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Restraining PI3K: mTOR signalling goes back to the membrane

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The lipid kinase phosphoinositide 3-kinase (PI3K) is activated in response to various extracellular signals including peptide growth factors such as insulin and insulin-like growth factors (IGFs). Phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] generated by PI3K is central to the diverse responses elicited by insulin, including glucose homeostasis, proliferation, survival and cell growth. The actions of lipid phosphatases have been considered to be the main means of attenuating PI3K signalling, whereby the principal 3-phosphatase – phosphatase and tensin homologue deleted on chromosome 10 (PTEN) – dephosphorylates PtdIns(3,4,5)P_3, reversing the action of PI3K. Recently, however, another pathway of regulation of PI3K has been identified in which activation of PI3K itself is prevented. This finding, together with earlier work, strongly suggests that a major form of negative feedback inhibition of PI3K results from activated growth signalling via mammalian target of rapamycin (mTOR) and the p70 S6 kinase (S6K) – a pathway that could have consequences for the development of type 2 diabetes and tuberous sclerosis complex.

Members of the three classes of the phosphoinositide 3-kinase (PI3K) family of enzymes have a central role in many cellular functions, including proliferation, differentiation, cell migration, survival, glucose homeostasis and control of cell growth [1]. PI3Ks are lipid kinases whose biological responses are evoked through their ability to phosphorylate phosphoinositides [1,2]. Class 1a PI3Ks, which are the focus of this review, are heterodimers consisting of a variable catalytic subunit (p110) and a regulatory subunit (p85, p55 or p50) that transduce signals from activated receptor tyrosine kinases (RTKs) [1].

After stimulation by various different growth factors and cytokines, PI3K is recruited, either directly or indirectly, to the membrane; this association activates the enzyme and brings it into close proximity with its lipid substrate, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] [3], thereby generating PtdIns(3,4,5)P_3 [1,2]. The tumour suppressor PTEN reverses the action of PI3K by dephosphorylating PtdIns(3,4,5)P_3 at the D-3 position and is thus an essential suppressor of PI3K signalling whose function is lost in various advanced-stage cancers [1].

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Here, we discuss evidence that indicates that signalling via both insulin-induced PI3K activation and mTOR signalling initiates a negative feedback loop which attenuates the ability of insulin to activate PI3K. Recent advances in the elucidation of the underlying mechanism of this negative-feedback regulation and its physiological and pathological implications are also discussed.

Insulin–PI3K signalling activates mTOR signalling

PtdIns(3,4,5)P_3 generated in the membrane by PI3K recruits members of intracellular signalling pathways containing pleckstrin homology (PH) domains to the plasma membrane, thereby coupling PI3K signals to downstream effector molecules [1]. Activation of one particular effector, protein kinase B (PKB; also known as AKT), seems to be essential not only in mediating the effects of insulin on glucose homeostasis, but also in regulating the profound effects of insulin and IGFs on mTOR signalling and cell growth.

PKB phosphorylates and inactivates various substrates involved in diverse processes including cell survival (the proapoptotic protein BAD), glycogen synthesis (glycogen synthase kinase-3) and gene transcription (FOXO transcription factors) [2–4]; however, PKB is also known to promote cell and organismal growth downstream of PI3K [5,6]. How the insulin–PI3K–PKB module induces growth has been recently clarified by the finding that PKB phosphorylates [7–9] and inactivates [8] an inhibitor of cell growth, tuberin [also known as tuberous sclerosis complex 2 (TSC2)], thereby inactivating the function of the TSC1–TSC2 tumour suppressor complex. TSC1–TSC2 is a complex of the proteins hamartin (TSC1) and tuberin [10] that shows inhibitory GTPase-activating protein activity on the small GTPase, Rheb [11–14]. Insulin-induced activation of PI3K has been shown to relieve this inhibitory activity [12], resulting in activation of Rheb. This induction of Rheb leads to activation of the mTOR pathway and an mTOR-regulated serine/threonine kinase, S6K.

The mTOR signalling pathway is crucial in promoting ribosome biogenesis and cell growth [15], and its induction after PI3K activation via PKB-mediated inactivation of TSC1–TSC2 might explain the sequential activation of PKB and S6K by insulin [16]. Notably, phosphorylation of tuberin by the p90 ribosomal S6 kinase 1 (Rsk1) [17] has been recently shown to have a similar inhibitory effect to that of PKB on TSC1–TSC2, thereby promoting mTOR signalling; by contrast, phosphorylation of tuberin by

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Table 1. Kinases implicated in IRS-1 phosphorylation and the effect on IRS-1 and/or insulin signalling[a,b]

Phosphorylated residue	Kinase	Effect of phosphorylation on IRS-1/insulin signalling	Refs
Ser302	S6K	Decreased InR–IRS-1 interaction	[50]
Ser302, Ser307	JNK?	Decreased InR–IRS-1 interaction	[69]
Ser307	JNK	Decreased IRS-1 P-tyrosine	[30]
		Decreased IRS-1-associated PI3K	[34]
Human Ser307, Ser323, Ser574 (≡rat Ser302, Ser318, Ser570)	PKCδ	Decreased IRS-1 P-tyrosine	[57]
Ser307 and others	IKK1, IKK2	Unclear	[73]
Ser307	?	Increased IRS-1 degradation	[27]
Ser789	SIK2	Unclear	[74]
Ser789	AMPK	Increased IRS-1-associated PI3K activity	[75]
Serine?	GSK3	InR RTK inhibited	[76]
Human Ser1101 (≡rat Ser1000)	PKCθ	?	[77]
Ser612	Unknown PMA-activated kinase	Decreased IRS-1 P-tyrosine by PMA	[78]
Peptide including Ser612	ERK2	Decreased IRS-1–PI3K interaction	[79]
Human Ser636, Ser639 (≡rat Ser632, Ser635)	Rapamycin-sensitive kinase(s)	Correlates with insulin resistance	[41]
Serine/threonine Ser318	PKCζ	Decreased IRS-1-associated P-tyrosine and interaction with the InR	[36,37,80]
		Decreased IRS-1-associated PI3K activity	[36]

[a]Rodent numbering unless otherwise stated.
[b]Abbreviations: AMPK, 5′ AMP-activated protein kinase; ERK2, extracellular-signal-regulated kinase 2; GSK3, glycogen synthase kinase-3; IKK, inhibitor of κB kinase; InR, insulin receptor; IRS-1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; P-tyrosine, tyrosine phosphorylation; S6K, p70 S6 kinase; SIK, salt inducible kinase.

5′ AMP-activated protein kinase in response to reduced cellular energy levels might act in the opposite manner to regulate TSC1–TSC2 function positively [18].

By analogy with other signalling pathways such as that of Ras and the mitogen-activated protein kinase (MAPK) [19,20], physiological control of PI3K signalling is likely to involve negative feedback acting to attenuate the strength or duration of PI3K activation. Indeed, such control has been demonstrated previously, although the precise molecular mechanisms involved and its physiological relevance have proved more elusive. Following on from studies examining the forward mode of mTOR signalling activated by the insulin–PI3K–PKB pathway, recent studies have begun to provide an insight into the particular role of growth signalling via mTOR and S6K in the reverse regulation of PI3K activation (negative feedback regulation), by establishing the mechanisms involved and illuminating the role that this type of feedback inhibition of PI3K might have *in vivo*.

Targeting the IRS family of adaptors inhibits PI3K

Previous work on activation of PI3K by insulin indicates that negative regulation of PI3K occurs mainly at the level of the proximal tyrosine kinase substrates of the insulin receptor (InR) RTK – namely, the IRS proteins, a family of adaptor proteins that are essential both for PI3K activation and for mediating the pleiotropic effects of insulin [3,21,22].

In a pioneering study, Haruta *et al.* [23] showed that prolonged exposure to insulin leads to a decrease in both the electrophoretic mobility and the level of IRS-1 protein. This decrease was shown to be functionally significant for signal transduction because activation of PKB, although initially stimulated by insulin exposure, decreased after prolonged treatment with insulin. The decrease in PKB activity closely followed the time course of the decrease in IRS-1 levels, strongly suggesting an attenuation of PI3K activation. The use of inhibitors enabled the signalling pathways required for these phenomena to be elucidated. Wortmannin, a PI3K inhibitor, and rapamycin, an mTOR inhibitor, were shown to prevent the prolonged insulin-induced decreases in mobility and IRS-1 protein. In addition, a constitutively active variant of PI3K, p110 CAAX, was found to be capable of mimicking the effect of prolonged insulin stimulation on IRS-1 [23].

Taken together, these observations indicated that a PI3K- and rapamycin-sensitive pathway is involved in an inhibitory mechanism impinging on the insulin–PI3K signalling pathway, and that this mechanism functions to decrease IRS-1 electrophoretic mobility and to reduce levels of IRS-1 protein. Additional reports have since confirmed the results of this study and have similarly indicated a decrease in IRS-1 protein in response to prolonged insulin treatment [24–29] – a decrease that is prevented by PI3K inhibitors and rapamycin [23–25,28]. Conflicting findings on the outcome of chronic insulin treatment on IRS-2 in different types of cell have been also reported [24–26], indicating that the effects of insulin on IRS-2 protein might be cell-type specific.

An increase in proteasomal degradation seems to be a common mechanism by which the reduction of IRS-1 protein is achieved after such prolonged insulin stimulation, because the effect is prevented by proteasomal inhibitors [23,24,27]. Similarly, blocking ubiquitination, by using cells expressing a conditional mutant of the ubiquitin-activating enzyme E1, also prevents the degradation of IRS-1 in response to prolonged treatment with insulin [24].

Serine/threonine phosphorylation and IRS protein function

The idea that the decrease in mobility of IRS-1 resulting from chronic insulin stimulation is at least contributed to by an increase in phosphorylation now seems to be in little

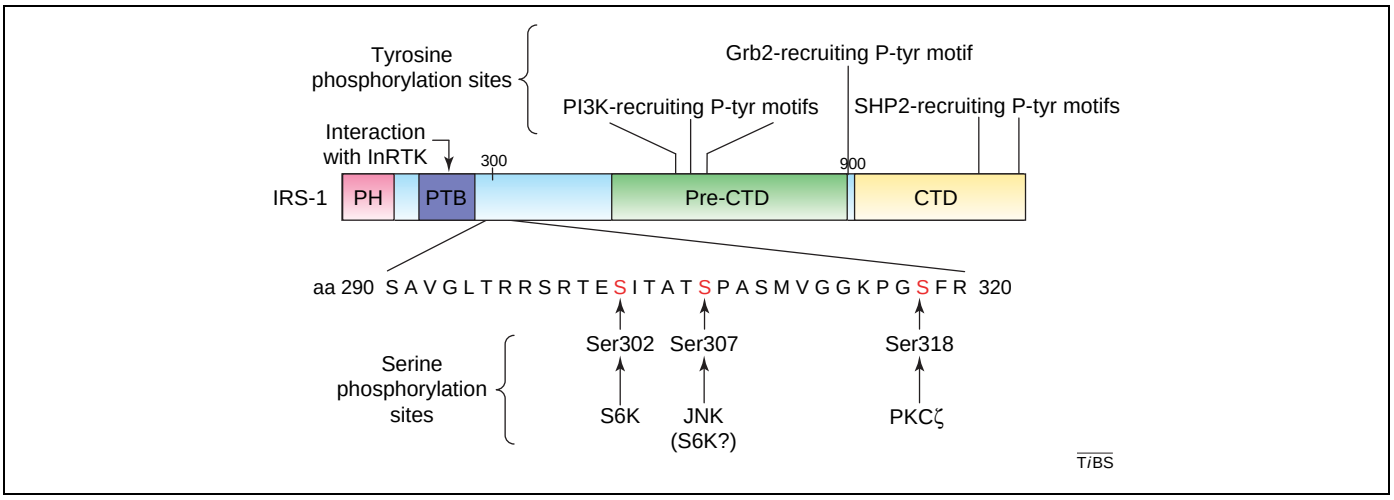

Figure 1. Diagram of rat insulin receptor substrate-1 (IRS-1). The protein modules of IRS-1 include a pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain, a pre-C-terminal domain (Pre-CTD) and a CTD. The phosphorylated tyrosine residues (P-tyr), which form docking sites for phosphoinositide 3-kinase (PI3K), growth factor receptor bound-2 (Grb2) and Src homology domain 2 (SH2)-containing protein tyrosine phosphatase (SHP2), are indicated. Also shown is the amino acid (aa) sequence of a section of IRS-1 following the PTB domain that contains three serine residues (highlighted in red) whose phosphorylation is implicated in the negative feedback loop that attenuates insulin signalling, in addition to the kinases thought to be responsible. An arrow indicates binding of the insulin receptor tyrosine kinase (InRTK) to the PTB domain. Abbreviations: JNK, c-Jun N-terminal kinase; PKCζ, protein kinase C-ζ; S6K, p70 S6 kinase.

doubt. Thus, the decrease in IRS-1 electrophoretic mobility after chronic insulin stimulation is reversed by dephosphorylation with alkaline phosphatase treatment [30] and mimicked by okadaic acid, a protein phosphatase 2A (PP2A) serine/threonine phosphatase inhibitor [28,31]. An increase in serine/threonine phosphorylation of IRS-1 has been shown to inhibit the activation of PI3K, because rapamycin, which inhibits the serine/threonine kinase mTOR and its effector S6K, reverses the mobility shift of IRS-1 resulting from prolonged insulin stimulation in many systems [23–25,28] and restores the activation of both PI3K [29] and its effector PKB [25].

What was less clear from those earlier studies, however, was the effect of the increase in IRS-1 phosphorylation on the ability of this adaptor to couple activation of the InR RTK to PI3K, particularly because IRS-1 is phosphorylated even under basal conditions and contains more than 50 serine/threonine residues with kinase consensus phosphorylation sites [32]. The identification of some of the kinases (rapamycin-sensitive or otherwise) that directly phosphorylate IRS-1 has helped to clarify this issue (Table 1, Figure 1 and reviewed in Ref. [33]).

For example, anisomycin, an activator of c-Jun N-terminal kinase (JNK), has been shown to induce a decrease in the electrophoretic mobility of IRS-1 [30] and to inhibit insulin signalling [34]. JNK has been shown to bind directly to IRS-1 and to phosphorylate it on Ser307 (amino acid numbering is that of rat IRS-1 unless otherwise stated) [30], and phosphorylation of Ser307 is reduced in cells lacking JNK1 and JNK2 [35]. Because JNK does not phosphorylate a Ser307→Ala mutant and anisomycin has no effect on the mobility of this mutant [30], it seems likely that the inhibitory effect of anisomycin on PI3K activation is mediated via the ability of JNK to phosphorylate IRS-1. Another report indicates that protein kinase C (PKC) ζ can also associate with IRS-1 and phosphorylate it both *in vitro* and *in vivo* [36], and more recently PKCζ was shown to phosphorylate Ser318 of IRS-1 [37]. A role for mTOR signalling in the phosphorylation of Ser307 has also been implied by the demonstration that treatment with rapamycin prevents the phosphorylation of Ser307 induced by insulin and osmotic stress [38–40].

How then does increased phosphorylation perturb IRS-1 function? Because increased serine/threonine phosphorylation seems to impair the ability of insulin to induce tyrosine

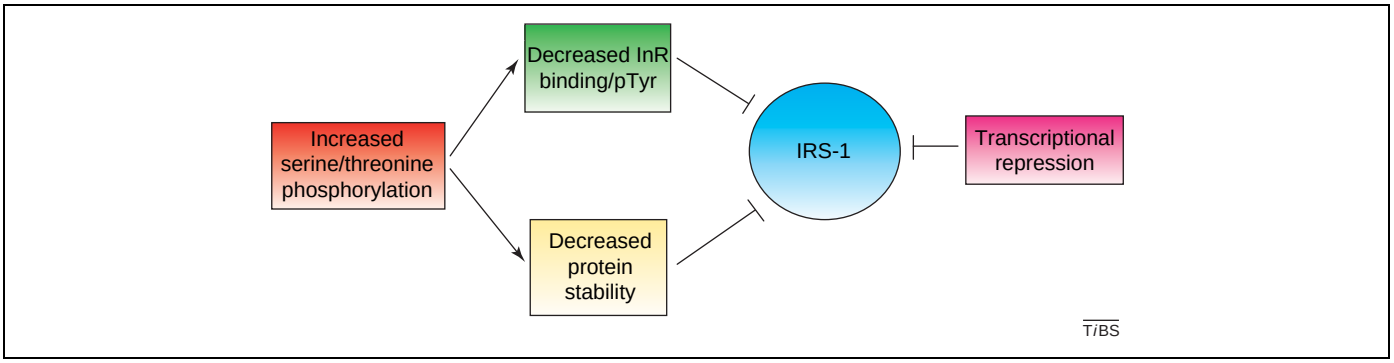

Figure 2. Regulation of insulin receptor substrate-1 (IRS-1) protein levels and function by serine/threonine phosphorylation. Serine/threonine phosphorylation of IRS-1 itself has a negative impact on the ability of insulin to activate phosphoinositide 3-kinase (PI3K). The consequences of IRS-1 phosphorylation identified so far include an increase in degradation of IRS-1 and a decrease in interaction of IRS-1 with the insulin receptor, leading to a reduction in IRS-1 tyrosine phosphorylation. In addition, the p70 S6 kinase (S6K) has been shown to have a negative effect on IRS-1 gene transcription, representing a novel mechanism of IRS-1 regulation.

phosphorylation of IRS-1 [36,41], a strong possibility is that binding of IRS-1 to the InR RTK is inhibited by phosphorylation. For JNK-mediated phosphorylation of Ser307 at least, IRS-1 binding to the InR RTK is indeed impaired by an increase in phosphorylation [34]. Similarly, an increase in serine/threonine phosphorylation of IRS-1, caused by inhibiting PP2A, decreases the ability of IRS-1 to interact with the juxtamembrane region of the InR RTK [31].

An additional mechanism of inhibition is likely to be increased proteasomal degradation of IRS-1 directed by site-specific phosphorylation. Thus a Ser312→Ala human IRS-1 mutant (equivalent to the Ser307 site in rat IRS-1) is partially resistant to insulin-induced degradation as compared with wild-type IRS-1, indicating that phosphorylation of this site might direct an increase in degradation of IRS-1 [27]. Taken together, these results suggest that specific serine/threonine phosphorylation of IRS-1 can negatively affect its function in PI3K activation in at least two ways: first, it can disrupt the interaction with the InR RTK and reduce the phosphotyrosine content of IRS-1; and second, it can destabilize IRS-1. Both mechanisms therefore reduce the pool of PI3K that is recruited to the InR RTK (Figure 2).

A role for mTOR signalling in PI3K inhibition

The rescuing effect of the mTOR inhibitor rapamycin in reversing the phosphorylation and inhibition of IRS-1 function resulting from prolonged insulin stimulation has suggested that a kinase (or kinases) that mediates mTOR signalling inhibits PI3K activation in addition to JNK and PKC.

mTOR is a component of two distinct large protein complexes that each contain at least three components. One complex is rapamycin sensitive, includes the mTOR kinase itself, raptor [42,43] and GβL [44], and is essential for an increase in ribosome biogenesis after cells are exposed to nutrients and growth factors [15]. The second, which has been very recently described in metazoans [45], is rapamycin insensitive, contains mTOR, AVO3/rictor and GβL, and seems to modulate the phosphorylation of PKCα and to regulate the actin cytoskeleton. Although the evidence of an increase in mTOR kinase activity in response to nutrients is conflicting, it is clear that nutrients such as amino acids stimulate the kinase activity of S6K and that full activation of S6K requires both PI3K and nutrients and is inhibited by rapamycin [46].

Notably, the presence of amino acids had been already shown to decrease the activation of PI3K after insulin treatment, an effect that is associated with both an increase in IRS-1 phosphorylation and a reduction in IRS-1 stability and association with PI3K [29,47]. Of particular note, this effect was found to correlate temporally with full activation of S6K, providing the first indication of negative feedback control of PI3K activity by this kinase [29].

TSC1–TSC2 promotes PI3K activation by inhibiting mTOR signalling

The negative feedback inhibition of PI3K has been most frequently observed in normal cells after prolonged stimulation with insulin. Two early reports intriguingly indicated, however, that cells lacking the TSC1–TSC2 tumour suppressor also fail to activate PKB in response to insulin, suggesting that a similar mechanism might be used to inhibit PI3K activation caused by constitutive mTOR-dependent signalling [48,49]. New studies have helped to clarify the mechanisms involved in this inhibitory role of mTOR-dependent signalling [50,51] and indicate that, as in chronic insulin stimulation, the crucial lesion occurs at the level of IRS proteins.

Consistent with the idea that IRS inactivation has an essential role in the effects of TSC1–TSC2 deficiency, these studies indicate that failure to activate PI3K in TSC1–TSC2-deficient cells is restricted to the insulin signalling pathway, because agonists such as epidermal growth factor and platelet-derived growth factor (PDGF), which activate PI3K directly, still activate the PI3K–PKB pathway [50–52]. Notably, however, two studies have shown that the activation of PI3K–PKB by PDGF is reduced in TSC1–TSC2-deficient cells [51,52], a result that might be due to reduced expression of PDGF receptors [52]. Both studies addressed the important issue of why PI3K is not activated in the absence of TSC1–TSC2 function; the results indicate that the protein levels of IRS-1, and possibly IRS-2, are maintained by the presence of wild-type TSC1–TSC2 function and reduced in its absence.

mTOR signalling inhibits IRS transcription

Unlike previous work indicating that mTOR signalling affects only IRS phosphorylation and protein turnover, the studies of Shah *et al.* [51] and Harrington *et al.* [50] have surprisingly indicated that TSC1–TSC2 deficiency and the resulting activation of the mTOR pathway lead to a reduction in IRS-1 transcription (Figure 2). A microarray screen [50], confirmed by northern blot analysis [50,51], revealed that the level of IRS-1 mRNA is reduced in TSC2-deficient cells as compared with TSC2-expressing cells. Similar to the effects on IRS-1 protein stability mentioned above, this reduction in IRS-1 gene expression also seems to be due to mTOR signalling, because sustained treatment of TSC-deficient mouse embryonic fibroblasts (MEFs) with rapamycin, or the restoration of TSC1–TSC2 expression, restores the IRS-1 mRNA level to that of normal cells [50,51]. Although the detailed mechanism is unclear, the likely target of mTOR signalling responsible for the reduction in IRS-1 gene transcription seems to be S6K, because suppression of S6K1 and S6K2 by RNA interference (RNAi) restores the abundance of IRS-1 mRNA in TSC2-deficient cells to that in TSC2-expressing cells [50].

The involvement of S6K in mediating at least some of the inhibitory effects of mTOR signalling on PKB activation was first indicated by work in *Drosophila*, in which removal of S6K was found to lead to a restoration of the reduced PKB activity resulting from loss of TSC1–TSC2 function [53]. Consistent with these data, in mammalian cells overexpression of the TSC1–TSC2 target Rheb, which induces activation of S6K [12,13], results in a decrease both in IRS-1 (and IRS-2) protein levels [51] and in PKB activation in response to insulin [12,51] that can

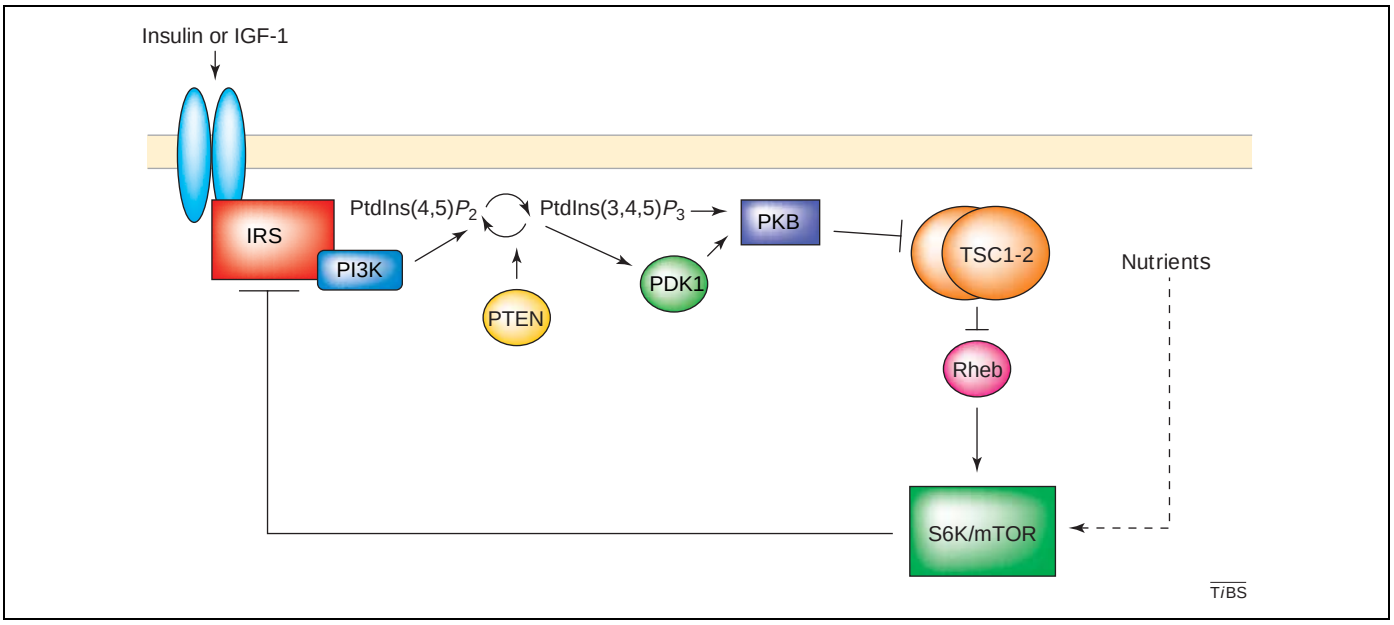

Figure 3. Control of insulin and phosphoinositide 3-kinase (PI3K) signalling by mammalian target of rapamycin (mTOR) and the p70 S6 kinase (S6K). Arrows indicate positive regulatory events, bars indicate inhibitory regulatory relationships. The mechanisms by which insulin or insulin-like growth factor-1 (IGF-1) and nutrients might activate mTOR and S6K are shown, along with the recently described feedback mechanism in which insulin receptor substrate (IRS) proteins are subject to negative regulation as a result of activation of mTOR and S6K. Activation of the cell-surface receptor (pale blue) leads to the recruitment of IRS-1 (red) and the activation of PI3K (blue). The phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] generated leads to the recruitment to the membrane of phosphoinositide-dependent protein kinase-1 (PDK1; dark green) and protein kinase B (PKB; purple). Active PKB phosphorylates and inhibits the function of tuberous sclerosis complex 1 and 2 (TSC1–2; orange), leading to activation of the Rheb GTPase (pink) and mTOR and S6K (pale green). S6K negatively regulates the ability of IRS-1 to transduce the insulin or IGF-1 signal. Abbreviations: PtdIns(,4,5)P_3, phosphatidylinositol (4,5)-bisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

be prevented by the co-transfection of a kinase-deficient S6K or kinase-dead mTOR variant [51].

In addition to its effects on gene transcription, mTOR signalling activated by TSC1–TSC2 deficiency seems to have important effects on IRS-1 expression and function. IRS-1 and IRS-2 proteins from TSC2-deficient cells show both decreased electrophoretic mobility [50] and increased turn over [51] and, as in prolonged insulin treatment of normal cells, both effects are reversed by rapamycin. Notably, recent work has shown that acute inhibition of mTOR signalling, although having no immediate effect on IRS protein levels, is sufficient to increase the electrophoretic mobility of IRS-1 and IRS-2, and to rescue partially the activation of PKB in TSC2-deficient cells [50]. Because this rescue of PKB activation has been shown to require both IRS-1 and IRS-2, it seems that an increase in mTOR-dependent phosphorylation of IRS-1 and IRS-2 is likely to inhibit the adaptor function of IRS proteins in PI3K activation.

Although the lack of activation of PI3K in TSC1–TSC2-deficient cells has been mainly observed in studies done *in vitro*, one report has suggested that the activity of PKB is reduced in TSC2-null tumours *in vivo* in rats [54]. Further work is clearly required to clarify this finding but, because TSC in humans is a non-malignant disease, it is possible that a failure to activate PI3K could potentially restrict the malignant potential of TSC hamartomas. Indeed, recent work has addressed this possibility directly by examining tumorigenesis in TSC heterozygous mice in which PtdIns(3,4,5)P_3 levels are raised by heterozygosity in PTEN, a manipulation that results in the formation of more aggressive tumours than are seen in mice with TSC heterozygosity alone (B.D. Manning, personal communication).

S6K and IRS-1 phosphorylation

The possibility that S6K might be the crucial rapamycin-sensitive effector responsible for directly phosphorylating IRS-1 and thereby inhibiting PI3K activation was first suggested by Tremblay and Marette [29] and has been subsequently investigated by Harrington *et al.* [50]. A specific serine residue in IRS-1, Ser302, has been shown to be phosphoryated by S6K *in vitro* [50] (Figure 1). Furthermore, phosphorylation of Ser302 in IRS-1 increases in the absence of TSC1–TSC2 function and is inhibited by RNAi of S6K1. Thus, S6K1 is likely to be the main rapamycin-sensitive kinase responsible for phosphorylation of Ser302, at least in TSC1–TSC2-deficient MEFs [50].

Because Ser302 lies close to the IRS-1 phosphotyrosine-binding (PTB), which is responsible for binding the InR RTK (Figure 1), phosphorylation of this site could inhibit the recruitment of IRS-1 to its receptor. Consistent with such a possibility, a phosphomimetic (Ser302→Glu) fragment of IRS-1 containing the PTB domain shows reduced binding to the activated InR RTK [50]. Increased phosphorylation of IRS-1 Ser302 is also observed in three models of insulin resistance, genetically obese *Lep*/*Lep* mice, diet-induced obesity and hyperinsulinaemia [55], further suggesting that there is a causal relationship between an increase in Ser302 phosphorylation and a failure to activate PI3K.

It remains possible that Ser302 can be phosphorylated by other kinases to inhibit IRS-1 function, although data on the role of JNK in such a phosphorylation step are conflicting [55,56]. Another AGC family kinase related to S6K, PKCδ, phosphorylates IRS-1 at Ser302 *in vitro* [57], but this modification remains to be confirmed *in vivo*.

Box 1. Unresolved issues

Some issues remain unresolved, particularly with regard to the mechanisms involved in mammalian target of rapamycin (mTOR)-mediated suppression of phosphoinositide 3-kinase (PI3K) activation. Because other signalling pathways are known to activate mTOR and the p70 S6 kinase (S6K), including phorbol ester [64], G-protein-coupled receptor [65] and brain-derived neurotrophic factor signalling [66] pathways, it is possible that constitutive activation and phosphorylation of S6K by mTOR could have inhibitory effects that are not on insulin receptor substrate (IRS) proteins. The identification of additional substrates of S6K, particularly those involved in regulating gene transcription, and the identification of other S6K-regulated genes, will undoubtedly increase our understanding of this type of chronic inhibition of signalling by S6K. In addition, clarification of the effects of activated mTOR signalling on IRS-1 versus IRS-2 is warranted because conflicting data exist [50,51] and particularly because these adaptors mediate PI3K activation to different extents in distinct tissues [67].

Finally, the similarities and difference between genetic mechanisms of insulin resistance such as tuberous sclerosis complex (TSC) deficiency and induced states of resistance caused, for instance, by a high-fat diet (HFD) – both of which have been argued to result in constitutive activation of the mTOR pathway [50,58] – need to be clarified in detail. For example, although the mechanism by which S6K1 inactivates insulin–PI3K signalling during HFD feeding also seems to involve perturbation of IRS-1 [58], it shows differences from the mechanism used after mTOR activation in TSC deficiency. Thus, under conditions of mTOR pathway activation [50,51], or in *Lep/Lep* obese mice [68], IRS-1 protein levels are downregulated – a finding that is not observed in normal HFD-fed mice [58], despite activation of S6K1 and increased phosphorylation of Ser307, which has been reported to destabilize IRS-1 protein [27]. This discrepancy can be explained if the negative feedback loop does not operate in the same way in all cells, because different tissues were examined in these two studies. Perhaps more significantly, the sites of phosphorylation reported to be increased by HFD and reduced when S6K1 is absent (Ser307, Ser636 and Ser639) are apparently not significantly phosphorylated by S6K1, at least *in vitro* [50].

Notably, the main site of IRS phosphorylation by S6K *in vitro* (Ser302), although not studied by Um *et al.* [58], has been recently shown to be phosphorylated to a similar extent as Ser307 in the same genetic model of obesity, *Lep/Lep* mice [69]. A possible resolution here is that *in vivo* S6K1 phosphorylates both Ser302 and Ser307 (which are both located in a motif similar to the S6 phosphorylation motif) to inhibit in full the association of IRS-1 with the insulin receptor (InR) receptor tyrosine kinase (RTK) and to effect inhibition of PI3K activation. This idea is consistent with the finding that complete inhibition of the binding of full-length IRS-1 to the InR RTK in a yeast assay seems to require phosphorylation of both Ser302 and Ser307 [69]. Phosphorylation of Ser636 and Ser639 is also apparently increased by HFD and dependent on S6K1 but, because these residues do not resemble the direct S6K sites present in S6, IRS-1 (Ser302 and Ser307) or other S6K substrates [70–72], their dependence on phosphorylation by S6K might be indirect.

Role of negative feedback regulation of PI3K *in vivo*

The physiological importance of negative feedback from mTOR and S6K to PI3K has been recently given impetus by a new study of S6K1-deficient mice [58]. This study suggests that at least some of the effects of a high-fat diet (HFD) on inhibiting the insulin–PI3K signalling pathway [59] might be mediated by S6K1 activation; thus, S6K1 is emerging as an attractive new therapeutic target in type 2 diabetes.

In an earlier study, S6K1-deficient mice were shown to be hypoinsulinaemic and mildly glucose intolerant owing to a reduction in pancreatic β-cell mass caused by a decrease in the growth of individual β-cells [60]. In the new study, S6K1-deficient mice show protection from the effects of age- and diet-induced obesity, apparently owing to enhanced lipolysis and metabolic rate [58]. Surprisingly, however, despite an increase in circulating free fatty acids (which might be expected to inhibit insulin action and glucose uptake [61]), S6K1-deficient mice show hypersensitivity to insulin in the activation of PI3K responses. Significantly, HFD conditions result in an impairment of insulin-induced PKB activation in three insulin-responsive tissues in normal but not S6K1-deficient mice, consistent with previous work showing the inhibitory effects of fatty acids and HFD on PI3K activity [62,63]. A direct role of S6K in the impaired activation of the insulin–PI3K pathway is inferred because normal mice under HFD conditions and two genetic models of obese mice (K/KA^y and *Lep*/*Lep*) show increased activation of S6K1, similar to that seen in cells with constitutive activation of the mTOR pathway [50].

Concluding remarks

In this review, we have focused mainly on the role of mTOR signalling in mediating negative feedback on insulin–PI3K signalling and on the principal role of IRS-1 proteins as recipients of negative regulation from kinases such as S6K, which are normally regulated by nutrient–mTOR and PI3K signalling (Figure 3). Through this type of negative feedback regulation, PI3K and the production of PtdIns(3,4,5)P_3 are selectively uncoupled from the stimulatory effects of insulin; this raises the obvious question: what is the normal function of this regulatory mechanism?

Despite a clear indication of the involvement of this inhibition of PI3K in insulin resistance in HFD-induced diabetes [58], it seems unlikely that the mechanism evolved solely to deal with excessive nutrient supply. An intriguing possibility for future study is that this type of uncoupling of PI3K might be necessary to attenuate the effects of insulin on cell growth once sufficient growth (and activation of mTOR and S6K) is achieved, thereby providing a mechanism to prevent further growth while leaving intact the activation of PI3K by other stimuli.

In conclusion, although unresolved issues remain (Box 1), in recent years we have learned much about the PI3K signalling pathway and its control of mTOR signalling, including the roles of PKB, TSC1–2 and Rheb (Figure 3). Clarification of how mTOR signalling goes back to the membrane to restrict activation of PI3K should provide further fruitful avenues for future research.

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