

Insulin signaling in health and disease

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Review Series

The molecular mechanisms of cellular insulin action have been the focus of much investigation since the discovery of the hormone 100 years ago. Insulin action is impaired in metabolic syndrome, a condition known as insulin resistance. The actions of the hormone are initiated by binding to its receptor on the surface of target cells. The receptor is an $\alpha_2\beta_2$ heterodimer that binds to insulin with high affinity, resulting in the activation of its tyrosine kinase activity. Once activated, the receptor can phosphorylate a number of intracellular substrates that initiate discrete signaling pathways. The tyrosine phosphorylation of some substrates activates phosphatidylinositol-3-kinase (PI3K), which produces polyphosphoinositides that interact with protein kinases, leading to activation of the kinase Akt. Phosphorylation of Shc leads to activation of the Ras/MAP kinase pathway. Phosphorylation of SH2B2 and of Cbl initiates activation of G proteins such as TC10. Activation of Akt and other protein kinases produces phosphorylation of a variety of substrates, including transcription factors, GTPase-activating proteins, and other kinases that control key metabolic events. Among the cellular processes controlled by insulin are vesicle trafficking, activities of metabolic enzymes, transcriptional factors, and degradation of insulin itself. Together these complex processes are coordinated to ensure glucose homeostasis.

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Insulin signaling in health and disease

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The molecular mechanisms of cellular insulin action have been the focus of much investigation since the discovery of the hormone 100 years ago. Insulin action is impaired in metabolic syndrome, a condition known as insulin resistance. The actions of the hormone are initiated by binding to its receptor on the surface of target cells. The receptor is an $\alpha_2\beta_2$ heterodimer that binds to insulin with high affinity, resulting in the activation of its tyrosine kinase activity. Once activated, the receptor can phosphorylate a number of intracellular substrates that initiate discrete signaling pathways. The tyrosine phosphorylation of some substrates activates phosphatidylinositol-3-kinase (PI3K), which produces polyphosphoinositides that interact with protein kinases, leading to activation of the kinase Akt. Phosphorylation of Shc leads to activation of the Ras/MAP kinase pathway. Phosphorylation of SH2B2 and of Cbl initiates activation of G proteins such as TC10. Activation of Akt and other protein kinases produces phosphorylation of a variety of substrates, including transcription factors, GTPase-activating proteins, and other kinases that control key metabolic events. Among the cellular processes controlled by insulin are vesicle trafficking, activities of metabolic enzymes, transcriptional factors, and degradation of insulin itself. Together these complex processes are coordinated to ensure glucose homeostasis.

While the 100th anniversary of insulin's discovery is a reminder of the astounding progress toward elucidating the molecular basis of insulin action, it also highlights the numerous gaps in understanding of the signaling pathways used by this important hormone. A major complexity is the pleiotropic nature of insulin's effects, which depend critically on tissue target, time course, and the presence of other hormones and biogenic amines. All of insulin's actions are mediated by its receptor, IR (encoded by *INSR*), a cell surface protein that signals via multiple pathways involving protein and lipid phosphorylation, activation of small G protein molecular switches, control of trafficking events, and regulation of a network of enzymes and transcriptional factors that together define insulin's unique actions (1–3). A central component of metabolic syndrome's pathophysiology is insulin resistance, produced by reduced responsiveness to insulin in fat, liver, muscle, and other tissues (4). Numerous longitudinal studies demonstrate that insulin resistance is an early step in the development of type 2 diabetes (T2D) and is closely linked to other health problems such as obesity, fatty liver, polycystic ovarian syndrome, hypertension, and atherosclerosis (5–7). Its central role in so many aspects of metabolic syndrome makes understanding insulin resistance, and hence insulin action, of great importance.

Insulin is a potent anabolic agent, promoting the cellular uptake, storage, and synthesis of nutrients, while blocking nutrient breakdown and release into the circulation. Insulin stimulates nutrient transport into cells, acutely regulates metabolic enzyme activity, controls transcription of metabolic genes, regulates cellular growth and differentiation, and controls its own clearance, all

through activation of its receptor. I will review current understanding of the insulin receptor, its interacting proteins, the proximal events responsible for signal initiation, and the specific pathways governing different aspects of insulin action. Throughout, I will comment on the negative regulation of insulin signaling that may be responsible for cellular insulin resistance, and review genetic evidence for the importance of specific pathways, while highlighting outstanding questions that remain enigmatic.

The insulin receptor

The insulin receptor is a glycosylated, disulfide-linked $\alpha_2\beta_2$ tetramer that belongs to a subfamily of receptor tyrosine kinases, including insulin-like growth factor 1 (IGF-1) receptor and insulin receptor-related receptor (IRR) (8, 9) (Figure 1A). The receptor's two subunits derive from a single precursor processed by a furin-like enzyme to produce an α/β subunit complex, which then undergoes disulfide linkage to form the tetramer (8, 10, 11). β Subunits in the mature receptor traverse the membrane via a helical structure, and the receptor is mostly found in the plasma membrane (12), although a fraction may be found in the nucleus (13, 14). However, the location of the insulin receptor and signal initiation remains controversial.

The receptor is activated upon binding of insulin to the α subunit, which derepresses the tyrosine kinase activity of the β subunit. Full activation requires one subunit to phosphorylate the other, causing a conformational change that further increases kinase activity, producing phosphorylation of exogenous substrates (15–18). Because α/β heterodimers of insulin, IGF-1, and IRR receptors undergo activation by transphosphorylation, a dominant-negative mutant form of one receptor subtype can inhibit the other's activity, explaining why individuals with insulin receptor mutations exhibit both metabolic insulin resistance and growth retardation (17).

Recent insights from x-ray crystallography and cryo-electron microscopy (cryo-EM) illustrate precisely how insulin bind-

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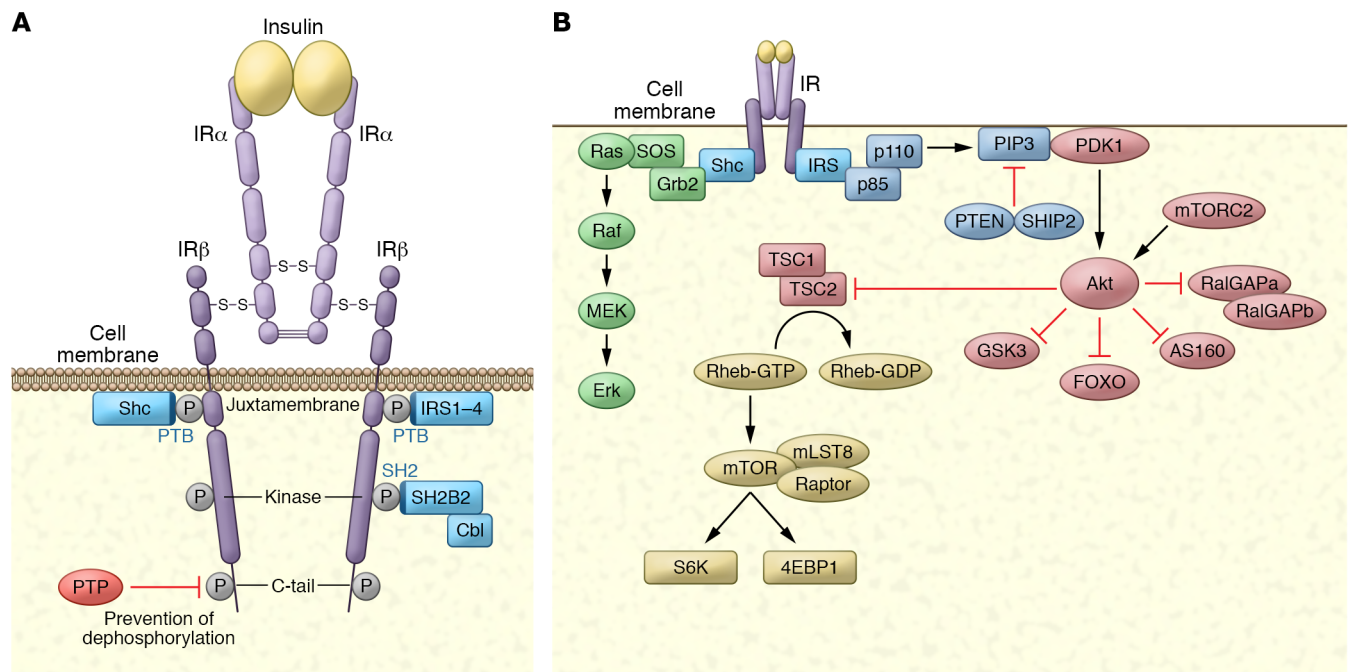


Figure 1. The insulin receptor, its substrates, and its activation of kinase cascades. (A) The insulin receptor is a disulfide-linked, α/β heterodimer glycoprotein that resides largely on the cell surface. The α subunit binds to insulin with high affinity, alleviating PTP-mediated repression of the β subunit's tyrosine kinase activity by inducing close proximity between the β subunits, permitting transphosphorylation on tyrosines in three β subunit domains. Phosphorylation of three crucial tyrosines leads to full activation of the receptor kinase. Once activated, the receptor kinase can phosphorylate exogenous substrates that act as adaptors: IRS-1–IRS-4 and Shc. Both are recruited to the juxtamembrane region via their PTB domains. SH2B2 is recruited to the kinase region's triple phosphorylation motif via its SH2 domain, serving as an adaptor protein for the substrate Cbl. **(B)** Activation of kinase cascades. Once phosphorylated, IRS and Shc activate lipid and protein kinases. IRS proteins are phosphorylated on tyrosines within specific motifs, recruiting the p85 subunit of PI3K, which binds to IRS through its SH2 domain. This results in activation of the p110 catalytic domain to generate polyphosphoinositides such as PI-(3,4,5)trisphosphate (PIP₃). These phosphoinositides can be degraded by the PI phosphatases PTEN and SHIP2. PIP₃ interacts with proteins containing PH domains, notably PDK1 and Akt. Once recruited to the plasma membrane, PDK1 and mTORC2 phosphorylate and activate Akt, which can phosphorylate a number of substrates, including the GAP proteins RalGAPa, AS160, and TSC2, as well as Foxo proteins, GSK3, and others. Upon phosphorylation, Shc interacts with the SH2/SH3 adaptor protein Grb2, which is constitutively associated with the GEF SOS. SOS is thus recruited to the plasma membrane, and catalyzes the exchange of GTP for GDP on Ras. In its active, GTP-bound state, Ras interacts with the protein kinase Raf, leading to activation of the MAPK cascade through sequential phosphorylation of MEK and ERK.

ing induces the conformational changes that lead to tyrosine kinase activation (19). Crystallography of the unbound receptor/ligand-binding domain dimer revealed an inverted U structure with about 115 Å distance between membrane insertion regions (20, 21). Importantly, single-particle cryo-EM of full-length insulin receptor corroborated this finding (22). Insulin binding converts the receptor extracellular domain into a T-like shape that draws the membrane-proximal regions together, leading to transphosphorylation in the β subunit (22).

The β subunit's cytoplasmic region consists of a juxtamembrane domain, a tyrosine kinase domain, and a carboxy-terminal tail. Tyrosine autophosphorylation sites have been mapped in all three regions (23, 24). While substrates can interact with each domain, phosphorylation of the three sites within the kinase domain are key for activation, as revealed by crystal structures of the tyrosine kinase domain determined in several different phosphorylation states and with bound substrates (25, 26). Crystallographic, biophysical, and biochemical evidence shows that the β subunits' phosphorylated kinase domains bring together the juxtamembrane region proximal to the kinase domain, cooperatively increasing transphosphorylation and activation.

To date, over 100 *INSR* mutations have been discovered (27) that cause reduced biosynthesis of the receptor, impaired transport or recycling to the plasma membrane, decreased binding affinity, or reduced tyrosine kinase activity. Rare *INSR* mutations cause inherited insulin-resistant syndromes such as Donohue syndrome and Rabson-Mendenhall syndrome. These recessive conditions are characterized by restricted intrauterine and postnatal growth, dysmorphic features, altered glucose homeostasis, and early death (28, 29). Mutations in the kinase domain can also alter insulin action and produce disease with a dominant pattern of inheritance. A Gly⁹⁹⁶Val mutation in a conserved Gly-X-Gly-X-X-Gly motif impairs tyrosine kinase activity and is associated with insulin resistance and acanthosis nigricans, suggesting a dominant-negative effect on the tyrosine kinase (30). Gain-of-function mutations in the kinase domain leading to familial hyperinsulinemic hypoglycemia have also been identified (31).

Insulin receptor substrates

Insulin-stimulated autophosphorylation of its receptor recruits several proteins for phosphorylation to initiate signaling pathways. At least nine intracellular substrates of insulin and IGF-1 receptor

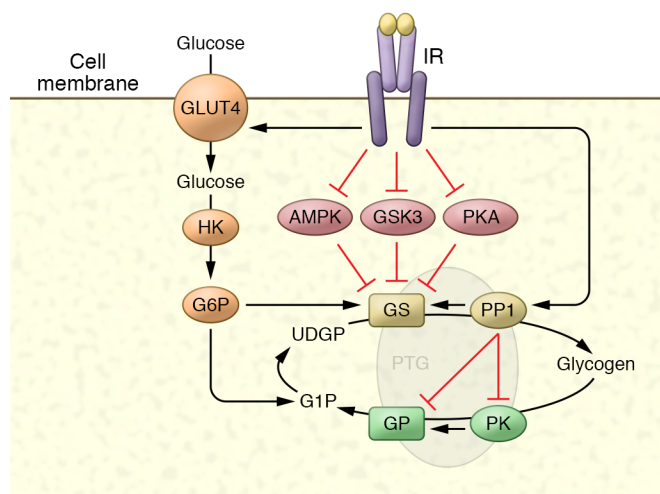


Figure 2. Regulation of glycogen metabolism by compartmentalized phosphorylation. Like other metabolic enzymes, control of glycogen metabolism is mediated by changes in phosphorylation of the enzymes glycogen synthase (GS) and glycogen phosphorylase (GP) through inhibition of kinases and activation of phosphatases. GS is inhibited by phosphorylation on up to nine amino acids, and insulin activates the enzyme by reversing this phosphorylation through a combination of kinase inhibition and phosphatase activation, primarily through protein phosphatase 1 (PP1). Similarly, GP is activated by phosphorylation, and insulin inhibits the enzyme by reducing phosphorylation. These events occur in discrete cellular compartments owing to the presence of scaffolding proteins such as PTG (Ppp1R3C) and others, by binding to GS, GP, phosphorylase kinase (PK), and AMPK, and targeting these proteins to glycogen itself. GS is also regulated by the binding of glucose-6-phosphate (G6P) to an allosteric site that increases activity.

tyrosine kinases have been identified (Figure 2). Four belong to the insulin/IGF-1 receptor substrate (IRS) protein family (32–34). Other direct substrates include Gab-1 (35), DOK1 (36), Cbl (37), SH2B2 (APS) (38), SHP2 (39), and the various isoforms of Shc (40), each of which initiates a signaling pathway. The tyrosine residues phosphorylated in each substrate occur in specific sequence motifs; once phosphorylated, they serve as docking sites for intracellular molecules containing SH2 (Src homology 2) domains (1). The best-characterized substrates are the IRS proteins Shc, SH2B2, and Cbl. Each contains either a phosphotyrosine-binding (PTB) domain (IRS proteins, Shc) or an SH2 domain (SH2B2, Cbl) that mediates receptor interaction. IRS protein and Shc PTB domains bind to the juxtamembrane autophosphorylation site pY⁹⁷² within a canonical PTB domain binding site (NPXpY). SH2B2's SH2 domain binds to the phosphorylated receptor kinase activation loop; once phosphorylated, SH2B2 binds to Cbl, permitting its phosphorylation on three tyrosines (38, 41).

Insulin receptor-mediated phosphorylation of IRS proteins occurs on at least nine tyrosines within sequence motifs that recognize and activate phosphatidylinositol-3-kinase (PI3K) and downstream protein kinases. IRS-1 and IRS-2 are widely distributed, whereas IRS-3 and IRS-4 expression is more limited (42). Although the IRS proteins are homologous and possess similar tyrosine phosphorylation motifs, knockout studies suggest complementary roles. IRS-1-deficient mice exhibit pre- and postnatal growth retardation due to IGF-1 resistance, as well as insulin resistance and impaired glucose tolerance, primarily in muscle and fat (43, 44). IRS-2-deficient mice exhibit hepatic insulin resistance, with some growth defects in the brain, β cells, and retinal cells (45).

Shc isoforms also undergo tyrosine phosphorylation by binding to the insulin receptor through their PTB domain. Upon tyrosine phosphorylation, Shc interacts with the SH2/SH3 adaptor protein Grb2 to activate the Ras pathway (40, 46). SH2B2 is the insulin receptor's highest-affinity substrate (38, 47). This adaptor protein interacts directly with the triad of phosphotyrosines in the activation loop as a homodimer, in which each member interacts with a separate receptor β subunit, then undergoes phosphorylation on a single tyrosine (47). SH2B2 thereupon serves as an adaptor for c-Cbl phosphorylation through the adaptor protein CAP (SORBS1), leading to downstream activation of G proteins includ-

ing TC10 (RhoQ) (see below). This pathway occurs largely in lipid raft domains of the adipocyte plasma membrane (38).

Control of glucose homeostasis requires a rapid on/off response to insulin to maintain blood glucose within a narrow range. Following dissociation of insulin, phosphorylation of the insulin receptor and its substrates is rapidly reversed by protein tyrosine phosphatases (PTPases). Although the substrate specificity of PTPases has proven difficult to evaluate, several identified PTPases can catalyze IR dephosphorylation, and some are upregulated in insulin-resistant states (48–50). Most attention has focused on the phosphatase PTP-1b (encoded by *Ptpn1*). Disrupting *Ptpn1* in mice increases insulin-dependent tyrosine phosphorylation of the insulin receptor and IRS proteins, leading to improved insulin sensitivity (51, 52). PTP-1b-deficient mice are resistant to diet-induced obesity, suggesting that PTP-1b deletion in the brain may influence energy uptake and expenditure via leptin signaling (52, 53).

IRS proteins and the insulin receptor also undergo serine phosphorylation that is generally associated with reduced insulin action (54). Serine phosphorylation of the receptor or substrates blocks insulin action by decreasing tyrosine phosphorylation and sequestering tyrosines by promoting interaction with 14-3-3 proteins (55). Multiple intracellular kinases are implicated in this serine phosphorylation (7, 56), including some activated by insulin, such as Akt (57), JNK (58), ERK (59), and PI3K (60), which potentially provide feedback inhibition. Moreover, serine kinases activated in obesity or by inflammation, especially PKC ϵ (7), can phosphorylate and inhibit substrate tyrosine phosphorylation (61). However, the physiological relevance of these negative phosphorylation events remains uncertain (62, 63).

Several polymorphisms in human *IRS-1* (G⁹⁷¹R and A⁵¹³P) observed in T2D produce decreased associated PI3K activity (64). These polymorphisms are associated with insulin resistance, hyperinsulinemia, adiposity, dyslipidemia, and risk of coronary disease, along with reduced IRS-1 protein levels and decreased IRS-1-associated PI3K activity (65). Other polymorphisms associated with obesity or T2D have been detected for *SH2B2*, *SORBS1*, and *Cbl* (66).

Control of phosphoinositide metabolism

The enzyme PI3K is pivotal in the metabolic and mitogenic actions of insulin (67) (Figure 3), and PI3K inhibitors or targeted deletion

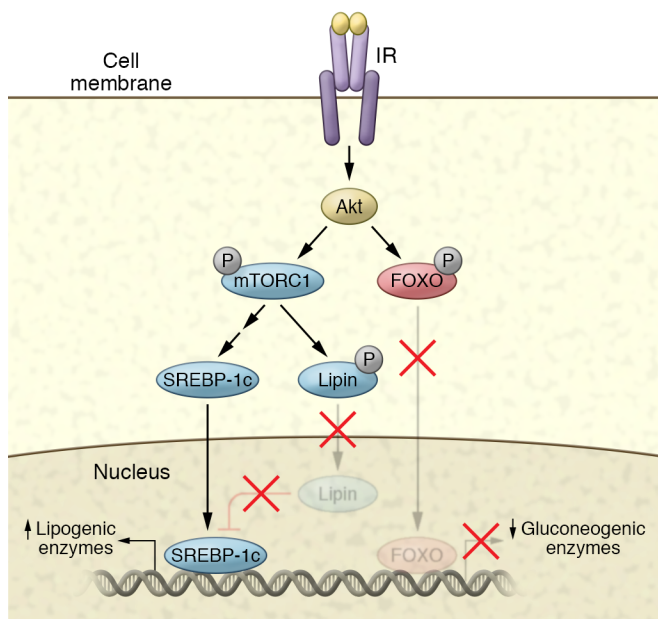


Figure 3. Transcriptional control of metabolism by insulin. Insulin increases the expression of lipogenic genes while inhibiting the expression of gluconeogenic genes in hepatocytes. Akt phosphorylates the transcription factor FOXO1, leading to the exclusion of the protein from the nucleus, and thus reducing transcription of gluconeogenic genes such as PEPCK, G6P, and others. Akt can also phosphorylate mTORC1, which in turn phosphorylates S6K. S6K activation leads to the activation of the SREBP pathway. mTORC1 also phosphorylates lipin, which inhibits SREBP action. Phosphorylation of this protein maintains a cytoplasmic localization, thus preventing its inhibitory activity.

block insulin's metabolic actions. The class 1 form of PI3K consists of a p85 regulatory unit (encoded by *PIK3R1*) and a p110 catalytic subunit (*PIK3CA*) and is activated by the two SH2 domains in the regulatory subunit interacting with tyrosine-phosphorylated IRS proteins (68, 69). The eight identified isoforms of regulatory subunits derive from three genes that undergo alternative splicing (70). p85 α and p85 β contain an SH3 domain, a BCR homology domain flanked by two proline-rich domains, two SH2 domains, and an inter-SH2 domain containing the p110 binding region (70). The shorter splicing variants of regulatory subunits p55 α and p50 α lack the N-terminal half. p85 α is ubiquitously expressed, while p55 α and p50 α play specific roles (71). In mice, disruption of all three *Pik3r1* isoforms is lethal within a few weeks of birth, indicating the enzyme's importance in normal growth and metabolism (72).

PI3K catalyzes the phosphorylation of phosphoinositides on the 3-position to generate PI-(3)P, PI-(3,4)P₂, and PI-(3,4,5)P₃. These lipids bind to the pleckstrin homology (PH) domains of target proteins, altering activity or subcellular localization. This pathway can be terminated by phosphoinositide phosphatases (73), such as PTEN (74) and SHIP2 (encoded by *Inpp1*) (75). PTEN dephosphorylates phosphoinositides on the 3'-position, while SHIP2 is a 5'-phosphoinositide phosphatase. Disrupting *Inpp1* yields mice with increased insulin sensitivity (75). Polymorphisms in *INPPL1* are associated with increased incidence of hypertension, obesity, T2D, and metabolic syndrome (76). A polymorphism in *INPPL1*'s catalytic domain identified in a Japanese population of diabetic subjects suggests possible protection from insulin resistance (77).

Regulation of serine kinase cascades

Serine phosphorylation events are initiated downstream of insulin receptor substrate tyrosine phosphorylation via PI3K and small GTPase activation (Figure 1B). PI-3 phosphates (PI3Ps) regulate three major classes of signaling molecules: the AGC superfamily of serine/threonine protein kinases, guanine nucleotide exchange proteins targeting the Rho family of GTPases, and the TEC family of tyrosine kinases, including BTK and ITK. The best-characterized

pathway in insulin signaling involves the AGC kinase Akt. Once recruited to the plasma membrane by PI3P, Akt is activated by a multistep process that requires phosphorylation of both Thr³⁰⁸ in the kinase domain's activation loop and Ser⁴⁷³ in the regulatory domain. PI-(3,4,5)P₃ recruits the serine/threonine kinase phosphoinositide-dependent kinase 1 (PKD1) to the plasma membrane through its PH domain and phosphorylates Thr³⁰⁸ on Akt (78, 79). Ser⁴⁷³ appears to be phosphorylated mainly by the rapamycin-insensitive mTOR complex mTORC2 (80). S⁴⁷³ phosphorylation may be partially redundant but stabilizes Akt's active conformation state (81). The mechanism of mTORC2 regulation remains uncertain.

Akt amplifies multiple pathways in insulin action (82). Targeted deletion of Akt isoforms produces insulin resistance and glucose intolerance (83, 84), and Akt mutations have been identified in patients with severe insulin resistance (85), whereas an activating mutation produced hypoglycemia (86). However, studies using Akt inhibitors and activators have not uniformly inhibited or mimicked insulin actions (87). In part, variability may reflect the presence of three Akt isoforms (88). Although Akt1 impacts cell survival and growth, Akt2 appears to play a more prominent role in the liver (84). Stable expression of a constitutively active, membrane-bound form of Akt in 3T3L1 murine adipocytes resulted in increased glucose transport and persistent localization of GLUT4 to the plasma membrane (89–91), but did not fully reproduce insulin action. Conversely, expression of a dominant-interfering Akt mutant inhibited insulin-stimulated GLUT4 translocation. Parenthetically, full expression of insulin action likely requires other signaling pathways (92). Other AGC kinases activated downstream of PI3K include the protein kinase C (PKC) family, particularly PKC- ζ . Overexpressing PKC- ζ or PKC- λ resulted in GLUT4 translocation (93, 94), whereas expressing a dominant-interfering PKC- λ blocked insulin action (95).

Akt phosphorylates a variety of substrates, including glycogen synthase kinase-3 (GSK3) (96), the forkhead (FOXO) transcription factors, cAMP regulatory element-binding protein (CREB) (87, 97, 98), and the GAP proteins TSC2, AS160, and RalGAP (99). Once activated at the plasma membrane, phosphorylated Akt can translocate to the cytoplasm or nucleus, depending on cell type (100).

Although PI3K activity is clearly necessary for insulin action, several lines of evidence suggest that additional signals may also be required. Activation of PI3K with other hormones, such as PDGF and IL-4, does not stimulate glucose transport in adipocytes (92). Likewise, adding a PI-(3,4,5)trisphosphate (PIP₃) analog alone did not effect glucose transport (101), and two insulin receptor mutants that produced complete PI3K activation failed to mediate full insulin action (102). As mentioned above, insulin

initiates additional pathways by recruiting other adaptor proteins, particularly SH2B2, which binds to the activated insulin receptor (47). Upon phosphorylation, SH2B2 recruits a complex of SORBS1 and c-Cbl (38, 103), triggering insulin receptor-catalyzed tyrosine phosphorylation of c-Cbl, which then interacts with the adaptor protein Crk in complex with C3G, a guanyl nucleotide exchange factor (GEF) (104). C3G in turn activates the small GTPase TC10 (RhoQ) (105, 106). SORBS1 expression correlates well with insulin responsiveness and increases when cells are treated with insulin-sensitizing thiazolidinediones (66, 107).

Insulin receptor-mediated tyrosine phosphorylation of Shc isoforms produces Ras/MAP kinase (Ras/MAPK) pathway activation (108). Phosphorylated Shc interacts with the SH2/SH3 domain-containing adaptor protein Grb2, which is constitutively associated with the Ras nucleotide exchange factor SOS, leading to Ras activation. Full activation of Ras by insulin also requires stimulation of the tyrosine phosphatase SHP2, which interacts with insulin receptor substrates including Gab-1 and IRS-1/2 (109, 110). Once activated, Ras operates as a molecular switch, converting upstream tyrosine phosphorylation into a serine kinase cascade via stepwise activation of Raf and the MAPKs MEK, ERK1, and ERK2 (111, 112). The MAPKs can phosphorylate cytoplasmic substrates or translocate into the nucleus and catalyze the phosphorylation of transcription factors (Elk1, p62^{TCF}, and others), initiating a transcriptional program that commits the cell to a proliferative or differentiative cycle. Blocking the Ras/MAPK pathway with dominant-negative mutants or pharmacologic inhibitors prevents insulin stimulation of cell growth but does not affect any metabolic actions of the hormone (113).

Insulin also controls protein synthesis via a process closely linked with nutrient sensing, involving the protein kinase mTORC1. mTORC1 is a PI3K family member but appears to serve primarily as a protein kinase. Insulin-mediated mTOR stimulation involves PI3K and other inputs (114–119). Akt phosphorylates and inhibits the GTPase-activating protein (GAP) tuberous sclerosis complex 2 (TSC2), which forms a complex with the scaffolding protein TSC1 that negatively controls the small GTPase Rheb, a key regulator of the mTORC1 complex (120, 121). mTOR regulates mRNA translation via phosphorylation and activation of the p70 ribosomal S6 kinase, as well as the phosphorylation of the eIF-4E inhibitor PHAS1 (also called 4E-BP1; *EIF4EBP1*). p70 S6 kinase phosphorylates ribosomal S6 protein, activating ribosome biosynthesis and increasing translation of mRNAs with a 5'-terminal oligopyrimidine tract. mTOR's phosphorylation of PHAS1 induces its dissociation from eIF-2, allowing cap-dependent translation of mRNAs with a highly structured 5'-untranslated region (122).

Small GTPases control vesicle trafficking and nutrient transport

Controlling transport processes, especially the uptake of nutrients into cells for storage, is a key aspect of insulin action. The rate-limiting step in insulin's control of glucose homeostasis is stimulation of glucose transport in fat and muscle (99, 123, 124). This occurs via the translocation of facilitative GLUT4 glucose transporters from intracellular sites to the plasma membrane. The GLUT4 protein consists of 12 transmembrane helices, with C- and N-terminal tails both oriented on the cytoplasmic side of the vesicle or

plasma membrane. GLUT4 continuously recycles between the cell surface and various intracellular compartments in the basal state. Insulin markedly increases the rate of GLUT4 vesicle exocytosis (99, 125). After endocytosis, GLUT4 returns to the plasma membrane via sorting endosomes or intracellular compartments. In the basal state, at least half of the GLUT4 population is found in a specialized vesicle compartment, and stimulation with insulin depletes a proportion of these GLUT4-enriched vesicles storage (GSVs), directing them to the plasma membrane.

Control of GLUT4 sorting and GSV trafficking relies on activity of several small GTPases that assemble effectors mediating vesicle budding, transport, tethering, and fusion. Small GTPases are active in the GTP-bound state, and inactive upon hydrolysis of GTP to GDP due to the intrinsic activity of the proteins. GTPases are activated by GEF recruitment and inhibited by GAPs. As a general rule, upstream GEFs and GAPs regulate GTPases that control different steps in GLUT4 sorting in adipocytes and muscle cells (126). Insulin activates TC10 via recruitment of the GEF C3G (106). The Akt substrate AS160 is a RabGAP that targets Rab8 and Rab14 in muscle cells, and Rab10 in adipocytes (126, 127). These Rabs have a positive role in GLUT4 translocation, suggesting that they may regulate GSV formation and/or intracellular retention (127–129). Insulin stimulates AS160 phosphorylation via Akt, relieving AS160's inhibitory effect on target Rabs (130, 131). Insulin-mediated activation of Rab8 and Rab14 was observed in muscle cells, but Rab10 activation has not been detected (129, 132). Nevertheless, Rab10 is a bona fide target of AS160 (132), and necessary for maximal GLUT4 exocytosis in response to insulin. Several lines of evidence indicate that Rab10 cycling may increase glucose uptake (127).

A tethering/docking step targets GSVs to regions of the plasma membrane that contain the fusion machinery. GLUT4 tethering relies on the exocyst, an evolutionarily conserved octameric complex that assembles at sites of exocytosis and tethers exocytic vesicles on the plasma membrane (133, 134). The exocyst mediates initial contact between exocytic vesicles and the plasma membrane and can thus tether GSVs before the final membrane fusion step. Inhibiting exocyst assembly in adipocytes disrupts GSV fusion without affecting their translocation, demonstrating that this complex is necessary for vesicle targeting to the plasma membrane (135).

Insulin regulates exocyst-mediated targeting through exocyst assembly, recognition of the exocyst by GSVs, and disengagement to enable fusion (135–137). Once activated, TC10 binds to the exocyst scaffolding subunit Exo70, which assembles the complex at the plasma membrane (135, 136, 138, 139). GSVs recognize the exocyst via the small GTPase RalA, which is present on GLUT4-containing vesicles. Insulin controls RalA activity primarily by inhibiting the RalGAP complex, comprising a regulatory subunit (RalGAPB) and a catalytic subunit (RalGAPA) that specifically inactivates Ral GTPases. RalGAP function requires RalGAPB, and deleting RalGAPB leads to RalGAPA instability (140). Akt-catalyzed phosphorylation of RalGAPA on three residues inhibits the complex and allows for RalA-GTP binding (140). Knockdown or overexpression of a dominant-negative RalA mutant blocks insulin-stimulated glucose uptake and GLUT4 insertion into the plasma membrane, while constitutively active RalA mutants increase insulin's effect (137). Moreover, targeted knockout of Exo70 blocks glucose uptake in vivo (138). Conditional knockout of RalGAPB

leads to RalA activation in both adipocytes (141) and muscle (142), along with a dramatic increase in glucose uptake and improved glucose tolerance (142). Once activated, RalA interacts with exocyst subunits Sec5 and Exo84 (137, 143, 144). Although the precise role of these two RalA-binding proteins remains uncertain, both are required for insulin-stimulated glucose uptake (137).

In addition to suppressing lipolysis, insulin stimulates fatty acid uptake (145). The fatty acid transporters CD36 (146) and FATP1 (147) are both implicated, and studies show that insulin can increase the translocation of these transporters from intracellular vesicles to the cell surface (146) to enhance fatty acid uptake in fat and muscle cells (142, 147). Likewise, although the pathways involved remain unclear, insulin can increase amino acid uptake in these cells, potentially reflecting increased protein synthesis downstream of mTORC1 activation (148).

Acute regulation of metabolic enzymes

Insulin acutely controls metabolic enzyme activity through a combination of changes in phosphorylation, gene expression, and interaction with allosteric regulators to coordinate an increase in energy storage and decrease in utilization. Upon entering the cell, glucose is rapidly phosphorylated by hexokinase and either stored as glycogen via the activity of glycogen synthase or oxidized to generate ATP synthesis via enzymes such as pyruvate kinase. In muscle, liver, and adipose tissue, glucose is stored as glycogen and triglycerides. In general, insulin regulates the rate-limiting enzymes involved in glycolysis and lipolysis, as well as in glycogen and lipid synthesis, by decreasing their serine/threonine phosphorylation state via a combination of protein kinase inhibition and phosphatase activation (149–151).

Insulin stimulates glycogen accumulation through coordinated increases in glucose transport and glycogen synthesis (Figure 2). Hormonal activation of glycogen synthase involves both allosteric interaction with glucose-6-phosphate (152) and dephosphorylation promoted by kinase inhibition (including PKA, AMPK, or GSK3; refs. 149, 153) and phosphatase activation (primarily protein phosphatase 1 [PP1]; ref. 154). Insulin's reduction in glycogen synthase phosphorylation is downstream of PI3K; Akt phosphorylates GSK3 (82, 155) and AMPK (156) to inactivate the kinases, resulting in decreased phosphorylation of glycogen synthase and an increase in its activity state. GSK3 inhibition is not sufficient for full activation of glycogen, since GSK3 does not phosphorylate all of the residues of glycogen synthase that are dephosphorylated in response to insulin (157), and knockins of GSK3 mutants have failed to support this enzyme's role in stimulating glycogen synthesis (158, 159). Insulin also reduces the activities of other GSKs, notably PKA and AMPK (156).

PP1 activation correlates well with changes in glycogen synthase activity (160). However, insulin does not appear to globally activate PP1, but rather targets specific pools of the phosphatase localized on the glycogen particle. The compartmentalized, insulin-mediated activation of PP1 is due to glycogen-targeting subunits that serve as molecular scaffolds, incorporating the enzyme with its substrates in a macromolecular complex (149). Four different proteins (G_M , G_I , PTG, and R_6) reportedly target PP1 to glycogen. Overexpressing these scaffolding proteins dramatically increases glycogen levels (161). Overexpressing PTG makes glycogen stores

refractory to breakdown by agents that raise intracellular cAMP levels, suggesting that PTG locks the cell into a glycogenic mode, whereas PTG knockouts dramatically reduce glycogen levels (162). The mechanism by which insulin activates glycogen-associated PP1 remains unknown. Although it was proposed that MAPK activation leads to phosphorylation of the targeting protein G_M to activate PP1 and dephosphorylate GS, blocking the pathway did not affect insulin's activation of glycogen synthase, and mutating the identified phosphorylation sites did not impair insulin action (113). However, PI3K inhibitors can block insulin's activation of PP1 (163), indicating that PIP_3 -dependent protein kinases are involved. Moreover, the genes encoding some of the scaffolding proteins are regulated, raising the possibility that transcriptional control constitutes a portion of glycogen synthesis regulation. Importantly, despite years of investigation, insulin's mechanism of regulating glycogen synthase remains uncertain.

In adipocytes, glucose is stored primarily as lipid, the result of increased uptake of glucose and activation of lipid synthetic enzymes, including pyruvate dehydrogenase, fatty acid synthase, and acetyl-CoA carboxylase, through dephosphorylation. Although insulin undoubtedly promotes dephosphorylation of these enzymes, the pathways mediating these effects are not well understood. Insulin also inhibits lipolysis in adipocytes, primarily by inhibiting the enzyme hormone-sensitive lipase (HSL) (164). PKA (150) and AMPK (165) also regulate HSL activation via phosphorylation, while insulin inhibits HSL via a combination of kinase inhibition and phosphatase activation, and a major pathway involves reductions in cAMP levels due to the activation of the cAMP-specific phosphodiesterases PDE4 and PDE3B in fat cells (150, 151). Although these phosphodiesterases can be phosphorylated and presumably regulated via Akt (166), Akt knockout did not compromise the insulin-mediated inhibition of HSL in adipocytes (167), indicating that the pathways responsible for this important action of insulin remain unknown.

Regulation of gluconeogenesis via changes in gene expression

Insulin inhibits hepatic and renal glucose production and release by blocking gluconeogenesis and glycogenolysis through phosphorylation and dephosphorylation (as described above), controlling substrates via crosstalk with other tissues, and regulating expression of genes encoding key hepatic enzymes (168–170). Controversy remains regarding the importance of direct insulin action in controlling hepatic glucose output. Several studies demonstrate that Akt2 is required for insulin's direct effect on glycogenolysis and gluconeogenesis (159). Insulin dramatically inhibits the transcription of *PEPCK*, encoding phosphoenolpyruvate carboxylase, the rate-limiting step in gluconeogenesis. Insulin also counteracts glucagon action by decreasing transcription of *FBP1* (encoding bisphosphatase) and *G6P* (glucose-6-phosphatase) and increases transcription of genes encoding glycolytic enzymes such as glucokinase and pyruvate kinase and lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase (169, 171).

Several transcription factors play a role in insulin's control of hepatic gene expression (Figure 3). Hepatic nuclear factor-3 (HNF3) and HNF4 both appear to be involved in regulating *PEPCK* expression (169, 172). Insulin regulates sterol regulatory element-

binding protein-1c (SREBP-1c), which may also contribute to insulin's negative effect on *PEPCK* transcription (173). However, the primary mechanism controlling expression of hepatic gluconeogenic genes appears to involve FOXO1 (174, 175). Both *PEPCK* and *G6P* promoter sequences contain Foxo binding sites, and FOXO1 overexpression markedly increases expression of glucose-6-phosphatase's catalytic subunit. Akt-mediated phosphorylation of FOXO1 inhibits its activity by retaining FOXO1 in the cytoplasm (176, 177). Both HNF4 and FOXO1 are modified by the coactivator PGC-1, which may also be a target of insulin action (178).

Interestingly, hepatic *FoxO1* deletion can normalize glucose tolerance and peripheral insulin sensitivity in mice lacking the insulin receptor, its substrates, or Akt isoforms in liver (179–181). Conversely, deleting *FoxO1* in liver eliminates the requirement for direct insulin action to suppress glucose output (179, 181, 182). In global insulin receptor–knockout mice, restoring expression of the insulin receptor in liver failed to correct systemic insulin resistance and glucose intolerance, despite restoring insulin signaling in hepatocytes (183). Thus, the relative importance of direct versus indirect control of hepatic glucose metabolism remains uncertain.

While insulin plays a key role in regulating the enzymes of gluconeogenesis, it can also indirectly influence glucose metabolism. This occurs via changes in the availability of gluconeogenesis substrates released from muscle and fat (184). Thus, when insulin levels are low, hydrolysis of muscle protein and adipocyte triglycerides increases levels of gluconeogenic substrates such as alanine and free fatty acids. In humans, the indirect pathway may contribute to diabetes pathogenesis, especially in individuals with central obesity, since visceral fat is less sensitive than subcutaneous fat to insulin inhibition of lipolysis, resulting in direct flux of fatty acids through the portal vein to the liver (185).

Transcriptional regulation of lipogenesis

Insulin action plays a key role in controlling hepatic lipid metabolism and the development of steatosis during insulin resistance (186). Despite pronounced insulin resistance, hepatocyte-specific knockout of the insulin receptor protects mice from fatty liver (187, 188). These mice have reduced serum triglycerides and increased fatty acid oxidation and fail to induce de novo lipogenesis and the lipogenic gene program. Observations that patients with insulin receptor loss-of-function mutations display extreme insulin resistance and hyperglycemia and are protected from developing fatty liver (189) support the essential role of direct insulin action in regulating overall hepatic lipid accumulation.

Insulin's stimulation of lipogenesis is largely mediated through the transcription factors SREBP-1c (190–192) and ChREBP (193). Dominant-negative forms of SREBP-1 can block lipogenic gene expression (191, 192), while SREBP-1 overexpression can increase it (194). Hepatic SREBP-1 levels are increased in rodent models of lipodystrophy, which is associated with coordinated increases in fatty acid synthesis and gluconeogenesis, mimicking the phenotype observed in genetic and dietary models of obesity-induced diabetes. These observations led Shimomura et al. (194) to speculate that increased expression of the isoform SREBP-1c might lead to “mixed insulin resistance” observed in the diabetic liver, with increased rates of both gluconeogenesis and lipogenesis. The pathways accounting for changes in SREBP-1c expression lie down-

stream of the IRS/PI3K pathway. However, the molecular basis for mixed insulin resistance remains uncertain. It remains possible that additional signals generated in obesity, such as nutritional factors (amino acids, sugars), ER stress, inflammation, or other hormones or transcriptional pathways (including LXR), drive the lipogenic pathway in a manner that still requires insulin (195).

mTORC1 is the major downstream effector of Akt in the control of lipogenic gene expression (196); the mTORC1 inhibitor rapamycin blocks insulin-stimulated SREBP activation and lipogenesis. Inhibiting S6 kinase, a downstream target of mTORC1, prevents SREBP-1 processing in TSC1/2-null MEFs, but fails to block insulin-induced SREBP-1c activation in primary rat hepatocytes, suggesting an additional S6 kinase-independent SREBP-1c step. The phosphatidic acid phosphatase and transcriptional coactivator lipin-1 is a direct substrate of mTORC1 and regulator of nuclear SREBP activity (197). mTORC1-mediated lipin-1 phosphorylation blocks its nuclear localization, thus permitting activation of both SREBP-1 and SREBP-2. Conversely, expression of a nonphosphorylated form of lipin-1 results in nuclear localization of lipin-1, reduced nuclear SREBP levels, and altered SREBP localization. Knockdown of lipin-1 in liver-specific Raptor-deficient mice restores SREBP-1 activation (197).

Finally, insulin also regulates SREBP activity by controlling SREBP ER-to-Golgi transport and proteolytic activation. Insulin stimulates ER-to-Golgi transport of SREBP-1c by promoting SREBP-1c phosphorylation and association with coat protein complex II (COPII) vesicles. In mouse liver, Akt also regulates SREBP transport through insulin-induced gene-2 (INSIG-2) levels. Insulin negatively regulates *Insig-2a* mRNA expression in an Akt-dependent manner, resulting in decreased INSIG-2 protein (198).

The role of ChREBP downstream of hepatic insulin action is less well defined. ChREBP is a glucose-responsive transcription factor that activates a lipogenic program similar to SREBP-1c, controlling *Fasn*, *Scd1*, and *Elovl6* gene expression (193). Dietary fructose administration induces hepatic steatosis, an effect dependent on ChREBP (199). Hepatic overexpression of ChREBP is sufficient to induce fatty liver in mice, and increased ChREBP has been correlated with nonalcoholic fatty liver disease in humans (200). Targeted deletion of ChREBP reduces fatty acid synthesis independently of changes in SREBP (200). Moreover, liver-specific knockdown of ChREBP reduces de novo lipogenesis and hepatic triglyceride in *ob/ob* mice (201). Although it is now well established that de novo lipogenesis and fat accumulation are increased in the livers of insulin-resistant people (202, 203), the precise activation status of SREBP-1c and ChREBP across a broad population remains to be determined. Future studies will be needed to better understand the specific effects of these key lipogenic transcription factors and how these molecules interact in regulating lipogenic gene expression and de novo lipogenesis in humans.

Regulation of insulin degradation

Once insulin is released into the abdominal portal vein via the pancreas, it immediately enters the liver. Interestingly, the liver clears approximately half of this insulin, and it never enters systemic circulation. Reduced hepatic clearance has been observed in patients with insulin resistance (204), and may correlate with ethnic differences in diabetes risk (203, 205), suggesting an important regula-

tory role. This is potentially important, since it remains possible that the hyperinsulinemia resulting from decreased clearance can potentiate or even cause insulin resistance through classic homologous desensitization pathways (206).

Several studies demonstrated that insulin is degraded in liver via a receptor-mediated mechanism that involves tyrosine phosphorylation (207). Although the precise details remain uncertain, this process is thought to involve tyrosine phosphorylation of CEACAM1 (207), a single-transmembrane protein highly expressed on the sinusoidal surface membrane of hepatocytes (208). This glycoprotein undergoes phosphorylation on a single tyrosine in response to insulin, and in turn forms complexes with other signaling molecules such as Shc and SHP1 (208). Insulin receptor activation is required for the rapid internalization of the insulin-receptor complex into clathrin-coated vesicles to be ultimately targeted for degradation (209–211). Whereas insulin undergoes degradation, the insulin receptor may either recycle back to the plasma membrane or translocate into lysosomes for degradation. CEACAM1 phosphorylation appears to accelerate receptor internalization and insulin degradation by increasing the complex's interactions with AP1-containing complexes in coated pits (207).

Final comments

The complexities of insulin action indicate the requirement for multiple signaling pathways. Indeed, as insulin maintains blood

sugar within a narrow range and controls the transport and metabolism of other nutrients in multiple tissues, the fine-tuning of metabolic changes in energy metabolism are unlikely to be explained by a single phosphorylation or protein interaction event. Years of investigations indicate that insulin controls a complex series of intertwining pathways that together instruct cells how and when to store energy. While extensive progress has been made, numerous gaps remain in our understanding of the precise molecular events involved in insulin action, and will remain the focus of much investigation for years to come.

Acknowledgments

I recognize that this is an incomplete review of the many signaling molecules and pathways that are controlled by insulin, and there are several important areas not covered in great depth, including the control of lipoprotein metabolism, amino acid transport and metabolism, and cellular growth and differentiation, involving additional signaling molecules, kinases, phosphatases, G proteins, and transcriptional factors; and the effects of insulin in additional tissues such as brain, kidney, vasculature, and others. I also apologize to the numerous investigators whose work was not mentioned or cited owing to space limitations. I thank members of my laboratory for useful discussions. The work of my laboratory is supported by NIH grants P30DK063491 and R01DK124496, -076906, -125820, -122804, and -117551.

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