From signal transduction to signal interpretation: an alternative model for the molecular function of insulin receptor substrates

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From signal transduction to signal interpretation:

An alternative model for the molecular function of insulin receptor substrates

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Abstract

The insulin receptor (IR) recruits adaptor proteins, so-called insulin receptor substrates (IRS), to connect with downstream signalling pathways. A family of IRS proteins was defined based on three major common structural elements: Amino-terminal PH and PTB domains that mediate protein-lipid or protein-protein interactions, mostly carboxy-terminal multiple tyrosine residues that serve as binding sites for proteins that contain one or more SH2 domains and serine/threonine-rich regions which may be recognized by negative regulators of insulin action. The current model for the role of IRS proteins therefore combines an adaptor function with the integration of mostly negative input from other signal transduction cascades allowing for modulation of signalling amplitude. In this review we propose an extended version of the adaptor model that can explain how signalling specificity could be implemented at the level of IRS proteins.
Introduction

Insulin resistance is most often associated with defective or insufficient intra-cellular signal transmission between the insulin receptor (IR) and its downstream mediator protein kinase B (PKB/Akt). Many studies have therefore been undertaken to elucidate the underlying molecular mechanisms and it has been postulated that insulin resistance might arise due to inappropriate activation of Ser/Thr kinases which is believed to inhibit insulin signalling at the level of insulin receptor substrate (IRS) proteins (Boura-Halfon and Zick, 2009; Siddle, 2011). In contrast to Tyr phosphorylation which is described as activating, Ser/Thr phosphorylation of IRS proteins is usually considered as inhibitory for insulin signalling. However, this rather simple concept cannot explain the great complexity of IRS functions. In this review we summarize the current view of how IRS proteins function in insulin signalling under homeostasis and how their failure is believed to contribute the development of insulin resistance. Based on theoretical considerations and some of our own new data we extend the current conventional view of how IRS proteins function and present an alternative hypothetical model to more accurately describe the molecular role of IRS proteins in signal transduction in general and in the control of glucose homeostasis in particular. According to our model IRS proteins do not merely act as adaptor proteins within individual pathways but rather as integrators of signalling information. We suggest that signalling information from different pathways and cues can be “encoded” on IRS proteins in the form of specific patterns of phosphorylation and that “decoding” is accomplished by the formation of corresponding protein complexes on IRS. In our model, not only the amplitude but also specificity of downstream signalling are a function of the composition of these complexes providing a molecular basis for the implementation of context-dependent signal transduction. We discuss implications of our model with a focus on the development of insulin resistance and type 2 diabetes.

Insulin signalling and insulin receptor substrates

The insulin receptor (IR) belongs to the superfamily of transmembrane receptor kinases and is composed of two α- and two β-subunits that are connected through disulfide bridges. The extra-cellular α-subunits each contain a binding site for insulin, whereas the transmembrane β-subunits contain insulin-regulated protein tyrosine kinases on their cytosolic part (Perz and Torlinska, 2001; White, 1997). Upon binding of insulin the tyrosine (Tyr) kinases of the IR are activated resulting in cross-autophosphorylation of tyrosine residues in the β-subunits: Tyr\textsubscript{960} in the juxtamembrane region, Tyr\textsubscript{1146}, Tyr\textsubscript{1150} and Tyr\textsubscript{1151} in the regulatory loop and Tyr\textsubscript{1316} and...
Tyr\textsubscript{1322} in the COOH-terminus (White, 1997). Insulin receptor substrates (IRS) bind to phosphorylated Tyr residues in the activated receptor and upon this interaction the receptor-intrinsic kinase activity phosphorylates Tyr residues within IRS. Besides IRS other target proteins of the IR Tyr kinase exist, such as Shc, Gab-1, Cbl, APS, and p60dok, however, these will not be covered here. IRS proteins do not contain any catalytic activity. Instead, they are thought to act as adaptors linking the activated IR to two major metabolically relevant downstream activities: phosphatidyl inositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK). A comprehensive review of the insulin/IGF signalling system and its components has been recently presented elsewhere (Siddle, 2011).

There are six main IRS family members (IRS1-6) of which IRS1-4 are the most studied sharing well conserved NH2 termini but fewer similarities in their C-termini. Relatively little is known about the function of IRS5 and IRS6. They are only distantly related to IRS1-4 and appear to be poor substrates for the IR (Versteyhe et al., 2010). While humans only express IRS1, IRS2 and IRS4, rodents also express IRS3. For metabolic regulation IRS1 and IRS2 appear to be the predominant isoforms, as demonstrated by the phenotypes of IRS-deficient mice (Araki et al., 1994; Fantin et al., 2000; Kubota et al., 2000; Liu et al., 1999; Tamemoto et al., 1994; Withers et al., 1998).

Binding of IRS proteins to the activated IR depends on two NH2-terminal regions, the phosphotyrosine-binding (PTB) and plextrin homology (PH) domains. Downstream signalling proteins, for example the regulatory subunit of PI3K (p85), GRB-2, and SHP2, bind to Tyr-phosphorylated IRS typically via src homology (SH2) domains (White, 2002). Indeed, a great number of Tyr are scattered mostly over the C-terminal parts of IRS1 and IRS2 and Tyr phosphorylation is essential for insulin signal transduction. There is some degree of conservation in the Tyr-motives of IRS1 and IRS2 but some sites are unique (Pirola et al., 2004; White, 1997). A comprehensive study has revealed the potential phospho-Tyr interactome of IRS1 and IRS2 by quantitative interaction proteomics (Hanke and Mann, 2009). Interaction partners for 52 out of 109 investigated sites were determined in a pulldown approach using lysates of the murine muscle cell line C2C12 with phosphorylated versus non-phosphorylated bait peptides, combined with quantitative proteomics. A large number of common interactors were identified in line with extensive functional redundancy between IRS1 and IRS2. However, several proteins involved in signalling and metabolism interacted differentially with IRS1 and IRS2 also indicating different physiological roles.
IRS proteins and the development of insulin resistance

Insulin resistance is a condition in which target organs show a reduced responsiveness to the action of insulin. Insulin resistance is associated with unusually high concentrations of circulating insulin and impaired glucose tolerance. Muscle and fat cells take up less glucose and store less glycogen and triglycerides in response to stimulation with insulin whereas liver cells fail to adequately reduce glucose production and its release into the blood stream. It can occur in the course of physiological fluctuations of insulin sensitivity during normal life cycle (puberty, pregnancy and aging). On the other hand, genetic predisposition, obesity and physical inactivity can result in persistent insulin resistance (Kahn et al., 2006). Altered secretion of cytokines and metabolites from fat tissue in obese subjects might contribute to the development of insulin resistance.

Although the principle molecular events of insulin signal transduction are well studied and understood in great detail, no conclusive explanation is available for how insulin resistance develops. A widely discussed concept, however, involves activation of Ser/Thr kinases that can phosphorylate numerous sites in IRS1 and IRS2. Phosphorylation of IRS proteins on Ser/Thr residues can uncouple the activated IR from downstream signal transduction (reviewed in (Boura-Halfon and Zick, 2009)). Ser/Thr phosphorylation might prevent docking of IRS to the IR, which either results in ubiquitination followed by proteolytic breakdown of IRS or in prevention of docking of downstream effectors such as PI3K. Several negative inputs converge at the level of IRS proteins. Highly relevant in the context of the metabolic syndrome appear to be increased levels of circulating pro-inflammatory cytokines produced and secreted by adipocytes (adipokines, (Sell and Eckel, 2010; Trayhurn et al., 2011)). Among the various pro-inflammatory cytokines, TNF-α was first recognised as an insulin resistance-inducing factor (Hotamisligil et al., 1995; Hotamisligil and Spiegelman, 1994). Pro-inflammatory cues can activate the inhibitor of κB kinase (IKK) /NF-κB axis which is now regarded as a critical pathway linking obesity-associated chronic inflammation with insulin resistance (Arkan et al., 2005; Shoelson et al., 2003). IKKβ is a Ser/Thr kinase that phosphorylates the inhibitor of NF-κB (IκB) during inflammation. Earlier studies (de Alvaro et al., 2004; Gao et al., 2002) indicated that IKK represses insulin signalling via serine phosphorylation of IRS1 at position 307, however, more recent results suggest that several other sites might also be involved (Herschkovitz et al., 2007). Mice that are hemizygous for IKK show lower fasting blood glucose and insulin levels and improved free fatty acid levels relative to littermate controls when placed on a high-fat diet (Yuan et al., 2001). Adipocyte-derived factors can act via IKK to induce insulin resistance in skeletal muscle (Dietze et al., 2004). Insulin-induced activation of Akt/PKB in myocytes was reduced after culture in adipocyte-conditioned medium and insulin sensitivity could be restored by inhibiting IKK.
Various cellular conditions known to be associated with the development of insulin resistance and type 2 diabetes mellitus, e.g. hypoxia, endoplasmic reticulum (ER) stress and the accumulation of reactive oxygen species, have been reported to increase the activation of Ser/Thr kinases with concomitant downregulation of IRS function. Kinases activated under these conditions are also called stress kinases. Also increased levels of circulating cytokines, as observed under systemic low-level inflammation during obesity, can result in activation of stress kinases (Boura-Halfon and Zick, 2009). Among them are p38 and several isoforms of the PKC family. The PKC family consists of 12 isoforms classified as follows: Atypical PKCs (ζ and λ), conventional PKCs (α, β and γ), novel PKCs (δ, ε, η and θ), and protein kinase Ns (PKN1, PKN2 and PKN3), from which PKCδ, PKCλ/ζ and PKCθ are known to target IRS. A well-studied case is the activation of JNK downstream of ER stress and the unfolded protein response (Ozcan et al., 2004; Xu et al., 2010). Obesity in humans and rodents was shown to be associated with the development of ER stress in hepatocytes and adipocytes leading to JNK-dependent phosphorylation/inhibition of IRS1 on Ser307 (Aguirre et al., 2000). Indeed, global or conditional loss of JNK in adipose tissue, skeletal muscle or brain attenuates diet-induced insulin resistance in mice (Sabio et al., 2010). However, mice in which the target site for JNK in IRS1 (Ser307) was replaced by alanine were less insulin sensitive, as were mice lacking JNK1 in hepatocytes (Copps et al., 2010; Sabio et al., 2010), indicating that JNK is also required for insulin action in hepatocytes. As in the case of JNK a significant number of IRS kinases are also required for insulin-dependent metabolic control. For example, ERK1/2 are believed to link insulin with cell proliferation, differentiation and the regulation of lipid metabolism, glycogen synthase kinase (GSK)-3 is required to regulate glycogen stores in muscle and liver, S6K to control protein synthesis, and isoforms of PKC may be required for insulin-induced glucose transport (Avruch, 1998; Bandyopadhyay et al., 2000; Bandyopadhyay et al., 2002; Copps et al., 2010; Kotzka et al., 2004; Martin and Parton, 2006; Roth et al., 2000; Ryden et al., 2004; Sajan et al., 2006).

**Complex roles of IRS proteins in signal transduction**

As described above, the interaction of IRS proteins with the activated IR and concomitant phosphorylation of Tyr sites has been recognized as the primary molecular mechanism underlying signal transmission via IRS proteins. However, recruitment of IRS proteins and activation of insulin signal transduction is never an isolated event but occurs in the presence of other extra-cellular cues, such as other hormones, cytokines or nutrients. Importantly, IRS proteins are not only recruited by the IR but they are also employed by other receptors for
transduction of their respective signal and, as pointed out above, also a significant number of
intra-cellular kinases target IRS proteins, mostly on Ser/Thr residues. Among other receptors
are IGF-I/IIIR, cytokine receptors like IL-4R, -9R, -13R and -15R, growth hormone (GH)
receptor, prolactin receptor, and integrin αvβ3 receptor (Yenush and White, 1997). How
specific signal transduction can be accomplished when different receptors recruit the same
substrates is an unresolved issue.

Another level of complexity was revealed by the analyses of genetic mouse models showing
that IRS1 and IRS2 have tissue-specific non-redundant roles in the regulation of glucose
homeostasis. While deficiency for IRS1 leads to predominantly peripheral effects, such as
reduced insulin-dependent glucose uptake into muscle and adipose tissue, loss of IRS2 in
addition is associated with β-cell failure (Araki et al., 1994; Kubota et al., 2000; Tamemoto et
al., 1994; Withers et al., 1998). The evidence for tissue-specific functions of IRS1 and IRS2
has been extensively reviewed (Thirone et al., 2006). It is important to point out that IRS1
and IRS2, when expressed in the same cell, can have different functions downstream of the
IR. For example, Huang et al. (Huang et al., 2005) showed that insulin-induced PKBα/Akt1
and PKBβ/Akt2 phosphorylation was reduced in myotubes transfected with siIRS1, but only
PKBβ/Akt2 phosphorylation was reduced in myotubes transfected with siIRS2. Similar
complexity regarding the functions of IRS1 and IRS2 in skeletal muscle was also revealed in
another study (Bouzakri et al., 2006). We observed increased proliferation in rat pancreatic
islets overexpressing IRS2, but not IRS1 (Mohanty et al., 2005). Another study utilised
shRNA to specifically knock-down IRS1 or IRS2 in liver of wild type mice (Taniguchi et al.,
2005). Reduction of IRS1 increased expression of gluconeogenic genes and decreased
expression of glucokinase, linking IRS1 to hepatic glucose metabolism. Downregulation of
IRS2 increased expression of important lipogenic genes showing that IRS2 is more closely
linked to lipid metabolism in liver. Specific functions of IRS1 and IRS2 might result due to
differential sub-cellular localization (Siddle, 2011; Taniguchi et al., 2006), however, the
molecular mechanisms that would specifically target IRS proteins to different cellular
compartments remain elusive. Specificity in function is difficult to reconcile with the molecular
structure of IRS1 and IRS2 since both contain highly homologous PH and a PTB domains
and similar sets of interaction sites for PI3K(p85), Grb2, JNK and other downstream targets.
How can specificity be implemented despite the use of the same downstream signal
transduction molecules? These observations are not easy to reconcile with a simple adaptor
model. We have therefore developed an alternative model to better describe the role of IRS
proteins in intra-cellular signal transduction. In essence we postulate that IRS proteins should
rather be described as integrators of multiple pathways and cues instead of being regarded
as adaptors within individual pathways.
A hypothetical model for the function of IRS proteins in signal transduction

Every cell in our body is exposed to a very large number of external cues at any given time. This complex “context” is processed using intra-cellular signalling pathways that convert multiple inputs into specific cellular decisions. Although individual receptors and pathways have been extensively studied, it is only poorly understood how multiple cues can be integrated. A simple example of integration over only two cues involves insulin and epidermal growth factor (EGF). The epidermal growth factor receptor (EGFR) and the IR can both regulate proliferation, differentiation, cell metabolism, survival, and apoptosis (Lemmon and Schlessinger, 2010; Taniguchi et al., 2006). However, insulin signalling mainly controls energy homeostasis, whereas EGF elicits mainly proliferative responses. Nevertheless, insulin can enhance EGF-stimulated ERK activation, DNA synthesis, and cell proliferation (Chong et al., 2004; Crouch et al., 2000; Ediger and Toews, 2000). Under some conditions, EGF can evoke metabolic responses, e.g. GLUT4 translocation (Gogg and Smith, 2002; Ishii et al., 1994), whereas insulin can be mitogenic (Ish-Shalom et al., 1997; Papa et al., 1997). In order to embrace such complexity and to account for interactions between pathways many authors describe signal transduction in terms of networks rather than in terms of individual pathways (Bhalla and Iyengar, 1999). Tanigushi et al. (Taniguchi et al., 2006) recently proposed that in order to understand any particular ligand-receptor system so-called “critical nodes” should be determined within this network. They postulated that critical nodes must “consist of several molecular isoforms that are involved in divergent signalling, they are highly regulated (both positively and negatively), they are essential for the biological actions of the ligand, and in many cases, they are points of crosstalk with other signalling systems”.

Network nodes as defined by Tanigushi et al. provide a basis for modulation of the amplitude of signal transduction but the question remains how signalling information is actually processed or interpreted. We have developed a model to address this problem. We propose that cells can integrate over different stimuli (context) by simultaneous modification of the numerous phospho-sites on IRS proteins leading to the formation of context-dependent phosphorylation patterns. We describe this process as encoding to which also post-translational modifications other than phosphorylation could contribute (e.g. O-linked N-acetylglucosamine modification (Klein et al., 2009)). Decoding is accomplished by the formation of specific protein complexes on individual IRS molecules dependent on these patterns. A context-specific phosphorylation pattern corresponds to a specific protein complex composed of a certain set of downstream mediators. Inclusion or exclusion of
mediators links or disconnects, e.g., the IR to or from insulin-dependent cellular functions, respectively. Mechanistically, phosphorylation sites do not necessarily represent binding sites but could instead mediate the induction of conformational changes of the IRS molecules to expose or conceal other interfaces that can be recognized by binding partners. The respective composition of the protein complexes reflects the context to which a cell is exposed at a given time. Our model provides a molecular explanation for how signalling information can be integrated and processed on a single molecule (a single IRS). Linking of pathways, modulation of amplitude and processing of information can occur simultaneously. Our model would also explain compartmentalization of IRS proteins and the tissue-specific use of IRS isoforms. For example, predisposition for the use of IRS1 and IRS2 in hepatocytes for glucose- and lipid metabolism, respectively (Taniguchi et al., 2005), could depend on specific phosphorylation patterns and corresponding protein complexes that incorporate the activities required for regulating the respective task and the specific binding partners for targeting them into the appropriate sub-cellular compartments. The many known binding partners of IRS are clearly good candidates to take part in the formation of the decoding complexes. Among them are SH2 domain-containing proteins such as PI3K(p85), Grb2, Nck, Crk, Fyn, Csk, Ship, and SHP2 as well as others lacking SH2 domains, e.g. Ca2+ ATPases (SERCA1 and SERCA2), and adaptor protein 14-3-3. There are four major isoforms of IRS each with many phosphorylation sites and, in addition, not all molecules of a single isoform of IRS in a given cell might carry the same pattern of modifications. Therefore, the coding/decoding system as we describe corresponds well to the very high complexity of cellular context. For example, IRS1 (mouse) contains 34 tyrosine and many more serine/threonine residues allowing for a very high number of possible phosphorylation patterns through combinatorial phosphorylation.

To some degree, the integration function of IRS proteins as described in our model is reminiscent of A-kinase anchoring proteins (AKAPs) that tether the cAMP-dependent protein kinase (PKA or A-kinase) holoenzyme (Welch et al., 2010). The AKAP family consists of over 40 members that can all bind to the regulatory subunit of PKA and act to concentrate PKA activity within certain sub-cellular compartments. Because AKAPs also interact with other signalling proteins they can integrate PKA-dependent signal transduction with other pathways. The versatility of AKAPs is further increased by alternative splicing which generates more than one isoform from single AKAP-encoding loci. In the case of AKAPs variation appears to arise to a large extent through a very large number of family members and isoforms while integration and processing of signalling information via IRS proteins as
described in our model mainly relies on combinatorial modification of individual IRS molecules.

**Identification of new IRS-interacting proteins reveals a high degree of interconnectedness**

In relation to the very high encoding potential, as estimated based on the large number of sites that could be phosphorylated, the number of known IRS-interacting proteins is small pointing out an apparent discrepancy between coding and decoding potential. Secondly, given that the known interaction partners are mostly kinases, phosphatases or other adaptor proteins we found it difficult to explain how the observed versatility of IRS proteins could be achieved or how differential interaction with these factors could result in differential specific sub-cellular localization. These considerations prompted us to search for more and previously unknown binding partners of IRS. To this end, 2-hybrid screening was performed with IRS2 as bait against a rat muscle cDNA library. Five different transcription units were identified in this manner: 14-3-3, an already known IRS-interacting protein, confirming the validity of the screen and four previously unknown interactors, filamin A, filamin C, glucose-regulated protein (Grp) 78, and titin. All interactions were confirmed in GST-pulldown assays and later in immuno-precipitations. The actin cross-linking proteins Filamin A and Filamin C as well as the glucose-regulated chaperon Grp78/Bip were confirmed to interact with both IRS1 and IRS2. Indeed, co-localization of both IRS1 and IRS2 with ER marker proteins was reported previously (Borge and Wolf, 2003). In contrast, titin, a sarcomer-spanning protein, interacted only with IRS2. We realize that only a small number of new interacting proteins was identified in our 2-hybrid screen, however, this might have resulted due to differential post-translational modifications of proteins in yeast compared to mammalian cells. Indeed, Ni-NTA-based pulldown of HIS-tagged IRS1 and/or IRS2 ectopically expressed in the rat-derived beta cell line INS-1E resulted in detection of a significantly greater number of co-purifying proteins (not shown). As suggested by the identity of the binding partners that we detected, IRS proteins might connect insulin signalling to cellular functions dependent on, e.g. the cytoskeleton (Filamins A/C), the sarcomere (Titin) and the endoplasmic reticulum (ER, Grp78/Bip). Regulated interaction between IRS isoforms and these proteins could underlie differential sub-cellular localization of IRS proteins for the targeted clustering of signal transduction complexes. It is well-known that insulin can induce actin remodelling and insulin-dependent glucose transport in skeletal muscle is sensitive to cytochalasin D, an inhibitor of actin polymerization, indicating that actin remodelling is required for GLUT4
Implications and conclusions

In the majority of studies insulin sensitivity at the signalling level is analysed by determining quantitative changes in the activation of perceived key elements of the canonical insulin signal transduction cascade as e.g. PKB/Akt or ERK1/2 by Western blotting or immunohistochemistry. However, if IRS proteins do indeed act as integrators in signal transduction thereby modulating specificity as described in our model, the amount of activation of PKB/Akt might not correlate well with a given read-out such as e.g. glucose transport. Cases in which insulin’s ability to elicit biological effects in target cells does not correlate well with the amount of intra-cellular PKB/Akt activity have been reported (Hoehn et al., 2008; Whitehead et al., 2001; Xu et al., 2010). For example, the maximal effect of insulin on GLUT4 translocation in L6 myotubes was observed at concentrations where only 5% of the total Akt/PKB pool was phosphorylated (Hoehn et al., 2008) and we described that insulin-induced...
glucose transport can be normal in 3T3-L1 adipocytes at very low levels of Akt/PKB activation (Xu et al., 2010), indicating that yet undefined qualitative differences might exist.

The molecular function of IRS proteins as proposed here provides a molecular basis for the implementation of context and specificity not only for insulin signal transduction but also for a significant number of other pathways. In this respect “encoding” of extra- and intra-cellular context by patterns of phosphorylation on IRS and “decoding” by the formation of pattern-specific protein complexes could be the physical basis for a number of phenomena observed in the context of the regulation of glucose homeostasis, such as multi-organ cross-talk and adipokine-dependent modulation of insulin sensitivity (Lehr et al., 2012; Rytka et al., 2011; Trayhurn et al., 2011).

According to our model insulin resistance is a consequence of the integration of many different intra-cellular and extra-cellular cues at the level of IRS proteins. In this sense, insulin resistance is not a result of defective insulin signalling but of proper integration of context into the cellular signal transduction network. Intervention to change the context should therefore be the most intuitive and straight forward approach to counteract insulin resistance. Indeed, often lifestyle change with concomitant improvement in blood parameters such as adipokines and lipid profile is most successful to improve metabolic control in individuals affected by the metabolic syndrome (Mikus et al., 2011; Roberts et al., 2006). Furthermore, specific interference with the interaction of IRS and its binding partners might open a way for safe therapeutic interventions to improve insulin sensitivity without unwanted global activation of proliferation factors such as PKB/Akt.

The hypothetical model described in this review can only be correct based on two premises: Both, IRS molecules with context-dependent phosphorylation patterns and correlating protein complexes, must exist. Mass spectrometry appears to be the method of choice to test both premises. However, even individual IRS isoforms might occur in more than one intra-cellular pool and each pool could represent a different pattern of phosphorylations. This would significantly complicate the analysis since in our model a pattern is defined as being associated with individual IRS molecules. Even though our model certainly needs to be consolidated, we are hopeful that its presentation will stimulate discussion and contribute to the development of new ideas about how cells process signalling information in general and how these might be linked to such phenomena as the development of insulin resistance.

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Declaration of interest

The authors report no conflicts of interest
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