model, IR from glucosamine-treated rodent cells demonstrate a decrease in phosphorylated tyrosine 974 (equivalent on the rodent IR to tyrosine 972 on the human IR), although it is not clear whether this represented a specific effect of O-glycosylation on this residue, or whether autophosphorylation was diminished at other sites as well [120].

It is tempting, then, to suggest a role for O-GlcNAc modification of IR function to explain the impaired IR function associated with glucose toxicity. Elevated levels of O-GlcNAc have been observed in tissues from animals with experimental diabetes. However, O-GlcNAc-modified IR have not been observed *in vivo*. While muscle does express OGT mRNA, the levels are significantly lower than in the pancreas [121], where O-GlcNAc modification has been induced *in vitro*. Further studies are needed to determine the extent to which O-glycosylation of the IR occurs and contributes to insulin resistance.

Inhibitors of IR tyrosine kinase activity

As discussed, a decreased IR autophosphorylation will naturally impair substrate tyrosine kinase activity by reducing the stability of the active receptor conformation. Yet numerous studies indicate normal IR autophosphorylation concurrent with reduced IRS-1 phosphorylation in tissues stimulated with insulin. As described previously, modification of IRS-1 can interfere with its ability to serve as a substrate independent of IR function. However, protein inhibitors of the IR have also been identified that can impair the substrate kinase activity of the IR independent of receptor autophosphorylation and activation.

Grb adaptor proteins

The activity of the IR, as with other growth factor receptors, appears to be regulated by members of the growth factor receptor bound-7 (Grb7) family of adaptor proteins. The structure, function, and regulation of the highly homologous Grb10 and Grb14, as well as their regulation of insulin action, have recently been thoroughly reviewed [122].

Interaction of Grb14 with the IR provides a mechanism that uncouples the activation/phosphorylation state of the IR from its substrate tyrosine kinase activity. The effects of Grb14 on IR signaling have been demonstrated in transfected cell lines and in knockout mice. In cells, Grb14 inhibits insulin-stimulated phosphorylation of IRS-1 and the biological effects of insulin [123–125] even though some investigators reported an increase in IR autophosphorylation [123]. This discrepancy results from a direct, insulin-mediated interaction between the IR and Grb14 that has been demonstrated by co-immunoprecipitation [123] and bioluminescence resonance energy transfer (BRET) [126]. This interaction impairs the association between the IR and PTP-1B in a manner that protects the phosphotyrosine residues in the kinase domain activation loop (tyrosines 1158, 1162, 1163) from dephosphorylation, but leads to enhanced dephosphorylation of tyrosine 972 [127] (Fig. 5a). The reduction in tyrosine 972 phosphorylation would explain the reduced IRS-1 binding [127] and phosphorylation [124], as well as the diminished cellular action [125] observed in cells overexpressing Grb14.

Consequently, Grb14 knockout mice have increased insulin sensitivity and enhanced glucose tolerance [128]. Examination of insulin signaling in tissues of these mice confirms the model developed by the *in vitro* and cell studies. In liver, the absence of Grb14 leads to a decrease in IR autophosphorylation but an increase in IRS-1 phosphorylation [128]. These results suggest that not only does Grb14 protect activation loop tyrosine phosphorylation, but that this effect occurs at physiological levels of Grb14 expression, at least in liver, and is not dependent on Grb14 overexpression. This effect is likely tissue-specific, as insulin signaling and action is enhanced in muscle but not adipose tissue from $Grb14^{-/-}$ mice [128].

Grb10 has demonstrated actions very similar to Grb14. Grb10 also binds to phosphotyrosine residues on the IR and IGF-1R kinase domain via SH2 and BPS (between PH and SH2) domains [122]. The possibility that Grb10 has a different function than Grb14 comes from studies indicating that Grb10 overexpression in cells potentiates insulin action and

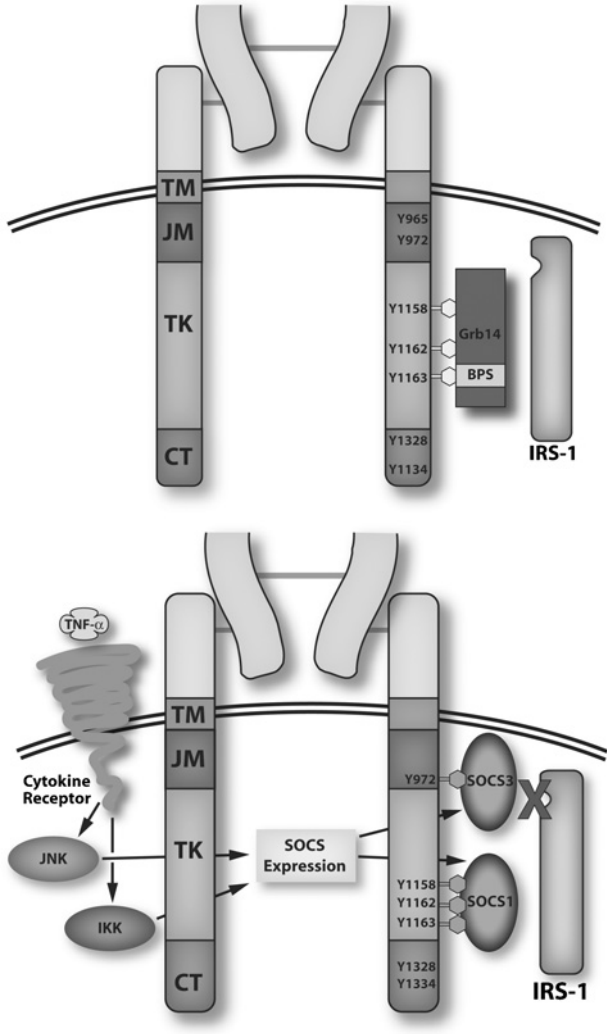

Figure 5. Models for inhibition of insulin receptor substrate tyrosine kinase activity. The BPS domain of Grb14 interacts directly with phosphorylated tyrosine 1163 on the IR (*a*). This interaction protects the tyrosines in the activation loop from dephosphorylation, but promotes dephosphorylation of tyrosine 972. This results in decreased interaction with, and phosphorylation of, IRS-1. Overexpression of Grb14 therefore results in a paradoxically hyperphosphorylated IR concurrent with decreased IRS-1 phosphorylation and insulin resistance. The SOCS proteins -1 and -3 bind to phosphotyrosine residues on the activated IR. SOCS-3 interacts with tyrosine 972, which would then interfere with association between the IR and IRS-1, while SOCS-1 interacts with the phosphotyrosines in the activation loop, which would then interfere with IRS-2 binding (*b*). As such, these compounds do not block IR activation, but have been shown to decrease phosphorylation of IRS proteins and downstream signal transduction in response to upregulation by cytokines such as TNF-α.

that dominant-negative Grb10 fragment peptides inhibit the biological effects of insulin in cells [129, 130]. However, these findings are controversial [122], and most studies in cells and *in vitro* demonstrate that, as with Grb14, interaction of Grb10 with the IR inhibits the substrate kinase activity of the receptor, and reduces insulin action [131–134]. Grb10 has also been implicated in mediating insulin-stimulated IR degradation [135]. However, several studies have not found any effect of Grb10 expression on IR content in cells [133, 136]. Knockout mice lacking Grb10 show increased growth rates, likely the result of enhanced IGF-1R signaling [137], and transgenic mice overexpressing Grb10 in a variety of tissues demonstrate growth retardation and showed signs of increased insulin resistance and susceptibility to diabetes [138]. While modulation of Grb10 and Grb14 expression can influence insulin action in tissues, it is not clear whether these adaptor proteins play a role in human insulin resistance. The increased IR autophosphorylation that has been reported for Grb14 overexpression [123] would be uncharacteristic for insulin resistance *in vivo*, but a reduction in IRS-1 phosphorylation concurrent with normal IR phosphorylation is a common finding in tissues from insulin-resistant humans or animals. There is evidence that Grb14 levels are increased in some animal models of diabetes, the ob/ob mouse and the non-obese KK (Goto-Kakizaki) rat [139]. However, the overexpression appears to be limited to adipose tissue, and not skeletal muscle or liver, sites of impaired IR function. While there is some evidence that insulin regulates the expression of Grb14 in adipocytes [139], there is no evidence to date for regulatory mechanisms that would explain alterations in Grb10 or Grb 14 expression or activity playing a primary role in the development of insulin resistance.

SOCS proteins

Two related proteins have been identified that bind to the activated IR and interfere with the phosphorylation of IR substrates without blocking receptor autophosphorylation. Suppressor of cytokine signaling (SOCS) proteins SOCS-1 and SOCS-3 are two of a family of eight proteins that are thought to regulate cellular response to cytokines in a negative feedback manner [140]. Studies have indicated that SOCS-1, SOCS-3 and SOCS-6 can bind to the IR in cells [141, 142]. SOCS-3 binds to phosphorylated tyrosine 972, whereas SOCS-1 interacts with the phosphorylated form of the C-terminus, containing tyrosines 1158, 1162, and 1163. These sites are essential for interaction of the IR with IRS-2 (Fig. 5b). Overexpression of SOCS-1 and SOCS-3 in cells results in decreased insulin-stimulated phosphorylation of IRS-1 and IRS-2 [142, 143]. These effects likely result from SOCS-1 or -3 directly blocking the ability of IRS proteins to interact with the IR.

As expression of SOCS proteins are increased by cytokines [140], SOCS-1 and SOCS-3 are obvious

candidates to mediate the insulin resistance involved in obesity and other conditions associated with a heightened inflammatory state. Treatment of muscle cells with TNF-α increases SOCS-1 and -3 expression [143], and SOCS-1 and SOCS-3 content is elevated in liver from obese insulin-resistant mice [144]. Knockdown of SOCS expression in adipocytes prevents much of the impaired phosphorylation of IRS-1 and IRS-2 induced by TNF-α [143]. Antisense-mediated knockdown of liver SOCS-1 or -3 expression reverses insulin resistance in obese diabetic mice [144], strongly supporting a role for SOCS proteins in obesity-related insulin resistance. Because SOCS proteins act to block the kinase activity of phosphorylated IR, the impairments in IR autophosphorylation observed in obesity and resulting from TNF-α treatment would necessarily result from other mechanisms.

Regulation of IR kinase activity by differential gene processing

Alternate splicing of the IR gene has been described as a potential mechanism whereby IR kinase activity can be modified. Slight differences in the tyrosine kinase activity of the A and B isoforms of the IR have been reported, despite the fact that their β-subunits are identical [145]. The A isoform of the IR, called IR-A or the fetal IR, lacks the 12-amino acid sequence coded for by exon 11. The IR-A is largely expressed during early development. The primary functional consequence of the absence of amino acids 717–729 is a change in the relative binding affinity for insulin versus the insulin-like growth factors [146]. The increased responsiveness of IR-A to IGF-I and IGF-II accounts for the role of this isoform in growth, and the potential for IR-A to contribute significantly to the development and proliferation of certain cancers. Along with small differences in the receptor's enzymatic activity, the low prevalence of the A isoform in tissues of the adult human make alternative splicing unlikely to contribute to insulin resistance in humans.

Pharmacologic regulation of IR function

Several small molecules have been developed that can reverse insulin resistance by interacting with the IR and enhancing IR signaling. One of these compounds, referred to as DMAQ-B1 was identified by Zhang and co-workers [147] as a naturally occurring benzoquinone that acts as an insulin mimetic. This compound has been shown to improve insulin sensitivity and lower blood glucose levels in a variety of animal models of diabetes and insulin resistance [147, 148]. Initial studies on the mechanisms of DMAQ-B1 on IR function suggested that the compound bound directly to the β-subunit, possibly in the ATP-binding domain [147]. How this interaction could induce a conformational change required to promote autophosphorylation and substrate kinase activity in the absence of insulin is not clear.

In contrast, the compound TLK16998, described by Manchem and co-workers [149] functions as an IR-sensitizing compound, as it can increase insulin-stimulated IR autophosphorylation but does not stimulate the receptor in the absence of insulin. In cells stimulated with insulin, TLK16998 increases both tris-phosphorylation of the activation loop and bis-phosphorylation of the C-terminus, while the phosphorylation of the juxtamembrane region of the IR is decreased [150]. TLK16998 had no effect on IR mutants lacking the connecting domain region of the α-subunit [150], but potentiated the kinase activity of peptides representing the active enzymatic domain of the IR [151]. The connecting domain IR mutants are unable to adopt an active conformation following ligand binding that would allow autophosphorylation to occur, while the kinase domain peptides are intrinsically in an active conformation in the absence of auto-inhibition by the α-subunits. Therefore, TLK16998 can directly stimulate the substrate phosphorylation capacity of the kinase domain of the IR, but apparently can do so only when this peptide is in an active conformation. In contrast DMAQ-B1, can activate the connecting domain IR mutant [150], suggesting an ability to induce an active conformation of the β-subunits.

We have explored the ability of TLK16998 to overcome specific defects in IR function in cultured cells [150]. Insulin stimulation of IR autophosphorylation was reduced by 50 % and 61 % in cells treated with TNF-α and the phorbol ester TPA, presumably due to serine phosphorylation of the receptor. Incubation with TLK16998 normalized the extent of insulin-stimulated IR autophosphorylation in cells treated with these compounds to that of control cells. In contrast, TLK16998 was not able to overcome inhibition of the IR induced by overexpression of ENPP1 [150]. This result is consistent with ENPP1 blocking the conformational change of the IR mediated by the connecting domain that activates the IR, and with the requirement of TLK16998 for an activated IR.

Single-dose administration of TLK16998 lowers blood glucose levels in two models of insulin-resistant diabetes, the obese db/db mouse and the high fat-fed, streptozotocin-treated mouse [149]. In addition, a single dose of TLK16998 in rats ameliorated the impairment in oral glucose tolerance induced by treatment with the protease inhibitor indinavir [152].

Together with data showing that drugs that inhibit IR and IGF-1R signaling raise blood glucose levels and impair glucose tolerance [153], these results demonstrate that modulation of IR sensivity to insulin (up or down) significantly impacts whole body glucose homeostasis.

Summary

An impaired capacity for IR autophosphorylation and/or substrate tyrosine kinase activity is a common finding in skeletal muscle from human and rodent models of insulin resistance. Importantly, although often considered to be equivalent readouts for IR signaling, autophosphorylation and substrate kinase activity are separate processes that are regulated by distinct mechanisms. Studies of cultured cells and transgenic animals have elucidated in detail these mechanisms whereby IR function can be up- or downregulated due to the actions of proteins that bind to or otherwise modify the IR. Some modifications, such as serine phosphorylation, can persist when IR are purified from the cellular environment and can be reversed *in vitro* with a corresponding increase in IR function. Modification of IR function in cells or *in vivo*, either by altered metabolism, genetic manipulation of protein expression, or even pharmacological targeting of the IR produces corresponding changes in cellular, tissue or whole-body insulin action. Thus, modulation of IR signaling must be considered a major means whereby insulin sensitivity is regulated over the short- and long-term.

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