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The Insulin Receptor and Its Cellular Targets*

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ABSTRACT

The pleiotropic actions of insulin are mediated by a single receptor tyrosine kinase. Structure/function relationships of the insulin receptor have been conclusively established, and the early steps of insulin signaling are known in some detail. A generally accepted paradigm is that insulin receptors, acting through insulin receptor substrates, stimulate the lipid kinase activity of phosphatidylinositol 3-kinase. The rapid rise in Tris-phosphorylated inositol (PIP_3) that ensues triggers a cascade of PIP_3-dependent serine/threonine kinases. Among the latter, Akt (a product of the *akt* protooncogene) and atypical protein kinase C isoforms are thought to be involved in insulin regulation of glucose transport and oxidation; glycogen, lipid, and protein synthesis; and modulation of gene expression. The presence of multiple insulin-regulated, PIP_3-dependent kinases is consistent with the possibility that different pathways are required to regulate different biological actions of insulin. Additional work remains to be performed to understand the distal components of insulin signaling. Moreover, there exists substantial evidence for insulin receptor substrate- and/or phosphatidylinositol 3-kinase-independent pathways of insulin action. The ultimate goal of these investigations is to provide clues to the pathogenesis and treatment of the insulin resistant state that is characteristic of type 2 diabetes. (*J Clin Endocrinol Metab* **86:** 972–979, 2001)

THIS YEAR MARKS the 50th anniversary of the seminal paper in which Levine and co-workers reported that insulin's effect on glucose utilization was mediated by increased membrane permeability to glucose (1). Twenty years later, Roth and colleagues discovered the insulin receptor (2), thus ushering in a new era of investigations that led to determination of the molecular basis of insulin action. Activation of the insulin receptor triggers complex biochemical reactions required for insulin's biological effects. However, a detailed road map of insulin receptor signaling is, with some noticeable exceptions, not available. Indeed, even in those instances where a defined chain of events from the receptor to its effector(s) has been established, such molecular mechanisms do not provide a complete explanation of the biological actions of insulin. Thus, despite enormous strides in understanding the elusive mechanism by which insulin regulates fuel homeostasis and growth, numerous questions remain unanswered.

Insulin receptor 1971–2000

The insulin receptor is necessary and sufficient to mediate insulin action. Humans and mice lacking insulin receptors are born at term, but do not survive long, suggesting that insulin receptors are essential for postnatal growth and fuel metabolism, but are not required for fetal metabolism (3, 4). Since the landmark paper describing specific insulin binding to rat liver membranes (2), structure/function relationships of the insulin receptor have been conclusively established using numerous approaches. These include site-directed mutagenesis (5) and a host of naturally occurring mutations identified in patients with genetic syndromes of extreme insulin resistance (6). In recent years, designer mice bearing constitutive or conditional null alleles of the insulin receptor have provided substantial insight into its *in vivo* function (4, 7–11). Moreover, determination of the crystal structure of the receptor's kinase domain has provided a mechanistic link between insulin binding and receptor activation (12, 13). The molecular basis of ligand binding to the receptor and its unique kinetic properties [negative cooperativity (14)] has proven more difficult to tackle due to the difficulty of crystallizing the carbohydrate-rich ectodomain.

The complete insulin receptor is a heterotetrameric membrane glycoprotein composed of two α- and two β-subunits, linked together by disulfide bonds (Fig. 1). Insulin binds to the receptor's extracellular α-subunit. Insulin binding presumably brings the two α-subunits closer together. This conformational change enables ATP binding to the β-subunit's intracellular domain. ATP binding activates receptor autophosphorylation (12, 13), which, in turn, enables the receptor's kinase activity toward intracellular protein substrates. There are numerous autophosphorylation sites in the β-subunit's intracellular domain. Three main clusters have been recognized to play a functionally important role. They include Y^{1158}, Y^{1160}, and Y^{1162} in the active loop of the catalytic domain, Y^{972} in the juxtamembrane domain, and Y^{1328} and Y^{1334} in the carboxyl-terminal domain. Phosphorylation of residues in the active loop is essential to promote the receptor's kinase activity. The carboxyl-terminal phosphorylation sites may play a role in the receptor's mitogenic

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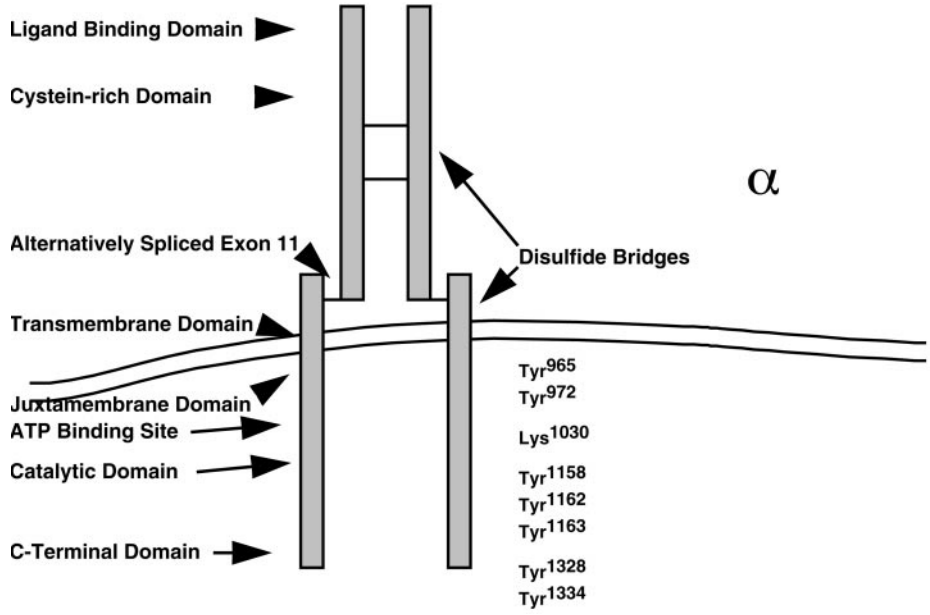

FIG. 1. Subunit structure of the insulin receptor. Schematic diagram of the insulin receptor subunit organization and major structural features. The insulin receptor is the product of a single copy gene located on chromosome 19. It is translated from messenger ribonucleic acid as a single chain polypeptide precursor, which undergoes posttranslational cleavage, followed by dimerization and export to the plasma membrane. The insulin-binding domain is localized to the N-terminus of the α-subunit. The β-subunit intracellular domain contains the tyrosine-specific protein kinase activity. Insulin binding to the extracellular domain causes a conformational modification in the intracellular domain, such that the receptor undergoes autophosphorylation and can bind ATP. Several tyrosine residues are phosphorylated in the receptor's juxtamembrane domain (Y^{965} and Y^{972}), catalytic loop (Y^{1158}, Y^{1162}, and Y^{1163}), and carboxyl-terminal domain (Y^{1328} and Y^{1334}). The variably spliced exon 11 is indicated at the COOH-terminus of the α-subunit.

activity. The juxtamembrane autophosphorylation site plays an important role in the interaction between the receptor and its intracellular substrates, providing a docking site to increase the stability of the receptor/substrate complex (5).

In addition to binding insulin, the insulin receptor can bind insulin-like growth factors (IGF-I and IGF-II). The affinity of IGF-I binding to the insulin receptor is in the high nanomolar range, approximately 100- to 1000-fold lower than insulin's affinity (15). However, as circulating IGF-I levels are approximately 100-fold higher than those of insulin, the potential exists for IGF-I binding and acting through the insulin receptor. Strongly supportive evidence to this effect comes from the observation that the growth of mice lacking both IGF-I receptor and IGF-II receptor is rescued by insulin receptors, presumably in response to IGF-I binding (16).

IGF-II binds with equal affinity to both IGF-I receptor and insulin receptor. In rodents, during embryonic growth, IGF-II binds to the insulin receptor to promote growth (17–19). The molecular basis for high affinity IGF-II binding to the insulin receptor has been proposed to reside within the alternatively spliced exon 11 of the insulin receptor gene. The insulin receptor is expressed as two isoforms, resulting from alternative splicing of exon 11. Isoform B contains a 12-amino acid peptide located at the carboxyl-terminal end of the receptor's α-subunit. Isoform A lacks this insertion. Frasca and colleagues have suggested that splicing of exon 11 to yield isoform A bestows on the insulin receptor the ability to bind IGF-II with high affinity (20).

Insulin receptor substrates (IRSs)

IRSs represent key elements in insulin and IGF actions (21). Insulin, IGF-I, and certain cytokine receptors phosphorylate IRSs at specific Y-x-x-M motifs. These motifs serve as molecular adhesives. Phosphorylation of their tyrosine residues increases the affinity with which IRS proteins bind other signaling molecules. Each tyrosine-phosphorylated motif binds to a specific signaling molecule. In this way, protein-protein complexes are formed, and various signaling pathways are engaged, providing a potential explanation for the diversity of insulin signaling (22) (Fig. 2).

Different roles of IRS proteins

The IRS family is composed of four closely related members (IRS-1 to -4) (23–26) and a more distantly related homolog, Gab-1 (27). Genetic ablation studies in mice have conclusively shown substantial differences in the abilities of various IRSs to mediate insulin action. Ablation of IRS-1 causes severe growth retardation with mild insulin resistance (28, 29), suggesting an important role of IRS-1 in both insulin and IGF actions. In contrast, ablation of IRS-2 causes combined insulin resistance in peripheral tissues and impaired growth of pancreatic β-cells (30). The findings in the IRS-2-deficient mouse recapitulate the natural history of type 2 diabetes and have led to the suggestion that IRS-2 is a diabetes-predisposing gene, a conclusion that is not borne out by genetic analyses carried out to date (31–33). Ablation of IRS-3 is devoid of a clear phenotype (34), whereas ablation of IRS-4 is associated with modest growth retardation and

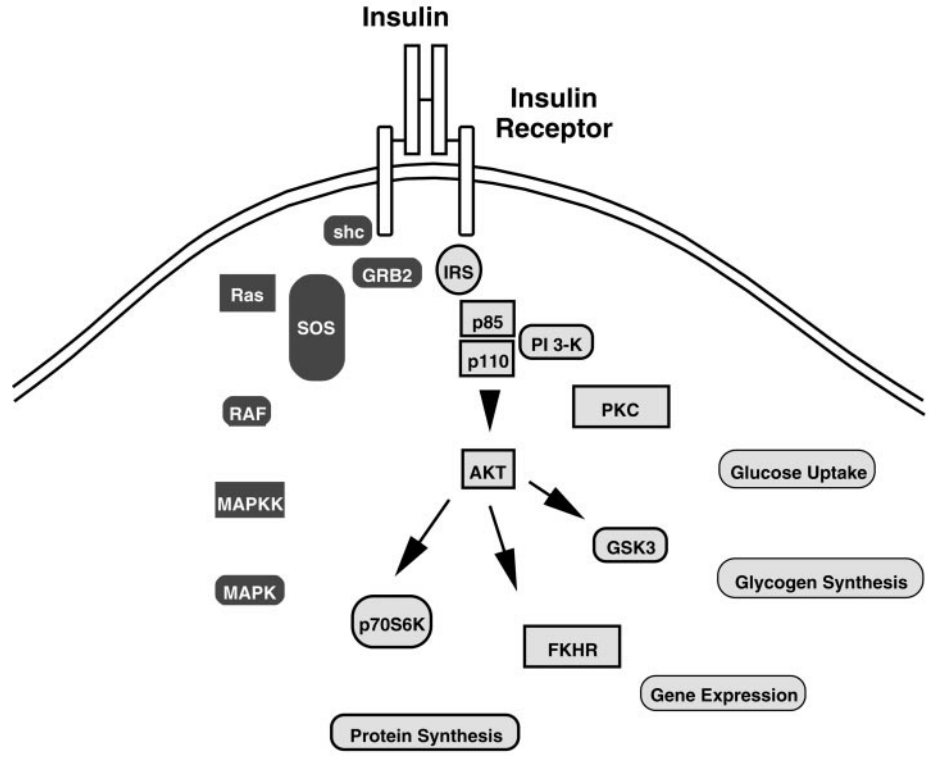

FIG. 2. Insulin signaling pathways. The diversity of insulin action can potentially be explained by the activation of multiple signaling pathways. The pathways emanating from activation of IRS proteins are described. The IRS/PI 3-K pathway leads to the generation of PIP_3 and the consequent activation of PIP_3-dependent kinases. The Ras/mitogen-activated protein kinase kinase pathway can be activated by insulin through the formation of complexes between the exchange factors SOS and growth factor receptor binding protein 2 (GRB2) and may play a role in certain tissues to stimulate the actions of insulin on growth and proliferation. SOS, son-of-sevenless; GAP, GTPase-associated protein; PDK, PI-dependent protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; GSK3, glycogen synthase kinase 3.

insulin resistance (35). Finally, inactivation of Gab-1 has an embryonic lethal phenotype that suggests a role in hepatic growth factor, rather than insulin, signaling (36).

As IRS-1 and IRS-2 have widely overlapping tissue distribution, these finding are consistent with the possibility that each molecule subserves a specific set of functions. We and others have proposed that IRS-1 is the main IRS in skeletal muscle, whereas IRS-2 is the main signaling molecule in liver (9, 30, 37).

An emerging area of investigation is the role of IRS proteins in β-cells. Ablation of IRS-2 is associated with impaired β-cell growth (30, 38). In contrast, lack of IRS-1 is associated with impaired coupling of glucose sensing to insulin secretion, suggesting that IRS-1 signaling is important for β-cell function (39). Interestingly, ablation of insulin receptors in β-cells results in abnormalities similar to those seen in IRS-1 knockout mice, whereas lack of IGF-I receptors increases the severity of the IRS-2 knockout phenotype. These findings have lead to the suggestion that insulin and IGF-I receptor signaling play physiological roles in β-cell function (10, 38, 40).

Are all the actions of insulin mediated through IRS proteins?

To address this question, it is interesting to compare the phenotypes of insulin receptor- and IRS-deficient mice. In every case, ablation of IRS proteins is associated with a much milder phenotype than lack of insulin receptors. Even the lack of IRS-2 does not have such a rapidly lethal effect as the lack of insulin receptors. Moreover, lack of IRS-2 is associated with a specific β-cell defect and does not lead to extreme insulin resistance. Even after three of four IRS-1 and IRS-2 alleles have been ablated (for example, in *Irs-1*$^{-/-}$*Irs-2*$^{+/-}$ mice), insulin resistance in newborn mice is not nearly as severe as that in insulin receptor knockout mice (38). On the other hand, mice lacking both IRS-1 and IRS-2 die before implantation, resulting in one of the most dramatic embryonic lethal phenotypes observed in mice with targeted gene mutations (38). This phenotype is substantially more severe than the phenotype due to combined lack of insulin and IGF-I receptors (18), suggesting that IRS proteins play additional roles to mediate the actions of other receptors, as predicted by studies of cytokine receptor signaling (22). The phenotype of insulin receptor-deficient mice indicates that multiple substrates are required to mediate insulin action. The conclusion of these studies is that the search for IRSs is not over.

Role of phosphatidylinositol 3-kinase (PI 3-kinase) in insulin action

The enzyme PI 3-kinase catalyzes the addition of phosphate on the D3 position of the inositol ring of phospoinositol, leading to the generation of PI 3-phosphate. The enzyme is composed of a regulatory subunit, which exists in several isoforms (p85-α, p85-β, p55/AS53, $p55^{PIK}$, and p50), and a catalytic 110-kDa subunit. 3-Phosphorylated inositides act as intracellular messengers, leading to activation of PI-dependent kinases, changes in intracellular trafficking, and growth stimulation (41). In addition, the enzyme has protein kinase activity, although there is no evidence yet for the latter's involvement in insulin action (42). Activation of PI 3-kinase is important for many of insulin's actions. Thus, blocking PI 3-kinase with the fungal inhibitor wortmannin is associated with inhibition of insulin-stimulated glucose uptake (43, 44); glycogen (45, 46), lipid (44), and protein (47, 48) synthesis;

and modulation of gene expression (49, 50). PI 3-kinase appears to play a permissive, rather than a necessary and sufficient, role in insulin action (51). The evidence generally offered to buttress this conclusion is that although several growth factors result in activation of PI 3-kinase, only insulin has the ability to stimulate processes such as glucose transporter 4 (GLUT4) translocation. This controversy has raged in the literature for the past decade and is not entirely settled. Convincing evidence for a direct role of PI 3-kinase in insulin action comes from mice carrying a deletion of the gene encoding the p85α subunit. These mice develop hypoglycemia due to increased basal levels of glucose uptake in several insulin-sensitive tissues (52). One possible interpretation of these data is that the p85α subunit exerts an inhibitory role on the kinase activity, and that its ablation increases the enzyme's catalytic activity, possibly through association with other regulatory subunits, such as p50 and p55. Although the latter point requires further investigation, these data indicate that PI 3-kinase is crucial for insulin action.

Targets of PI 3-kinase

Arguably, the most important question in insulin action is to identify targets of PI 3-kinase that may account for the specificity of insulin signaling. The rapid increase in Tris-phosphorylated inositol (PIP_3) concentration in response to insulin stimulation activates several PIP_3-dependent serine/threonine kinases, such as PI-dependent protein kinase-1 and -2 (53), Akt (a product of the *akt* protooncogene) (54), salt- and glucocorticoid-induced kinases (55), protein kinase C (PKC) (56), wortmannin-sensitive and insulin-stimulated serine kinase (57), and others (58). Among the PIP_3-dependent kinases, Akt has received much attention. The Akt kinase exists as three different isoforms, all of which are activated by phosphorylation on T^{308} and S^{473} (59, 60). Upon growth factor stimulation, Akt localizes near the plasma membrane, where it becomes phosphorylated. The activated enzyme has the ability to translocate to the nucleus (61) (Fig. 3). Expression of constitutively active Akt stimulates glucose uptake in 3T3-L1 adipocytes (62–64), whereas Akt inhibition through the use of dominant negative mutants does not completely inhibit the insulin effect on glucose transport (65). These results suggest that regulation of glucose transport may involve multiple kinases. Akt has the ability to phosphorylate proteins that regulate lipid synthesis (66), glycogen synthesis (67, 68), cell survival (69), and protein synthesis (70, 71). This mechanism provides a direct link between insulin receptor signaling and biological effects. Nevertheless, it is not clear whether Akt plays a unique or redundant role in insulin action.

Members of the PKC family of serine/threonine kinases have been implicated in several of insulin's actions. There are four subgroups of PKCs; the classical ones are activated by calcium binding, whereas the other three groups can be activated by diacylglycerol or other phospholipids, such as PIP_3 (atypical PKCs). Different isoforms of PKC have been shown to undergo translocation from the cytosol to the membrane in response to insulin stimulation in different tissues (72). Atypical PKCs (ζ and λ) have been proposed to play a role in insulin-dependent glucose transport (73, 74) and protein synthesis (75). It is also known that PKCs can activate the mitogen-activated protein kinase pathway and the transcription factor nuclear factor-κB, leading to increased gene expression and protein synthesis.

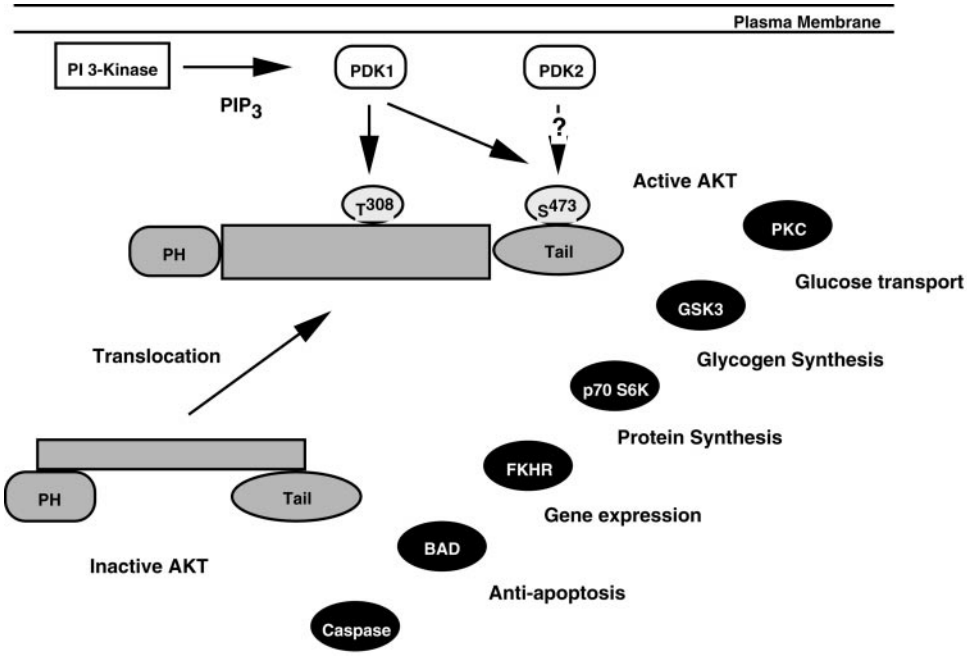

FIG. 3. Akt in insulin action. The rapid increase in intracellular PIP_3 leads to activation of the PI-dependent kinase-1 and -2 (PDK1 and PDK2). PDK1 and PDK2 phosphorylate Akt on amino acid residues T^{308} and S^{473}, leading to its activation. The identity of PDK2 has not been established, and there is evidence that PDK1 is the S^{473} kinase. After insulin stimulation, Akt undergoes phosphorylation and nuclear translocation. Some of the known cellular targets of Akt are indicated.

An insulin receptor/PI 3-kinase pathway regulates metabolism and survival in Caenorhabditis elegans

The nematode *C. elegans* has provided novel insight into the mechanism of insulin action (76). The life span of *C. elegans* consists of four developmental stages leading to the maturation of larvae into adult hermaphrodites. When larvae are grown at high density or in the presence of high levels of pheromone, they enter the *dauer* stage, a reversible arrest of development characterized by reduced metabolic activity, increased fat content, and a near doubling of life span (77). A constitutive *dauer* stage can be brought about by mutations in specific genes. The alleles causing a constitutive *dauer* phenotype have been dubbed Daf alleles. Mutations of the Daf-2 gene cause a constitutive *dauer* phenotype, as do mutations of the Age-1, Akt-1, and Akt-2 genes (78). The Daf-2 gene encodes the *C. elegans* homolog of the insulin/IGF-I receptor gene, whereas the Age-1 gene is the homolog of PI 3-kinase, and the two Akt genes represent the homologs of mammalian Akt (79–81) (Fig. 4). A parallel pathway implicates transforming growth factor-β signaling through SMAD proteins (Daf-1, Daf-4, Daf-8, and Daf-14 mutations) in the same process (82). Considerable interest has been generated by the study of mutations that suppress the effects of the Daf-2, Age-1, Akt-1, and Akt-2 mutations. Two of them are especially relevant to insulin signaling through PI 3-kinase: Daf-16 and Daf-18. Daf-16 mutations completely suppress the *dauer* phenotype due to Daf-2 mutations, whereas Daf-18 mutations have a less complete ability to rescue Daf-2 mutations. Daf-16 encodes a transcription factor with homology to the mammalian forkhead transcription factors (83), whereas Daf-18 encodes a phosphoinositide phosphatase with homology to the mammalian phosphatase- and tensin-homolog deleted on chromosome 10 tumor suppressor gene (84). Daf-16 is a substrate of Akt (85). The observations that Daf-16 and Daf-18 are important for insulin receptor signaling in *C. elegans* and are regulated in a PIP_3-dependent manner suggest that similar mechanisms play a role in the regulation of mammalian metabolism.

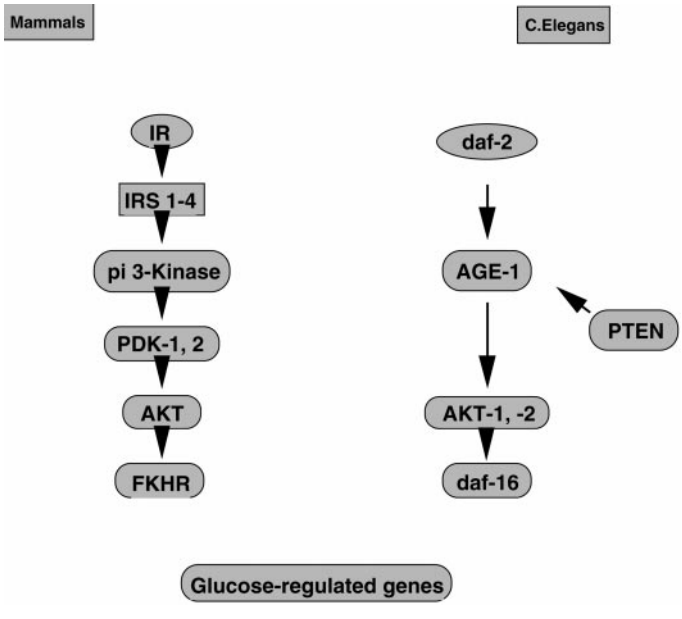

FIG. 4. Conserved insulin signaling pathways in *C. elegans* and mammals. The pathways regulating cellular metabolism and survival in mammalian cells are conserved in the nematode *C. elegans*. Activation of the insulin receptor ortholog Daf-2 leads to stimulation of Age-1, the PI 3-kinase ortholog. Targets of Age-1 include Akt-1 and -2, which, in turn, phosphorylate Daf-16. Age-1 is regulated by Daf-18, the PI phosphatase phosphate- and tensin-homolog deleted on chromosome 10 ortholog.

FKHR, the mammalian homologue of the C. elegans Daf-16 gene, regulates insulin-dependent gene expression

The Daf-16 gene product belongs to the forkhead family of transcription factors. These proteins were first identified as the homeotic gene product of the forkhead mutation in *Drosophila* (86). They contain a highly conserved DNA-binding domain, the forkhead or winged helix domain (87). A subgroup of forkhead proteins known as FKHR is the closest mammalian homolog of the Daf-16 gene product. These proteins were first identified as the products of chromosomal translocations associated with alveolar rhabdomyosarcoma, hence the acronym *F*or*K*head in *H*uman *R*habdomyosarcoma (88). The family includes three expressed genes, *FKHR*, *FKHRL1*, and *AFX*, and two pseudogenes (89).

Indeed, based on the presence of binding sites for the forkhead transcription factor HNF-3, Unterman was the first to propose that such transcription factors might represent transcriptional regulators of insulin-responsive genes such as IGF-binding protein-1, phospho-enolpyruvate carboxykinase, and glucose-6-phosphatase (49, 90). Several groups have shown that FKHR is phosphorylated in an insulin-responsive manner by PIP_3-dependent kinases, such as Akt and others (91–100). FKHR is a transcriptional enhancer, the targets of which include genes regulating apoptosis, glucose production, and entry into the cell cycle (69, 101). Under basal conditions, FKHR and its homologs, FKHRL-1 and AFX, reside within the nucleus. When cells are exposed to insulin or other known stimulators of PI 3-kinase, these transcription factors become phosphorylated at Akt consensus sites. Phosphorylation is followed by nuclear exclusion and cytoplasmic retention (69, 101). It follows that FKHR phosphorylation is a powerful mechanism by which insulin inhibits gene transcription. The full array of FKHR target genes as well as the spectrum of FKHR kinases in addition to Akt remain to be determined.

What makes GLUT4 tick

Insulin stimulation of glucose uptake is mediated by translocation of an intracellular pool of GLUT4 to the plasma membrane (102, 103). Two approaches are being employed to identify elements in the signal transduction chain leading to GLUT4 translocation: a forward approach, starting from the insulin receptor, and a backward approach starting from GLUT4. Like two teams digging up a tunnel starting at both sides of a mountain, the two approaches will hopefully merge at some point, although none can predict when. In the worst-case scenario, we may end up with two tunnels.

The status of the forward approach is summarized in the IRS/PI 3-kinase and CAP-cbl sections of this review. What do we know about the distal components of this pathway? Two models have been proposed to account for insulin's effect on

GLUT4 translocation: a retention model and a synaptic vesicle model (104). The former would predict that GLUT4 molecules are prevented from joining the constitutive cellular recycling compartment from an inhibitory mechanism, or molecule, that would be inactivated by insulin. The latter envisions a mechanism analogous to neurotransmitter release, in which a specialized GLUT4 vesicle would dock onto and fuse with the plasma membrane via a v-SNARE protein (*V*esicle *SNAP Re*ceptor) pairing with appropriate target membrane or t-SNAREs. It is easily realized that the two models need not be mutually exclusive.

Considerable progress has been made in identifying the v-SNAREs and t-SNAREs that facilitate GLUT4 vesicle translocation. VAMP-2 is the main v-SNARE found in GLUT4 vesicles. The main t-SNAREs found in the plasma membrane of insulin-sensitive tissues are syntaxin 4 and SNAP-23 (104). Many of the accessory components of GLUT4 vesicles have been identified, and intensive efforts are underway to isolate every single constituent of this important subcellular organelle. Adapter molecules that regulate the interaction between VAMP-2 and syntaxin-4 in an insulin-dependent manner have been cloned from protein-protein yeast interaction libraries from 3T3-L1 adipocytes. Synip is a syntaxin 4-binding protein (105). Insulin catalyzes Synip dissociation from syntaxin 4. Moreover, inhibition of Synip dissociation by a dominant negative mutant results in inhibition of GLUT4 translocation. The mechanism by which insulin causes Synip dissociation remains unknown. Another syntaxin 4-binding protein is Munc18. Insulin inhibits binding of Munc18c to syntaxin 4, thereby increasing binding of VAMP2 to syntaxin 4 (106).

PI 3-kinase independent pathways of insulin signaling

As stated above, the possibility exists that not all of the actions of insulin are mediated through the IRS/PI 3-kinase pathway. One such example is the pathway mediated through the protein Cbl. Cbl is a substrate of the insulin receptor kinase in differentiated 3T3-L1 adipocytes, but not in preadipocytes (107). This differential phosphorylation is due to expression of a Cbl-associated protein (CAP) (108). Although CAP is not phosphorylated in response to insulin, it is able to target Cbl to the insulin receptor. After phosphorylation, Cbl translocates to caveolae, a specialized subdomain of the plasma membrane. Inhibition of the CAP-Cbl interaction by dominant negative CAP correlates with inhibition of insulin-stimulated glucose transport and GLUT4 translocation in a wortmannin-independent fashion, suggesting that Cbl participates in a PI 3-kinase-independent mechanism whereby insulin stimulates GLUT4 translocation in adipocytes (109).

Role of tyrosine phosphatases in insulin action

Tyrosine phosphatases play a key role in terminating the signal generated through tyrosine kinases. This family of enzymes comprises more than 100 different genes. Therefore, it has proven difficult to identify physiological phosphatases that regulate insulin signaling by dephosphorylating the insulin receptor and its targets. Experiments in various cell types have suggested that the receptor type leukocyte common antigen-related phosphatase is an insulin receptor phosphatase (110). Accordingly, mice lacking leukocyte common antigen-related phosphatase exhibit a complex syndrome of insulin sensitivity and insulin resistance (111). Likewise, mice lacking protein tyrosine phosphatase 1b present with an insulin sensitivity syndrome that has suggested that this phosphatase represents an important modulator of insulin action. Ablation of PTP-1b in mice is associated with failure to develop insulin resistance when exposed to a high fat diet (112, 113). These studies implicate PTP-1b as a physiological mediator of insulin action and as a potential therapeutic target to develop therapies against diet-induced obesity.

Conclusions

Substantial progress has been made in understanding how insulin mediates its effects on fuel metabolism. Through a combination of approaches, from cellular and molecular techniques to transgenic and knockout mice, many pathways of insulin signaling have been reconstructed in detail. As outlined in this review, significant questions remain unanswered. First and foremost is the identification of the complete chain of events leading from IRS phosphorylation and PI 3-kinase activation to the biological effects of insulin. Second and not less important is to dissect the role of PI 3-kinase-dependent and -independent pathways of insulin action. Finally, it remains to be determined how these complex pathways interact *in vivo* and how different tissues contribute to the pathogenesis of the insulin resistant state of type 2 diabetes.

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