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Phosphorylation of IRS proteins, insulin action, and insulin resistance

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Boura-Halfon S, Zick Y. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* 296: E581–E591, 2009. First published August 28, 2008; doi:10.1152/ajpendo.90437.2008.—Insulin signaling at target tissues is essential for growth and development and for normal homeostasis of glucose, fat, and protein metabolism. Control over this process is therefore tightly regulated. It can be achieved by a negative feedback control mechanism whereby downstream components inhibit upstream elements along the insulin-signaling pathway (autoregulation) or by signals from apparently unrelated pathways that inhibit insulin signaling thus leading to insulin resistance. Phosphorylation of insulin receptor substrate (IRS) proteins on serine residues has emerged as a key step in these control processes under both physiological and pathological conditions. The list of IRS kinases implicated in the development of insulin resistance is growing rapidly, concomitant with the list of potential Ser/Thr phosphorylation sites in IRS proteins. Here, we review a range of conditions that activate IRS kinases to phosphorylate IRS proteins on "hot spot" domains. The flexibility vs. specificity features of this reaction is discussed and its characteristic as an "array" phosphorylation is suggested. Finally, its implications on insulin signaling, insulin resistance and type 2 diabetes, an emerging epidemic of the 21st century are outlined.

insulin receptor substrate

INSULIN RESISTANCE IS A STATE in which the sensitivity of target cells to respond to ordinary levels of insulin is reduced. It plays a central role in the development of type 2 diabetes, an emerging epidemic of the 21st century. A variety of agents and conditions that induce insulin resistance, such as TNFα and free fatty acids, activate a number of protein kinases that target elements along the insulin-signaling pathway. Some of these kinases phosphorylate the insulin receptor substrate (IRS) proteins. Ser/Thr phosphorylation of IRS proteins inhibits their function and interferes with insulin signaling in a number of ways (Fig. 1), thus leading to the development of an insulin resistance state.

In this review, we focus on the key molecular links between Ser/Thr phosphorylation of IRS proteins and the impairment in insulin signal transduction. We outline the relations between inflammation, stress responses, the activation of IRS kinases, and the induction of insulin resistance, and we propose a few directions for future studies in this field.

Insulin Signaling

Insulin is one of the anabolic hormones which promotes proper metabolism, energy balance and maintenance of normal body weight (56). Binding of insulin to its receptor activates the intrinsic tyrosine kinase activity of the receptor (IRK), which phosphorylates Tyr residues of target proteins such as the insulin receptor substrates (IRS-1 to -6), Shc proteins, Cbl, p60dok, APS, and Gab-1 (92, 112). Three major signaling pathways are propagated in response to activation of the IRK: phosphatidylinositol 3-kinase (PI3K), MAP kinase, and the Cbl/CAP pathway (92). The MAP kinase cascade leads to enhanced cell growth, while the Cbl/CAP cascade mediates glucose transport through activation of the GTP-binding protein TC10 and the recruitment of the CIP4/Gappex-5 complex to the plasma membrane (73). The PI3K cascade is activated by the IRS proteins to trigger the metabolic functions of insulin. Tyr-phosphorylated IRS proteins, the major insulin receptor substrates, function as signaling scaffolds that propagate insulin action through binding of Src homology 2 (SH2) domain-containing proteins. These include the p85 regulatory subunit of PI3K, Nck, Fyn, Grb-2, and SHP2, which mediate various aspects of insulin action (58, 64).

PI3K is one of the best-characterized downstream effector of IRS proteins (14). It associates with Tyr-phosphorylated IRS proteins following insulin stimulation and catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate, which stimulates phosphoinositide-dependent kinase (PDK-1) activity and initiates the activation of its downstream effectors protein kinase B (PKB, Akt), mammalian target of rapamycin (mTOR), and p70 S6 kinase (S6K1) as well as the atypical isoforms of PKC (PKCζ/λ), leading to glucose transport and protein and glycogen synthesis (8, 108).

Control over insulin signaling can be achieved by autoregulation, whereby downstream components inhibit upstream elements (negative feedback control) (39, 80). Alternatively, signals from apparently unrelated pathways can inhibit insulin signaling. The insulin receptor (IR) and the IRS proteins are targets for such feedback control mechanisms (53, 72, 116, 117), with phosphorylation of IRS proteins on Ser residues being a key step in these feedback control processes (39, 80, 116, 117). Many of the insulin-stimulated Ser/Thr kinases that

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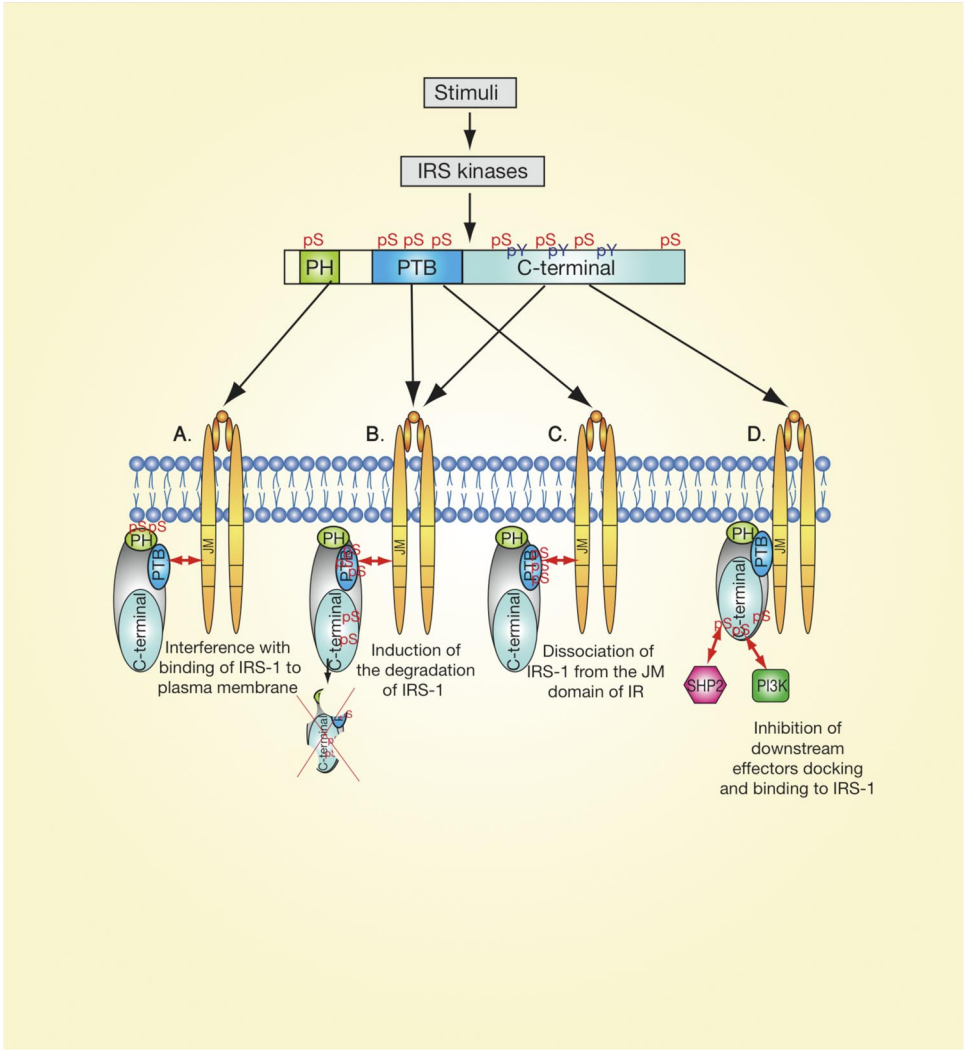

Fig. 1. Potential consequences of Ser/Thr phosphorylation of IRS proteins. IRS proteins contain a conserved pleckstrin homology (PH) domain at their NH_2 terminus followed by a phosphotyrosine-binding (PTB) domain. The latter promotes interaction of IRS proteins with the juxtamembrane (JM) domain of the insulin receptor (IR), which phosphorylates the IRS proteins at their COOH-terminal region. Tyr-phosphorylated IRS proteins then serve as docking molecules for downstream effectors such as PI3K and phosphotyrosine phosphatase 2 (SHP-2). Prolonged insulin stimulation and other stimuli triggered by inducers of insulin resistance activate IRS kinases that phosphorylate the IRS proteins, such as IRS-1, on Ser/Thr residues. Such phosphorylation has several consequences. *A*: Ser phosphorylation of residues located at the PH domain interferes with binding of IRS-1 to the plasma membrane and to the receptor. *B* and *C*: phosphorylation of Ser/Thr residues within the PTB domain or at the COOH-terminal region of IRS-1 induces dissociation of IRS-1 from the receptor concomitant with IRS-1 degradation. *D*: phosphorylation of Ser residues at the COOH-terminal region interrupts with binding of IRS-1 to its downstream effectors.

are downstream effectors of IRS proteins serve as negative modulator of IRS proteins function. The activity of these kinases is blocked by inhibitors of the PI3K pathway, implicating Ser/Thr kinases, downstream of PI3K as potential IRS kinases (72). Interestingly, it is becoming apparent that inducers of insulin resistance such as tumor necrosis factor-α (TNFα), free fatty acids (FFAs), and cellular stress make use of similar mechanisms by activating a set of IRS kinases that phosphorylate the IRS proteins and inhibit their function (109, 115).

IRS Kinases and Insulin Resistance

Insulin resistance is defined as the failure of ordinary levels of insulin to trigger its downstream metabolic actions and is closely associated with obesity and the development of type 2 diabetes (55). It is becoming clear that obesity promotes a state of chronic low-grade inflammation and insulin resistance (51, 52, 98). This is attributed to the release from the adipose tissue of FFA, glycerol, hormones (e.g., leptin, adiponectin, endothelin-1), proinflammatory cytokines (e.g., TNFα, IL-1β, IL-6), and additional products of macrophages that populate adipose tissue in obesity (27, 98, 109). It has been shown already a hundred years ago that high doses of salicylates lower glucose levels in diabetic patients implicating the involvement of inflammation in type 2 diabetes. Additionally, increased release of FFAs decreases insulin-mediated glucose transport in skeletal muscle and impairs suppression of glucose production by the liver, a characteristic of insulin resistance (3, 9, 47).

Many inducers of insulin resistance activate IRS kinases that negatively regulate insulin signaling and action. The list of IRS kinases implicated in the development of insulin resistance is growing rapidly, concomitant with the list of potential Ser/Thr phosphorylation sites in IRS proteins (22, 117). Recent studies have focused on IRS-1 as a major target for IRS kinases (22). However, it is now becoming evident that IRS-2 serves as a target as well (40, 97, 99). IRS kinases can be divided into two groups. One includes kinases that are mediators of insulin signaling. These kinases negatively regulate IRS proteins upon prolonged insulin stimulation [e.g., mTOR/S6K1 (105), MAPK (21), and PKCζ (65, 72, 90, 100)]. The other group consists of kinases that are activated along unrelated pathways to inhibit insulin action [e.g., glycogen synthase kinase (GSK)-3β (25, 69) IKKβ (33), c-Jun NH_2-terminal kinase (JNK) (2, 66), mouse Pelle-like kinase (mPLK) (59), and AMPK (103)]. Of note, several IRS kinases (e.g., S6K1, PKC) are activated both in response to insulin and as inducers of insulin resistance (80, 93, 104, 115).

Ser Phosphorylation of IRS Proteins and Its Potential Consequences

IRS proteins share a similar structure characterized by the presence of an NH_2-terminal pleckstrin homology (PH) domain adjacent to a phosphotyrosine-binding (PTB) domain followed by a variable-length COOH-terminal tail that contains a number of Tyr and Ser phosphorylation sites. The PH domain is critical for IR-IRS interactions. Plasma membrane phospholipids, cytoskeletal elements, and protein ligands mediate these interactions (31, 37). In contrast, the PTB domain interacts directly with the juxtamembrane (JM) domain of the insulin and IGF-I receptors (88, 107), and hindrance of these interactions (by Ser/Thr phosphorylation) negatively affects insulin signaling (107). A third domain, the kinase regulatory loop binding (KRLB) is found only in IRS-2 (43, 95). This domain interacts with the phosphorylated regulatory loop of the IR, whereas the phosphorylation of two Tyr residues within the KRLB are crucial for this interaction (94).

The COOH-terminal end of each IRS protein contains a set of Tyr phosphorylation sites that act as on/off switches to recruit downstream effector molecules. IRS-1 and IRS-2 have the longest tails, which contain ~20 potential Tyr phosphorylation sites. Many of the Tyr residues gather into common Tyr-phosphorylated consensus motifs (YMXM or YXXM) that bind SH2 domains of their effector proteins. Ser/Thr phosphorylation adjacent to these Tyr phosphorylation sites impedes binding of the SH2 domains of these effctors, thus inhibiting insulin signaling.

Effects of autologous vs. "cross-talking" signaling cascades on IRS kinases that phosphorylate the PTB domain, leading to the dissociation of IRS proteins from the JM domain of IR. An obvious necessity for a successful protein-protein binding is a spatial matching. Therefore, it is becoming apparent that Ser/Thr phosphorylation of IRS proteins in close proximity to their PTB (receptor-binding) region affects insulin signaling. We could show that mutation of seven Ser sites located within or in close proximity to the PTB domain of IRS-1 protects it from autologous desensitization as well as desensitization induced by IRS kinases triggered by inducers of insulin resistance (71). Autologous desensitization is exemplified by the atypical PKCζ, which is activated in response to insulin to mediate glucose uptake in adipocytes (7) and skeletal muscle (12, 70) downstream of IRS-1 and PI3K (70). In addition to its role as a mediator of insulin action, PKCζ is involved in a self-attenuated mechanism induced by insulin to negatively regulate the function of IRS proteins upon prolonged insulin stimulation (72, 90). It involves Ser/Thr phosphorylation of IRS proteins, mediated by PKCζ, which leads to the dissociation of IR:IRS (86) and IRS:PI3K (81) complexes. This inhibits the ability of IRS proteins to undergo further insulin-stimulated Tyr phosphorylation and, as a result, terminates insulin signaling (72). A direct interaction between IRS-1 and PKCζ was demonstrated in rat adipose tissue (90), implicating PKCζ as an IRS-1 kinase. The time line of action of PKCζ is still unclear. It is conceivable to assume that PKCζ acts first on its target proteins along the glucose transport machinery to stimulate this process and promote insulin action before it acts on IRS-1 to dissociate it from the receptor and thus terminate insulin action.

Support for this conclusion is provided by studies (30) where muscle-specific knockout of PKCλ, a postulated mediator for insulin-stimulated glucose transport, was accompanied by systemic insulin resistance, impaired glucose tolerance, and islet β-cell hyperplasia while maintaining intact insulin signaling and actions in muscle, liver, and adipocytes of these mice. These findings demonstrate the importance of aPKC in insulin-stimulated glucose transport in muscles of intact mice (28, 29). They further demonstrate that the stimulatory roles of aPKCs in insulin action override their inhibitory actions. They might further suggest that the inhibitory actions of PKCλ on insulin signaling have been overtaken by another kinase. Still, the molecular mechanism that coordinates these contradictory actions of atypical PKCs remains to be explored.

Ser^{318} of IRS-1 (numbering of Ser sites is based on mouse IRS-1 and IRS-2 sequences unless otherwise indicated), is a potential target for PKCζ (78), JNK, and kinases along the PI3K-mTOR pathway (82). It is located in close proximity to the PTB domain. Therefore, its phosphorylation presumably disrupts the interaction between IR and IRS-1. Phosphorylation of Ser^{318} is not restricted to insulin stimulation. Elevated plasma levels of leptin, an adipokine produced by adipocytes (4), also stimulates the phosphorylation of Ser^{318}. This down-regulates insulin-stimulated Tyr phosphorylation of IRS-1 and glucose uptake (44).

Desensitization of insulin signaling is also triggered by several unrelated signaling cascades. Two major cascades are activated in response to inflammatory signals: one is mediated by the stress-activated JNK, and the other is mediated by IKKβ (114). JNK1 promotes the phosphorylation of Ser^{307} of IRS-1 in response to TNFα (1, 2). Ser^{307} is adjacent to the PTB domain of IRS-1, and its phosphorylation interferes with the interaction of IR and IRS-1, thus preventing Tyr phosphorylation of IRS-1 (1). Indeed, JNK1-deficient mice show decreased adiposity and significantly improved insulin sensitivity (49). Palmitic acid induces JNK activation in pancreatic β-cells, resulting in the inhibition of pivotal gene transcription, including the insulin gene (99). This inhibition results in part due to phosphorylation of IRS-1 at Ser^{307} and of IRS-2 at Thr^{347}, which most likely are inhibitory Ser/Thr sites (99).

Signaling via NF-κB is another key process during inflammation. IKKβ is a Ser/Thr kinase that is part of the IKK complex that phosphorylates the inhibitor of NF-κB, IκB. This results in degradation of IκB, allowing the activation of NF-κB (57). Heterozygous deletion of IKKβ ($IKK\beta^{+/-}$) protects against the development of insulin resistance during high-fat diet (HFD) and in obese $Lep^{ob/ob}$ mice (61, 114). These findings support a pivotal role for IKKβ in the induction of insulin resistance and diabetes. IKKβ exerts its effects both globally (systemic) and locally (in selected tissues). Mice whose IKKβ was selectively deleted from their myeloid cells preserve their whole body insulin sensitivity and are protected from insulin resistance induced by HFD (5). Similarly, hepatic expression of the IκBα superrepressor reverses the type 2 diabetes phenotype induced by low-level activation of NF-κB (13). In contrast, selective loss of IKKβ in hepatocytes retains insulin sensitivity in liver but not in muscle or fat tissues in response to HFD (114). The reason why expression of IκBα superrepressor, but not deletion of IKKβ in hepatocytes, reverses systemic insulin sensitivity of mice on HFD is not clear but could be attributed to different strategies employed to

attenuate IKKβ signaling. Nonetheless, IKKβ seems to have a central role in hepatic insulin resistance (114) and in the development of systemic insulin resistance (13). At the molecular level, studies have yielded conflicting results. Interactions between IKKβ and IRS-1 were shown, whereas Ser^{307} was implicated as a potential phosphorylation site for IKKβ (20, 33). Support for this conclusion was provided by showing that the motor protein Myo1c and its receptor protein NEMO act cooperatively to form the IKKβ:IRS-1 complex that functions in TNFα-induced insulin resistance (83). In contrast, a reduction in IKKβ levels using specific small interfering (si)RNA failed to prevent TNFα-mediated IRS-1 phosphorylation on Ser^{312} (human equivalent of mouse Ser^{307}) in primary human skeletal muscle (6). This apparent discrepancy could be attributed to the fact that Ser^{307} is subjected to phosphorylation by a number of IRS kinases in addition to IKKβ (Fig. 2) Furthermore, IKKβ can phosphorylate sites different from Ser^{307}. We have shown that mutations of seven Ser sites in IRS-1 ($Ser^{265/302/325/336/358/407/408}$) confer protection from the action of IKKβ when the mutated IRS-1 ($IRS\text{-}1^{7A}$) is overexpressed in Fao cells or primary hepatocytes (46). Interestingly, Ser^{307} was not one of the seven mutated sites in $IRS\text{-}1^{7A}$, implicating other Ser residues as potential IKKβ-mediated phosphorylation sites.

Inducers of insulin resistance also activate PKCθ, a novel-type PKC. PKCθ is activated upon increased content of intramuscular long-chain fatty acyl-CoA. An increase in PKCθ activity occurs concomitantly with a decrease in insulin-stimulated Tyr phosphorylation of IRS-1 and a reduction in glucose transport (38, 113). PKCθ-deficient mice are protected against fat-induced defects in insulin signaling (e.g., reduced insulin-stimulated Tyr phosphorylation of IRS-1 and glucose uptake) in skeletal muscle, further supporting the role of PKCθ in mediating fatty acid-induced insulin resistance (60). Notably, in the latter study, no direct evidence on Ser phosphorylation of IRS-1 or activation of other Ser/Thr kinases such as IKKβ or JNK was provided. The notion that PKCθ plays a role in fatty acid-mediated insulin resistance was challenged by the findings that transgenic mice with muscle-specific expression of a dominant negative PKCθ have shown age- and obesity-associated glucose intolerance, implicating PKCθ as a protective rather than a negative regulator of insulin function (96). These discrepant findings may be due to the different technical approaches that were used. For example, a dominant negative PKCθ could bind (and titrate out) proteins necessary for the proper function of other PKC isoforms. At the molecular level, PKCθ, a known activator of IKKβ and JNK (60, 101), may attenuate insulin signaling directly or via the activation of these IRS kinases. Ser^{1101} (68) and Ser^{307} (113) were suggested as potential target sites of PKCθ in IRS-1.

Autologous vs. heterologous feedback mechanisms can sometimes act in antagonistic manner. This is exemplified by GSK-3, an important mediator of insulin signaling, which phosphorylates IRS proteins on Ser residues to attenuate insulin actions. Ser^{332} in IRS-1 is a potential GSK-3 phosphorylation site, with Ser^{336} being the priming site (69). GSK-3 activity is inhibited by insulin upon its phosphorylation by insulin-stimulated PKB (19). Hence, GSK-3 activity is not part of an autologous insulin-induced negative feedback control mechanism. In contrast, inducers of insulin resistance stimulate the activity of GSK-3 (18, 45), which is elevated in diabetic tissues (26). Accordingly, GSK-3α KO mice display enhanced glucose and insulin sensitivity accompanied by reduced fat mass. Hepatic insulin signaling in these mice is increased, and so is the IRS-1 expression (76). Moreover, a reduction in GSK-3α in human muscle cells results in an increase in insulin-stimulated glucose uptake, glycogen synthase activity, and IRS-1 expression (16), whereas treatment with GSK-3 inhibitors enhances insulin actions in vitro and in vivo (17, 85). Of note, insulin-mediated phosphorylation of PKB and glycogen synthase were similar in skeletal muscle from both wild-type and GSK-3α KO mice, which may indicate different roles for GSK-3α in mice vs. human (16, 76). Similarly, McManus et al. (77), have generated homozygous knock-in mice of constitutively active (CA) GSK-3α, GSK-3β, or GSK-3αβ. Although GSK-3β had a major role in regulating glycogen synthase activity in muscle of CA GSK-3β knock-in mice, these animals were not diabetic, and their insulin-stimulated PKB activation and glucose uptake were not changed. These findings cast a certain doubt as to the critical role of GSK-3β in the attenuation of insulin signaling, at least in mouse models, although constitutive activation of GSK-3β under normal conditions (e.g., regular diet, low BMI) might be necessary, but it is insufficient to promote systemic insulin resistance. Hence, with respect to GSK-3, insulin acts to prevent its action as an IRS kinase, whereas heterologous signaling cascades activate it.

Interference with IRS:IR complex formation mediated by the PH domain. IRS kinases, triggered by inducers of insulin resistance, can phosphorylate sites located within the PH domain of IRS proteins. An example is the mouse Pellet-like kinase (mPLK, homolog of human IL-1 receptor-associated kinase) (59). Overexpression of mPLK-WT impairs, apparently via Ser^{24} phosphorylation, insulin-stimulated Tyr phosphorylation of IRS-1 and its association with p85α. Ser^{24}, located within the PH domain of IRS-1, seems to be critical for IR:IRS-1 complex formation (31). Interestingly, insulin can also trigger conventional and novel PKCs that phosphorylate this site. Conventional and novel PKCs were shown to be activated by insulin under certain conditions (91), and their role as transducers and modulators of insulin signaling is discussed in a recent review (93). Ser^{24} is a potential phosphorylation site for PKCα (84). Similarly, insulin-stimulated phosphorylation of Ser^{24}, apparently by PKCδ, diminishes the ability of IRS-1 to bind phosphatidylinositol-4,5-bisphosphate (PIP_2) further supporting the hypothesis that Ser^{24} is a negative regulatory phosphorylation site in IRS-1 (36). In agreement with this prediction, knockout of PKCα enhances insulin signaling (67). In a recent study using mass spectrometry analysis, Ser^{67} (rat numbering), located at the PH domain of IRS-1, was shown to be phosphorylated upon prolonged insulin stimulation. This site as well may be considered an inhibitory site (34). Consequently, Ser phosphorylation within the PH domain of IRS proteins could account for the development of insulin resistance state.

Inhibiting the ability of downstream effectors to dock and bind to specific tyr residues at the COOH-terminal tail of IRS proteins. Many of the Tyr-phosphorylated consensus motifs (YMXM or YXXM) of IRS-1 are located at its COOH-terminal tail. Consequently Ser phosphorylation within this area could interrupt with the binding of downstream effector proteins of IRS-1 such as p85α and the phosphotyrosine

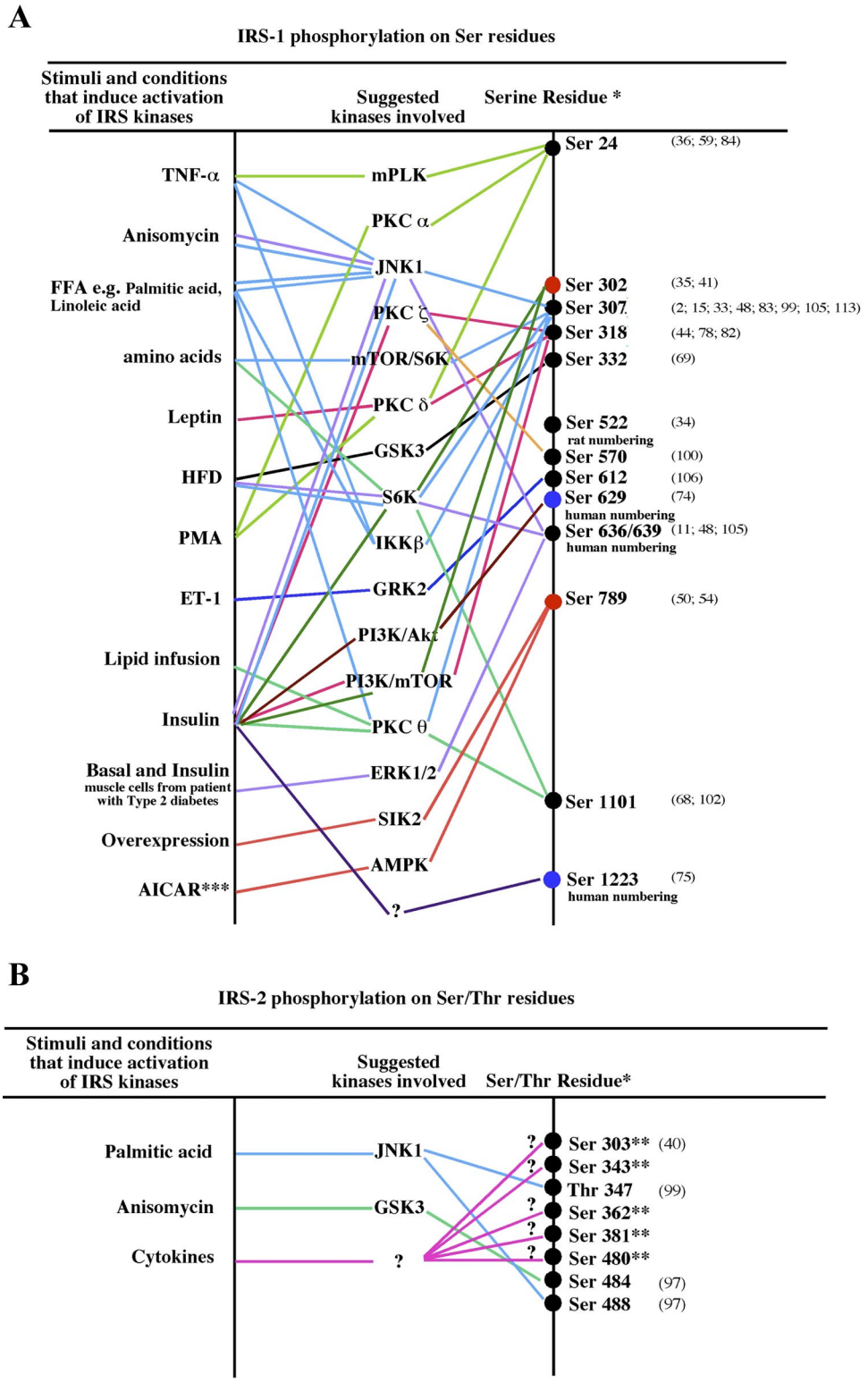

Fig. 2. *A*: Ser phosphorylation of IRS-1: is it an array phenomenon? Representative list of Ser residues of IRS-1 that were shown to undergo phosphorylation as a result of different stimuli. *B*: Ser/Thr phosphorylation of IRS-2. IRS-1/2 kinases (*middle*) are activated by several stimuli (*left*) to phosphorylate IRS-1/2 on a number of Ser/Thr residues (*right*). *Mouse numbering. **Sites marked with star were mutated as a group. ***5-Aminoimidazole-4-carboxamide riboside. Black, blue, and red filled circles represent, respectively, a suggested negative, positive, or negative/positive regulatory phosphorylation site.

phosphatase SHP-2. Indeed, Ser^{570} of IRS-1, located in the vicinity of the PI3K interaction motif, was shown to be a potential PKCζ phosphorylation site that, upon phosphorylation, disrupts the IRS-1-p85α complex (31). IRS-3 and IRS-4 but not IRS-2 are also substrates for PKCζ (65). Potential MAP kinase phosphorylation sites, Ser^{612}, Ser^{632}, Ser^{662}, and Ser^{731} in IRS-1, located next to Tyr phosphorylation YMXM motifs, were shown to be negative regulators for PI3K activity associated with IRS-1 (81). A twofold increase in the basal phosphorylation of IRS-1 on Ser^{636} (human numbering) was observed in muscle biopsies from patients with type 2 diabetes, concomitantly with higher basal activity of extracellular signal-regulated kinase (ERK)1/2. Defects in insulin signaling were evident, including reduced PI3K activity, decreased association

of PI3K with IRS-1, and reduced Tyr phosphorylation of IRS-1 during insulin stimulation. Inhibition of ERK1/2 by a specific inhibitor strongly inhibited Ser^{636} phosphorylation, thus implicating ERK1/2 in the phosphorylation of Ser^{636} and in the attenuation of insulin signaling (11).

mTOR and S6K1 kinases are downstream effectors of PI3K and potential candidates for negative regulation of IRS proteins. Indeed, mTOR complex 1 (mTORC1), an integrator of nutrient and insulin signaling, and its downstream target S6K1 are critical components in mediating the nutrient effects on insulin resistance (15, 63). Um et al. (104) clearly demonstrated that S6K1 negatively modulates insulin's effects by phosphorylating IRS proteins. The pivotal role played by S6K1 is indicated by the fact that when $S6K1^{-/-}$ mice are placed on HFD, their levels of glucose and FFA rise, and they fail to fully autophosphorylate and activate their IRs; still, they maintain their capacity to activate downstream effectors such as PKB, as opposed to their WT counterpart. S6K1-deficient mice remain sensitive to insulin, owing to apparent loss of a negative feedback loop from S6K1 to IRS-1, which blunts Ser^{307} and $Ser^{636/639}$ phosphorylation; sites involved in insulin resistance (105). In a recent publication, Ser^{1101} was identified as another S6K1 site in IRS-1, the phosphorylation of which is increased upon nutrient overload and obese setting. Phosphorylation of Ser^{1101} was increased in liver of obese (*db/db*) or WT but not of $S6K1^{-/-}$ mice maintained on HFD, implicating S6K as the kinase involved (102). The potential Ser residues implicated in the negative feedback regulation of S6K1 are located at the PTB domain [Ser^{307}, Ser^{302} (41)] and in close proximity to Tyr-phosphorylated consensus motifs at the COOH-terminus of IRS-1 ($Ser^{636/639}$). Such phosphorylation leads to dissociation of IR:IRS complexes concomitant with an inhibition of downstream effectors to dock and bind to IRS-1. The findings that persistent activation of mTOR/S6K might be responsible for impaired insulin actions lead to the obvious question whether inhibition of these kinases improves insulin sensitivity in insulin resistance states. Consistent with these findings, in a short-term in vivo study, in which insulin resistance was introduced by infusion of a high concentration of amino acids in humans, rapamycin, an mTOR inhibitor, improved insulin actions (62). On the other hand, long-term treatment with rapamycin increased rather than decreased the insulin resistance of *Psammomys obesus* that was induced by high-energy diet (32). This could be attributed to the increased activity of stress-response kinases in muscle and islets. Hence, either sustained activation of the mTOR/S6K1 pathways or their inhibition could result in insulin resistance albeit through different mechanisms.

An additional Ser site at the COOh terminus of IRS-1 is Ser^{789}. Ser^{789} was shown to be a target for the salt-inducible kinase-2 (SIK2, a novel member of the AMPK family) in adipose cells (50). The activity and content of SIK2 are elevated in white adipose tissue of *db/db* diabetic mice, suggesting that overexpression of SIK2 induces phosphorylation of Ser^{789} of IRS-1 and as a result negatively regulates insulin signal transduction (50). Tzatsos et al. (103) could show that AMPK is activated upon energy depletion (glucose deprivation, hypoxia) to phosphorylate IRS-1 on Ser^{794} (human equivalent of mouse Ser^{789}), which further inhibits PI3K/Akt signaling. Similarly, it was suggested that SIK2 may function like AMPK to turn off lipogenesis in low-energy states (23). Qiao et al. (89), however, claim that an unknown Ser/Thr kinase rather than AMPK phosphorylates IRS-1 on Ser^{789} in an insulin-resistant animal model. In contrast, phosphorylation of this site was shown to promote insulin signaling (54) in the muscle C_2C_{12} cell line. The latter study invovled the use of AICAR (5-aminoimidazole-4-carboxamide riboside), a selective inducer of AMPK, and showed a correlation between Ser^{789} phosphorylation of IRS-1 and activation of PI3K. Still, we cannot rule out the possibility that AMPK activates IRS-1 by a different mechanism while the phosphoryaltion of Ser^{789} reflects a delayed inhbitory response.

Induction of degradation of IRS proteins. Sustained insulin treatment that resembles hyperinsulinemia in insulin resistance states induces IRS-1 degradation. Downstream targets of the PI3K and mTOR pathways were implicated in mediating IRS-1 phosphorylation under these conditions (24, 42, 48). Activation of G protein-coupled receptor kinase-2 (GRK-2) upon chronic endothelin-1 treatment induces phosphorylation and degradation of IRS-1, most likely at Ser^{612} (53, 106). Insulin-stimulated degradation of IRS-1 via the PI3K pathway depends in part on phosphorylation of Ser^{307}. Indeed, a mutated form of IRS-1, in which Ser^{312} (human equivalent of mouse Ser^{307}) was replaced with Ala is protected from insulin-stimulated IRS-1 degradation (37). Of note, phosphorylation of Ser^{307} might be necessary, but it is insufficient to promote IRS-1 degradation. In accord with this conclusion we could show that insulin-induced degradation of a mutated form of IRS-1 was significantly reduced even when Ser^{307} phosphorylation remained unchanged (10). We could further show that elimination of an entire Ser/Thr-rich domain of IRS-1, proximal to its PTB domain protects IRS-1 from chronic-insulin induced degradation (10). Collectively, these findings support the notion that Ser/Thr phosphorylation is involved in insulin-stimulated IRS-1 degradation.

Positive regulation of IRS-1 by insulin-mediated ser phosphorylation. Tyr phosphorylation of IRS-1 positively regulates IRS-1 activity. Ser phosphorylation mainly negatively regulates IRS-1 function, as discussed above, although it might have some positive roles. The first indication linking Ser phosphorylation of IRS-1 and improved IRS-1 function (e.g., increased Tyr phosphorylation) was observed (87) when an IRS-1 mutant lacking four potential PKB phosphorylation sites (Ser^{265}, Ser^{302}, Ser^{325}, Ser^{358}) markedly enhanced the rate of Tyr dephosphorylation, implicating one or more of these sites as positive regulator of IRS-1 function (87). In agreement with these findings, Ser^{302} phosphorylation was implicated as a positive mediator of nutrient availability that promotes mitogenesis and cell growth (35). Using mass spectrometry techniques, Luo et al. (75) could demonstrate that phosphorylation of Ser^{1223} or Ser^{629} (human numbering) resulted in an increased IRS-1 function in respond to insulin. Two distinct mechanisms were suggested. Phosphorylation of Ser^{1223} reduces association of IRS-1 with SHP-2, a Tyr phosphatase, thereby increasing IRS-1 Tyr phosphorylation. Phosphorylation of Ser^{629} was proposed to attenuate the phosphorylation of a second Ser site, Ser^{636}, which was implicated as a negative regulator of insulin signaling, and thereby phosphorylation of Ser^{629} enhanced insulin signaling (74).

These findings suggest that, upon acute insulin stimulation, IRS-1 is phosphorylated on Tyr residues to propagate insulin signaling, a reaction accompanied by its phosphorylation on

Ser residues, which serve as "guardians" of the phosphorylated Tyr residues, namely, by inhibiting Tyr phosphatases and/or phosphorylation at "inhibitory" Ser sites. Phosphorylation of IRS-1 on these inhibitory Ser sites then commences with a delayed onset.

Phosphorylation of IRS Proteins in Human Subjects

Most information related to the regulation of IRS protein function is based on studies in cell cultures and in mouse models. Still, accumulating evidence of in vivo studies in humans supports the concept that increased Ser/Thr phosphorylation of IRS proteins might turn subjects prone to the development of insulin resistance (80). The IRS kinases involved seem to be those already implicated as negative regulators of insulin signaling. In a recent study using skeletal muscle biopsies from 11 humans, the mTOR-S6K pathway was shown to negatively modulate glucose metabolism under nutrient abundance (62). In agreement with previous studies, phosphorylation of Ser^{312} and Ser^{636} of IRS-1 was implicated as part of this negative regulation (62, 102). Increased phosphorylation of Ser^{636} of IRS-1 was observed in myotubes of patients with type 2 diabetes. Inhibition of ERK1/2 with PD-98059 reduced this phosphorylation, thereby implicating ERK1/2 in the phosphorylation of Ser^{636} in human muscle (11).

To unveil the importance of phosphorylated Ser/Thr residues of human IRS-1, Yi et al. (111) adopted a mass spectrometry approach. Although this system may suffer some drawbacks, a number of the potential Ser/Thr residues involved in the regulation of IRS proteins function could be identified. More than 20 Ser residues of IRS-1 were found to undergo insulin-stimulated phosphorylation in human muscle biopsies, three of which were newly identified sites: Thr^{495}, Ser^{527}, and Ser^{1005} (human numbering). This report validates previous in vitro and in vivo studies in animal models and suggests that the same strategy could be employed to identify phosphorylated Ser/Thr sites under conditions of insulin resistance, obesity, or type 2 diabetes.

Ser Phosphorylation of IRS Proteins: Is It an Array Phenomenon or an Event Confined to Few Selected Ser Sites?

Data collected so far suggest that an array of Ser/Thr phosphorylation sites of IRS-1 attenuates insulin signaling rather than phosphorylation of single selected sites (Fig. 2). Ser/Thr phosphorylation disrupts at least three interactions of IRS-1: with the plasma membrane, the receptor, or its downstream effectors. Therefore, many of the potential Ser residues distributed along the IRS proteins could be involved. IRS-1 contains more than 70 Ser residues at potential consensus phosphorylation sites, and each signal could induce the phosphorylation of a different set of residues. Hence, numerous optional phosphorylation combinations are possible. To obtain an accurate analysis, all potential sites phosphorylated in response to various stimuli should be examined as opposed to analysis of a small number of residues limited by availability of phosphospecific antibodies. It has already been shown that quite a number of Ser residues undergo phosphorylation upon insulin treatment in human muscle biopsies (111) and in $CHO^{IR/IRS}$ cells (34), indicating a robust phosphorylation of an array of Ser sites of IRS-1 in an in vivo setting. In both studies, Ser/Thr phosphorylation of IRS-1 was analyzed upon prolonged insulin stimulation (30–120 min) using mass spectrometry, and around 20 sites were suggested as potential phosphorylated sites in each study. Of note, these lists were not identical, thus suggesting that an even longer list of Ser sites are phosphorylated upon insulin stimulation or upon treatment with inducers of insulin resistance.

Specificity vs. Flexibility

The identity of the Ser residues, the phosphorylation of which results in specific alteration in IRS proteins structure and function is still poorly understood. Ser^{307} is considered to be involved in IRS-1 degradation (37), while we have shown that deletion of a Ser-rich domain of IRS-1 protects it from insulin-stimulated degradation despite the fact that this IRS-1 mutant is still highly phosphorylated on Ser^{307} (10). We could also show that mutation of seven Ser sites of IRS-1, different from Ser^{307}, confers upon the mutant protein protection from the inhibitory action of sustained insulin treatment or the inhibitory effects of proinflammatory cytokines (46, 71). These findings support the notion that phosphorylation of IRS proteins at selected domains (e.g., their PTB domain), rather than phosphorylation of selected sites (e.g., Ser^{307}), is required to inhibit IRS protein function. Accordingly, mutations of seven Ser residues within a given domain confer stronger protection from IRS kinases than mutations of three or a single residue (46, 71). This conclusion is supported by a recent study that made use of muscle-specific knock-in mice that express a mutant IRS-1 where three Ser residues were replaced by alanine (Ser^{302}, Ser^{307}, Ser^{612}) (79). The transgenic mice were partially protected from fat-induced insulin resistance. These studies therefore indicate that mutation of a number of Ser/Thr phosphorylation sites, rather than single site-specific mutation, sensitizes IRS-1 and protects it from negative feedback regulation of insulin signaling. The issue of dominance should also be considered, namely, what happens when an array of "positive" and "negative" Ser sites is mutated. For example, we could show that when such "mixed" mutation is performed the net effect is potentiation of insulin signaling (46, 71), suggesting the dominance of "inhibitory" Ser sites over the "stimulatory" sites in this particular case. All together, these findings indicate the existence of a cross talk between Ser phosphorylation sites and suggest that the overall phosphorylation pattern dictates IRS-1 functions.

Ser/Thr Phosphorylation of IRS-2 Protein

Most of the data accumulated so far focus on Ser phosphorylation of IRS-1 rather than IRS-2. Although IRS-2 was cloned more than a decade ago, there were not too many studies of its regulation by Ser/Thr phosphorylation (*schema 2B*). Recently, it was shown that palmitic acid induces JNK activation in pancreatic β-cells, resulting in the inhibition of pivotal gene transcription, including insulin. This inhibition resulted partly due to phosphorylation of IRS-2 at Thr^{347}, which was implicated as a potential inhibitory Thr site (99). Sequential in vitro phosphorylation of IRS-2 on Ser^{484} and Ser^{488} by GSK-3 and JNK, respectively, was suggested to promote hepatic insulin resistance (97). We recently mutated into Ala five potential inhibitory Ser sites located proximal to the PTB domain of IRS-2 (Ser^{303}, Ser^{343}, Ser^{362}, Ser^{381}, Ser^{480}). We were able to

show that cytokine-treated pancreatic islets overexpressing the mutated IRS-2 were protected from apoptosis and secreted significantly more insulin in response to glucose compared with islets overexpressing IRS-2^{WT}. Consequently, these five Ser residues can be considered negative regulators of IRS-2 function (40). IRS-2 function is also regulated by mechanisms distinct from Ser/Thr phosphorylation. For example, the kinase regulatory-loop binding (KRLB) region (aa 591–733), which is unique to IRS-2, serves as a negative regulatory element to control the extent of Tyr phosphorylation of IRS-2. Hence, inhibition exerted by KRLB domain attenuates insulin signaling and action independent of Ser/Thr phosphorylation (94, 110).

Future Perspectives

Impaired regulation of insulin signaling is a critical factor in the development of insulin resistance, and a better understanding of this process may lead to the development of novel therapies. IRS proteins are major targets for Ser/Thr phosphorylation-based negative regulation that uncouples them from their upstream receptors and their downstream effectors, leading to the termination of insulin signaling. A positive role for Ser phosphorylation was implicated as well; however, data on stimulatory Ser sites is less comprehensive and the underlying mechanisms are yet elusive.

It is becoming clear, however, that Ser phosphorylation of IRS proteins involves a number of IRS kinases. Hence, Ser/Thr phosphorylation of IRS proteins represents combinatorial consequences of several kinases, activated by different pathways, acting in concert to phosphorylate multiple sites to generate a rather complicated network. This model is supported by studies that show that one stimulus could increase the phosphorylation of many Ser residues in IRS-1. Second, elimination of a number of Ser residues of IRS-1 confers upon IRS-1 better protection from inducers of insulin resistance than elimination of single sites. Major questions that remain to be addressed are: which kinases directly phosphorylate IRS proteins, thereby affecting insulin signaling; which Ser residues are the most critical in regulating IRS function; and are there Ser/Thr-rich domains whose phosphorylation at a number of sites inhibits IRS proteins function?

The spatial and temporal regulatory elements that control this complex phosphorylation network need to be revealed. It is conceivable to assume that Ser kinases activated along the insulin pathway (e.g., S6K1, PKCζ) will be allowed first to execute their action as promoters of insulin signaling before they induce the phosphorylation of IRS proteins, as part of a negative feedback mechanisms that will terminate their own activation. Then, how these kinases are targeted toward their different substrates (e.g., S6 the "positive" signal vs. IRS-1 the "negative" signal) is currently unknown. The issue of stimulatory vs. inhibitory Ser sites of IRS-1 also needs further clarification. Does phosphorylation of stimulatory sites precede that of inhibitory sites; and if so, what regulates this process? The issue of "priming" deserves attention. For example, does phosphorylation of IRS proteins at stimulatory Ser sites "tag" the protein to further phosphorylation at inhibitory Ser sites?

The array of Ser/Thr phosphatases that dephosphorylate the different Ser residues and reset the system to its basal state have not been elucidated. It is only conceivable to assume that the activity of these phosphatases is regulated to no lesser extent than the activity of the IRS kinases.

Several strategies could be applied to address these questions. The fast-developing field of mass spectrometry now enables the identification of arrays of Ser sites of IRS proteins that are subjected to phosphorylation under in vivo conditions. Samples of human tissues, processed to isolate their IRS proteins and subject them to such analysis, could be most insightful in defining the extent of phosphorylation of each site under physiological or pathological conditions.

Introduction of siRNA technology could help decipher the role of individual kinases, or a kinase combination thereof, in regulating IRS protein function. Combined with tissue-specific knockout of given kinases, this strategy could enlighten our understanding about the IRS kinases activated under different biological conditions. Then, knock-in into IRS-1/2-null mice of IRS proteins mutated at selected Ser phosphorylation sites should provide insight into the role of selected Ser sites in the regulation of IRS proteins function.

Addressing these and related questions awaits further studies that will lead us to a better understanding of this complex process. Such research has much clinical relevance and physiological importance, as it might direct us toward new potential therapeutic strategies to treat insulin resistance and diabetes.

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