

Insulin Receptor Substrate-1 Regulation in Health and Disease

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IRS-1 regulation in health and disease

Insulin is the major anabolic hormone and its primary function is to maintain glucose homeostasis. Insulin's ability to achieve this is impaired in states of insulin resistance (characterised by a failure of peripheral tissues to respond appropriately to a given dose of insulin) and Type 2 diabetes. The latter is further complicated by an inability of the pancreatic β -cells to compensate for peripheral insulin resistance. The incidence of these conditions, which occur as a result of a complex interplay between genetic and environmental factors, is increasing to epidemic proportions. This is attributed to changes in our environment, especially the adverse changes in our diet and exercise, which are also strongly associated with increasing obesity.

Insulin employs intricate signalling pathways to elicit its effects in responsive tissues, including skeletal muscle, liver and fat. The molecular coordination of this signalling is complex, occurring at multiple levels. This allows integration of inputs from multiple hormonal and nutritional systems, and so facilitates both coarse and fine-tuning of insulin action. However, this also renders insulin action susceptible to impaired regulation as a result of perturbations in these systems. Thus, as our comprehension of insulin signalling expands, so too does our understanding of the ways these pathways may become dysregulated in today's environment. By consider-

ing the regulation of Insulin Receptor Substrate-1 (IRS-1) function, a protein central to insulin signalling, we aim to highlight important regulatory elements that function at the molecular level. We also discuss how analogous mechanisms may be employed by pathogenic factors resulting in impaired IRS-1 function, a common finding in studies of people with insulin resistance or diabetes.

Regulation of IRS-1 in insulin signalling - a story of phosphorylation and localisation

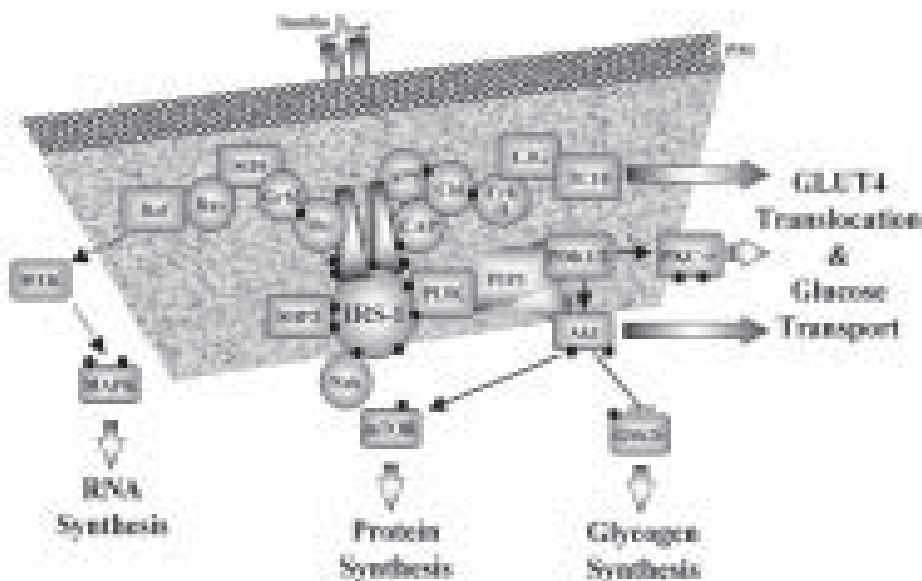
Tyrosine phosphorylation

The IRS proteins play a central role in insulin signalling (Fig. 1). To date, four members of this family of scaffold molecule have been identified in mammalian cells (IRS-1 to IRS-4), with each sharing a similar structure. Evidence suggests that IRS-1 functions primarily in skeletal muscle and fat whilst IRS-2 plays the dominant role in liver. Like many scaffold proteins, IRS-1 contains discrete modular domains. The best characterised of these are the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains at the N-terminus (Fig. 2), which appear to couple IRS-1 to the activated IR (1).

The C-terminal region of IRS-1 contains a number of consensus tyrosine phosphorylation motifs that are phosphorylated directly by the IR. Whilst there may be some degree of redundancy, it is clear that specific phosphotyrosines are involved in recruitment of certain

Fig. 1. Insulin signalling pathways.

Insulin signalling begins at the cell surface when insulin binds its cognate receptor, a transmembrane tyrosine kinase receptor, resulting in insulin receptor (IR) autophosphorylation and activation. The activated IR phosphorylates several adaptor/scaffold molecules including the Insulin Receptor Substrate (IRS) family of proteins, Shc, APS, CAP and c-Cbl. Tyrosyl phosphorylation of these proteins within specific motifs serves to recruit downstream effectors. Recruitment of the Grb2/SOS complex to tyrosyl phosphorylated Shc leads to activation of the MAPK cascade, which promotes transcription. Phosphorylation of IRS-1 (or IRS-2) leads to recruitment of effectors such as the protein phosphatase SHP2, the adaptor protein Nck and the lipid kinase, PI3K. Activation of PI3K leads to generation of PIP_3 in the inner leaflet of the plasma membrane, resulting in recruitment and activation of Ser/Thr kinases including PDK1/2, the atypical PKCs ($PKC\lambda/\zeta$) and Akt. Phosphorylation of downstream substrates, including the mammalian Target of Rapamycin (mTOR) and Glycogen Synthase Kinase-3 (GSK3 β) promotes increased protein and glycogen synthesis. Phosphorylation of APS, CAP and c-Cbl leads to recruitment of CrkII and C3G which promotes the activation of TC10. The activation of Akt, $PKC\lambda/\zeta$ and TC10 are all required for maximal insulin-stimulated translocation of the sugar transporter GLUT4 (not shown) from an intracellular compartment to the plasma membrane where it facilitates increased glucose transport into the cell.



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effectors, by virtue of their Src homology 2 (SH2) domains. For example, PI3K (phosphoinositide 3-kinase) binds principally to phosphotyrosine 608 and 941 whilst the protein tyrosine phosphatase SHP2 binds at phosphotyrosines 1172 and 1222 (2). Importantly, recruitment to IRS-1 serves to promote translocation and activation of these enzymes.

Localisation

As with other signalling pathways, the appropriate localisation of signalling molecules is necessary for efficient insulin signalling. It has been known for some time that IRS-1 exhibits a characteristic subcellular distribution. This appears to be important for function and subject to regulation. Upon subcellular fractionation by differential centrifugation, the majority of IRS-1 is found in an ill-defined fraction termed the High Speed Pellet (HSP). Also present in this fraction are low-density microsomes and vesicles, including the insulin responsive GLUT4 vesicles, as well as cytoskeletal elements (3). IRS-1 appears to be anchored to this fraction by virtue of protein-protein interactions, leading to the suggestion that IRS-1 may be present in large pre-assembled complexes, possibly in association with the cytoskeleton (3). Evidence suggests that such anchoring of IRS-1 affords it close proximity to the activated IR ensuring rapid and efficient tyrosine phosphorylation. Redistribution of IRS-1 from the HSP to the cytosol is promoted by several treatments including chronic insulin and occurs concomitant with a reduction in insulin-stimulated tyrosine phosphorylation of IRS-1 (4). Further evidence that this HSP fraction represents the active pool of IRS-1 comes from the observation that PI3K is recruited and activated specifically in the HSP in response to insulin (3).

Serine/threonine phosphorylation

In addition to tyrosine phosphorylation IRS-1 is also subject to extensive Ser/Thr phosphorylation. Given the large number of putative Ser/Thr phosphorylation sites (over 50) and the likelihood that Ser/Thr phosphorylation may be organised in a hierarchical manner that is also coupled to the tyrosine phosphorylation status, understanding how these events are coordinated at the molecular level represents a formidable task. That said, we are beginning to understand how such events impact on the overall function of IRS-1 and insulin signalling. Many recent studies have focused on the role of Ser/Thr phosphorylation of IRS-1 as a mechanism of negative feedback or signal attenuation that uncouples IRS-1 from upstream and downstream effectors. Before considering these events we will briefly describe evidence that suggests that Ser/Thr phosphorylation of specific, as yet unidentified, sites may serve as a positive effector of IRS-1 function in insulin signalling. First, IRS-1 isolated

from quiescent cells is phosphorylated on Ser/Thr residues (5). Removal of these phosphates, by treatment with alkaline phosphatase, reduces the ability of IRS-1 to act as a substrate of the IR *in vitro* (6). This suggests that basal Ser/Thr phosphorylation of IRS-1 may play an important positive role in subsequent tyrosine phosphorylation by the IR. Second, following insulin stimulation IRS-1 is phosphorylated at serine residues within the PTB domain by Akt (otherwise known as PKB, protein kinase B; further abbreviations are defined in the legend to Fig. 1, and also in the text below), a Ser/Thr kinase that is activated downstream of PI3K (Fig. 1). This phosphorylation appears to protect IRS-1 from the action of tyrosine phosphatases, so maintaining IRS-1 in the tyrosine phosphorylated active form (7).

A growing body of literature provides compelling evidence that Ser/Thr phosphorylation of IRS-1 represents an important mechanism involved in insulin signal attenuation. Upon insulin treatment, IRS-1 exhibits a characteristic retardation of mobility on SDS-PAGE. Although not often formally tested, this is generally assumed to be indicative of hyperphosphorylation on Ser/Thr residues and occurs subsequent to tyrosine phosphorylation. Inhibition of PI3K prevents this, whereas expression of constitutively active PI3K is sufficient to mimic this effect. Multiple Ser/Thr kinases are activated downstream of PI3K upon insulin treatment (Fig. 1). Of these, several have been shown to phosphorylate IRS-1. These include PI3K itself, Akt (see above), atypical PKC ζ and mTOR (serine 632, 662 & 731) (see ref. 8 for review). In contrast to the positive effects of phosphorylation by Akt, phosphorylation by atypical PKC ζ or mTOR seems to inhibit insulin-stimulated tyrosine phosphorylation of IRS-1, in part by reducing the interaction between the IR and IRS-1 (9). Logically, the action of these Ser/Thr kinases should be orchestrated temporally to enhance activation of IRS-1, by Akt phosphorylation, followed by signal termination, by mTOR and atypical PKC ζ phosphorylation.

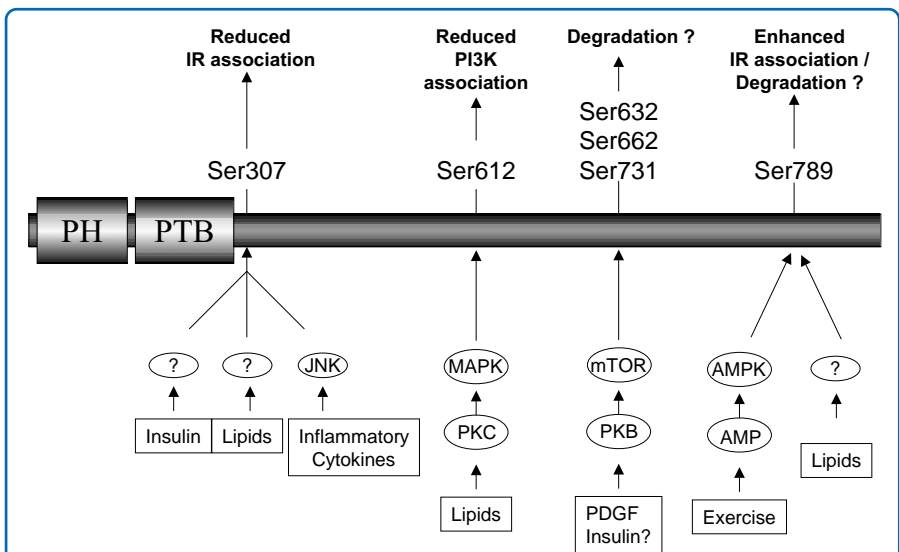


Fig. 2. Known sites of IRS-1 Ser/Thr phosphorylation: potential pathways and consequences.

The effects of insulin may represent not only negative feedback regulation but also aberrant signal downregulation in the case of chronic hyperinsulinaemia. The latter is a consequence of insulin resistance prior to overt diabetes, and so can be considered as a secondary effect that compounds defective IRS-1 action (IRS-1 numbering refers to the rat primary sequence).

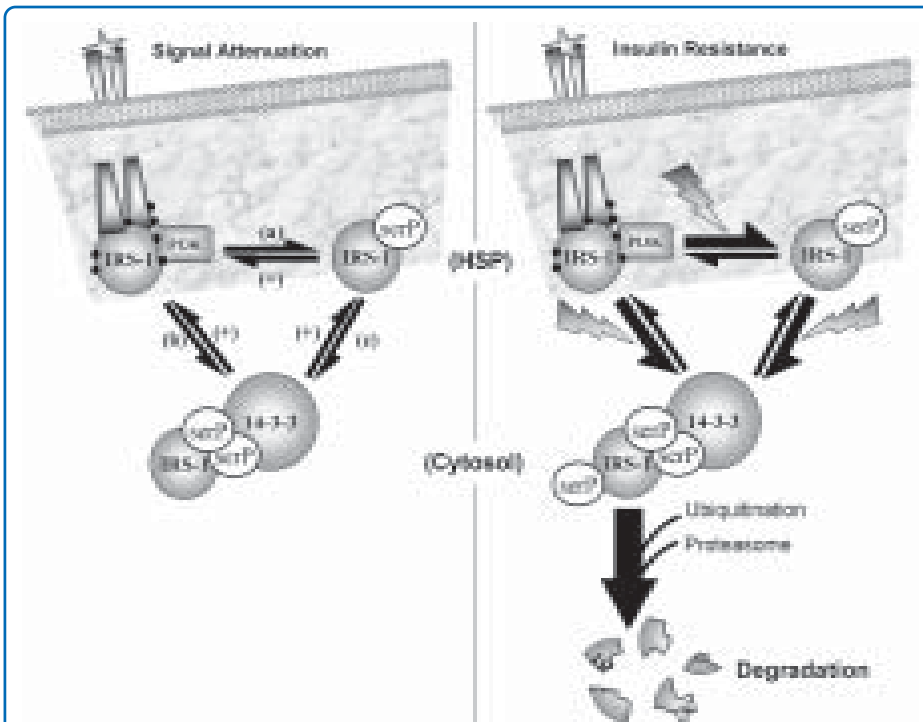


Fig. 3. Regulation of IRS-1 function: signal attenuation and insulin resistance.

Under normal conditions, IRS-1 is thought to be in a dynamic equilibrium between the HSP and cytosol, favouring the HSP. Following insulin stimulation, IRS-1 activates PI3K in the HSP fraction. This results in PI3K dependent serine phosphorylation of IRS-1, promoting dissociation of IRS-1 from the IR (a). This may be sufficient to promote translocation of IRS-1 to the cytosol (b) or may require further phosphorylation of IRS-1 at additional key serine residues (c). 14-3-3 binds to serine phosphorylated IRS-1 in the cytosol and may also serve to trigger its translocation to the cytosol. The action of Ser/Thr phosphatases would reverse these events (+). In states of insulin resistance, such as hyperlipidemia, the equilibrium is shifted promoting hyper-Ser/Thr phosphorylation of IRS-1. This leads to increased translocation of IRS-1 to the cytosol, resulting in ubiquitination and subsequent proteasomal degradation of IRS-1.

As mentioned above, the appropriate localisation of IRS-1 is important for coupling to the IR. Treatments that promote redistribution of IRS-1, such as chronic insulin, also promote its Ser/Thr hyperphosphorylation. Thus, translocation of IRS-1 may represent another feature of signal attenuation. Recently, the 14-3-3 phosphoserine binding protein has been implicated in this process (10). In response to insulin, or other agents that promote Ser/Thr phosphorylation of IRS-1, the interaction of 14-3-3 with IRS-1 was increased. The IRS-1/14-3-3 complex was found exclusively in the cytosolic fraction. Finally, the addition of a phosphorylated 14-3-3 binding peptide to cell homogenates was sufficient to promote an increase in the abundance of IRS-1 in the HSP fraction and a decrease in the cytosol. This last point is of note, not least because it implies that the association of IRS-1 to the HSP is dynamic, even *in vitro*.

Regulation of IRS-1 in insulin resistance

Given that insulin signalling is often impaired at the level of IRS-1 in humans with insulin resistance or Type 2 diabetes (11, 12), it is clearly of interest to determine whether Ser/Thr phosphorylation of IRS-1 contributes to this. Indeed, studies from rodent and cell culture models of insulin resistance suggest that this is the case. Efforts are now focussed on identifying and characterising factors and signalling pathways that promote dysregulation of IRS-1, especially those factors associated with obesity such as increased lipid availability.

Protein kinase C

In the past decade, a wealth of evidence has accumulated in support of a role for protein kinase C (PKC) isoforms. The best characterised activator of PKC is diacyl-glycerol (DAG), although other lipid species also contribute, making PKCs obvious suspects. Translocation (indicating activation) of specific PKCs, especially PKC ϵ and PKC θ , to the membrane fraction of muscle from the high-fat-fed rat (13), a well-characterised model of insulin resistance, correlate with the content of DAG. Increased PKC activity has also been observed together with diminished insulin action in several other animal and human studies as well as in lipid-treated cells (14), strongly supporting a link between muscle insulin resistance and activation of PKC by increased lipid availability.

Indirect action of PKC

Findings in cultured cells and livers from insulin resistant ob/ob mice suggest that MAP kinase, activated by PKC, is able to inhibit IRS-1 tyrosine phosphorylation and PI3K activation, through increased phosphorylation at Ser612 (which lies close to a PI3K binding site, see Fig. 2) (15). While

enhanced MAP kinase activity has not been observed in other studies of insulin resistance, this highlights the possibility that PKC may act upstream of other kinases to mediate IRS-1 phosphorylation indirectly.

More recently, re-examination of the beneficial effect of high-dose aspirin in the treatment of glucose intolerance, originally described over 100 years ago, has led to the identification of a role for I κ B kinase β (IKK β) in the inhibition of IRS-1 signalling. Activation or overexpression of IKK β attenuates insulin signalling in cultured cells, whereas IKK β inhibition reverses insulin resistance (16). Importantly, deletion of IKK β prevents insulin resistance in fat-fed mice (16). While the mechanism of IKK β activation is unclear, various PKC isoforms, including PKC ϵ and PKC θ have been shown to lie upstream of IKK β . This suggests that there is a pathway linking fat oversupply to IRS-1 modulation through PKC and IKK β , although direct phosphorylation of IRS-1 by IKK β again remains to be shown.

These kinases could also mediate insulin resistance by activation of NF κ B and enhanced transcription of inducible nitric oxide synthase (iNOS): NO production has been linked to insulin resistance in skeletal muscle, and targeted deletion of iNOS in muscle prevents insulin resistance in fat-fed mice (17). While it is therefore possible to link increased lipid availability, via PKC, IKK β , and/or iNOS, to insulin resistance at the level of IRS-1, the IRS-1 phosphorylation sites involved remain unknown.

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Phosphorylation of IRS-1 Ser307 and Ser789

In contrast, potential 'key' or 'gatekeeper' phosphorylation sites have been identified, whilst the kinases responsible remain controversial. Ser307 was initially identified as a direct phosphorylation site of JNK in response to inflammatory cytokines, which cause insulin resistance, and subsequently shown to disrupt the interaction between the IR and the PTB domain of IRS-1 (18). More recently Ser307 phosphorylation has also been reported to accompany insulin resistance upon hyperinsulinaemia (19) or acute lipid infusion (20). However, hyperinsulinaemia promotes Ser307 phosphorylation in a PI3K-dependent, JNK-independent manner suggesting that other kinases may also play a role.

A second site of interest is Ser789, which is located in a 5' AMP activated protein kinase (AMPK) consensus phosphorylation sequence. Consistent with this, AMPK phosphorylated IRS-1 on Ser789 *in vitro* and in cells treated with the AMPK activator AICAR (21). This phosphorylation enhanced insulin-stimulated activation of PI3K providing a possible mechanism by which exercise, itself a potent activator of AMPK, may contribute to improved insulin-stimulated glucose disposal. However, other studies have found an association of Ser789 phosphorylation with insulin resistance. Ser789 phosphorylation was increased in tissues from insulin resistant rodents and these tissue extracts contained increased Ser789 kinase activity (22). Although the kinase responsible has yet to be identified it does not appear to be AMPK or any of those kinases that have previously been implicated in the negative feedback mechanisms (see above).

The role of IRS-1 Ser/Thr phosphorylation in its degradation

As discussed above, Ser/Thr hyperphosphorylation of IRS-1 promotes increased translocation from the HSP to the cytosol where it is found in a complex with the phosphoserine binding protein 14-3-3 (Fig. 3). Studies from cells treated chronically with insulin, mimicking the hyperinsulinaemia observed in subjects with insulin resistance, suggest that once in the cytosol, IRS-1 is targeted for ubiquitination and subsequent degradation by the proteasome (23). In addition, cultured cells subject to nutrient oversupply in the form of excess amino acids show increased IRS-1 degradation by a PI3K-dependent pathway, possibly involving mTOR (24). Whilst incomplete, this model (Fig. 3) provides a framework to examine the precise mechanisms responsible for the targeted degradation of IRS-1. Elucidating the molecular details of this process may allow us to explain and combat the reduction of IRS-1 protein levels observed in humans (and rodent models) with obesity and/or Type 2 diabetes.

Perspectives

In conclusion, Ser/Thr phosphorylation of IRS-1 regulates the function of this central signalling molecule in several ways, ranging from IR association and tyrosine phosphorylation to localisation and degradation. An emerging consensus suggests that diabetogenic factors, such as hyperlipidaemia, promote insulin resistance at the level of IRS-1 by the inappropriate activation of feedback mechanisms employed in normal signal attenuation and by additional mechanisms.

Future advances are likely to include the identification of novel molecules that serve to anchor IRS-1 in the appropriate subcellular location and the molecular details of how such interactions are regulated, both temporally and spatially. The identification of 'gatekeeper' kinases and pivotal phosphorylation sites, and the signalling pathways that regulate these events, also represents key objectives. With this in mind, the increasing availability of phosphorylation site-specific antibodies will aid in the rapid assessment of the significance of such phosphorylation events in insulin resistant and diabetic states. The ultimate goal in such work is to define novel targets for the treatment of diabetes. While specific PKCs or IKK β may represent such molecules, further as yet unsuspected players may come to light.

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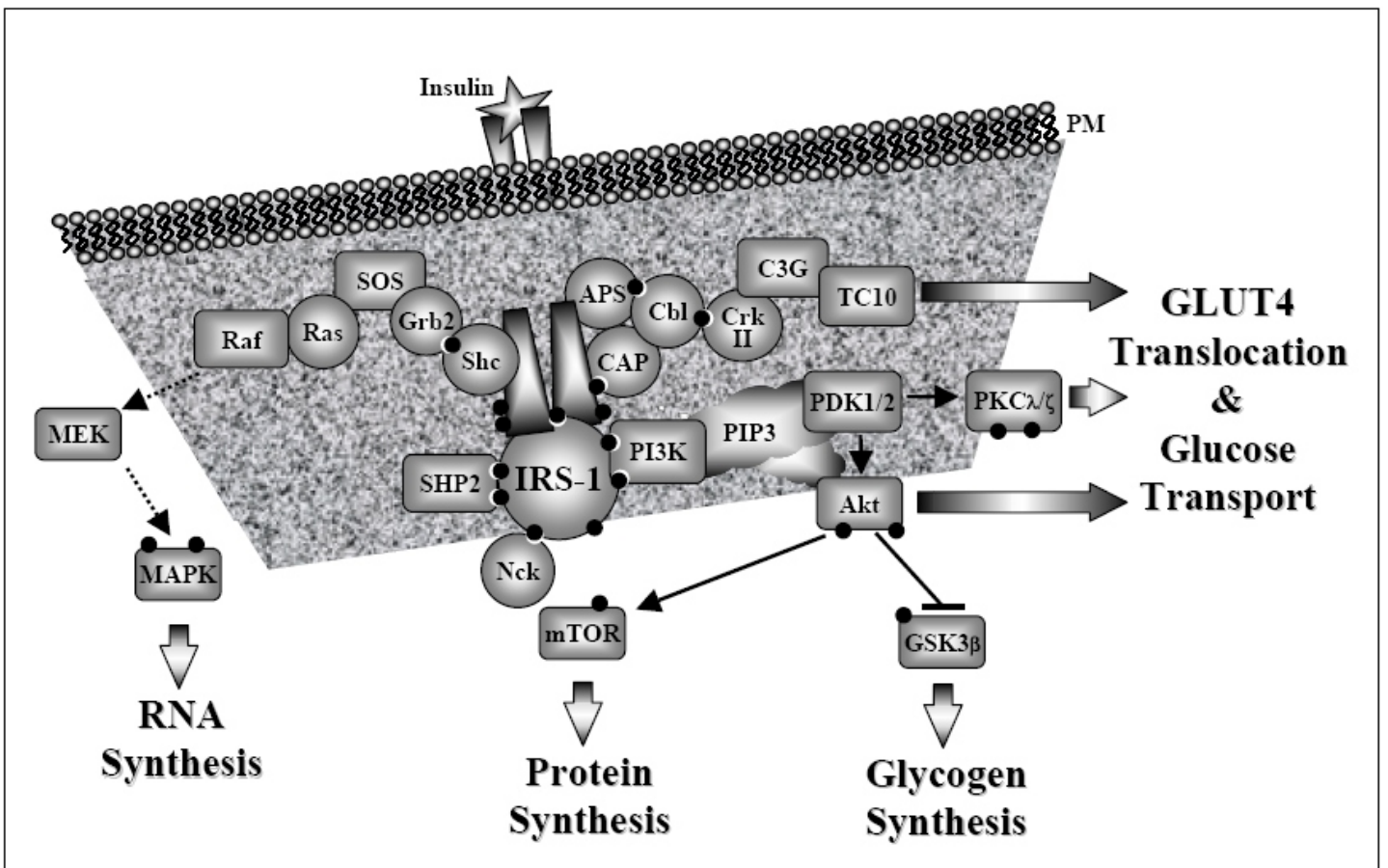


Fig. 1

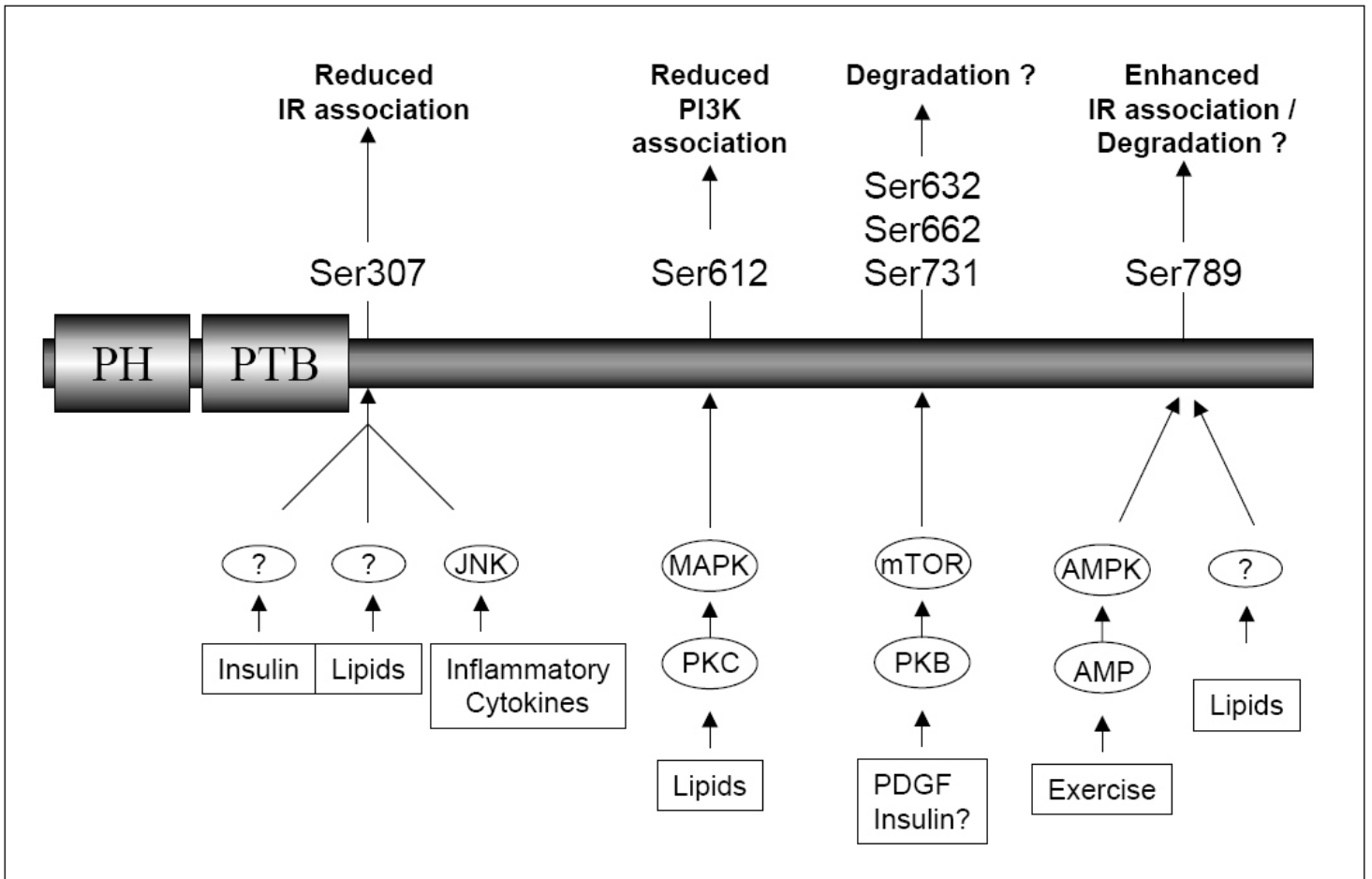


Fig. 2

