REVIEW ARTICLE

Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling

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Insulin plays a key role in regulating a wide range of cellular processes. However, until recently little was known about the signalling pathways that are involved in linking the insulin receptor with downstream responses. It is now apparent that the activation of class 1a phosphoinositide 3-kinase (PI 3-kinase) is necessary and in some cases sufficient to elicit many of insulin's effects on glucose and lipid metabolism. The lipid products of PI 3-kinase act as both membrane anchors and allosteric regulators, serving to localize and activate downstream

enzymes and their protein substrates. One of the major ways these lipid products of PI 3-kinase act in insulin signalling is by binding to pleckstrin homology (PH) domains of phosphoinositide-dependent protein kinase (PDK) and protein kinase B (PKB) and in the process regulating the phosphorylation of PKB by PDK. Using mechanisms such as this, PI 3-kinase is able to act as a molecular switch to regulate the activity of serine/threonine-specific kinase cascades important in mediating insulin's effects on endpoint responses.

BACKGROUND

It has been known for some time that the cell-surface receptor for insulin is a receptor tyrosine kinase and is highly homologous with the insulin-like growth factor-1 (IGF-1) receptor. However, the steps lying between receptor activation and stimulation of end-point responses have only recently begun to be unravelled. Signalling pathways initiated by receptor tyrosine kinases depend on the recruitment of signalling proteins which bind to the autophosphorylated kinases or their tyrosine-phosphorylated substrates, this binding being mediated by protein modules known as Src homology (SH2) domains [1]. Phosphatidylinositol 3-kinase (PI 3-kinase) was one of the first, and remains one of the best understood, examples of a protein recruited in this way, although the signalling pathways downstream of PI 3-kinase have until recently remained very elusive [2–4]. A great deal of evidence has accumulated to suggest that PI 3-kinase plays a central role in a diverse range of cellular responses, including cell growth and differentiation [4,5], synthesis and degradation of carbohydrates, lipids and proteins [6,7], platelet activation [8], cytoskeletal organization and cell motility [5,9] and membrane trafficking [6,10,11]. Here we review the evidence that has been accumulated establishing that PI 3-kinase plays a pivotal role in signal-transduction pathways linking insulin with many of its specific cellular responses.

THE PI 3-KINASE FAMILY OF ENZYMES

The first evidence linking PI 3-kinase to growth-factor signalling was the observation that PI kinase activity and an 85 kDa phosphoprotein were recruited into anti-phosphotyrosine im-

munoprecipitates following stimulation by platelet-derived growth factor (PDGF) [12]. This PI kinase was shown to be distinct from the abundant and previously recognized PI 4-kinase activity, and was found to make a novel series of 3-phosphorylated inositol phospholipids [13,14]. Subsequent work identified a number of 3-phosphorylated inositol lipids in the cell, and a role for these in growth-factor signalling was suggested by the finding that growth factors stimulated an acute increase in levels of two of these, namely phosphatidylinositol 3,4,5-trisphosphate (PIP₃) and phosphatidylinositol 3,4-bisphosphate [PI(3,4)P₂] [15,16]. Insulin also causes an acute increase in PIP₃ levels although its effects on PI(3,4)P₂ levels have been less well documented [17–21]. It is currently thought that PIP₃ is the major product formed by the PI 3-kinases activated by growth factors and that the PI(3,4)P₂ produced is in fact a breakdown product of PIP₃ [16,22].

A number of enzymes possessing PI 3-kinase activity have been identified and all share significant homology within the catalytic domain (Figure 1; for a review, see [3]). However, the catalytic domains of PI 3-kinases share a great deal of homology with PI 4-kinases and even with some enzymes that function only as protein kinases. There is also significant functional heterogeneity amongst enzymes with established PI 3-kinase activity, and these have been grouped into categories on the basis of sequence homologies and similarities in function, structure and substrate specificities [3].

Activation of the insulin-receptor tyrosine kinase results in the rapid recruitment of PI 3-kinase activity into signalling complexes, and current evidence indicates that class 1a PI 3-kinases account for the bulk of this increase. The class 1a

Abbreviations used: PI3P, phosphatidylinositol 3-phosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PI 3-kinase (4-kinase), phosphoinositide 3-kinase (4-kinase); PH, pleckstrin homology; PDK, phosphoinositide-dependent protein kinase; PK, protein kinase; PKA, PKB and PKC, protein kinases A, B and C; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; GAP, GTPase-activating protein; BH, Bcr/Rac GAP homology; IRS, insulin-receptor substrate; PTB, phosphotyrosine-binding domain; EGF, epidermal growth factor; MAP, mitogen-activated protein; MEK, MAP kinase kinase; GEF, guanine-nucleotide exchange factor; PIP3-5'-phosphatase, phosphatidylinositol phosphate 3-5'-phosphatase; GSK3, glycogen synthase kinase-3; mTOR, mammalian target of rapamycin; JNK, jun-N-terminal kinase; PP1 and PP2A, protein phosphatase-1 and -2A; CHO, Chinese-hamster ovary; PFK-2, phosphofructokinase-2; PEPCK, phosphoenolpyruvate carboxykinase; HSL, hormone-sensitive lipase; PDE3b, phosphodiesterase 3b; eEF2, eukaryotic elongation factor-2; eIF-4E, eukaryotic initiation factor 4E; 4EBP-1, eIF-4E binding protein-1; PLC, phospholipase C; SH, Src homology; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.

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Figure 1 Domain structure of PI 3-kinase catalytic subunits and homologues

SH3 domains are shown in pale pink, the P1 and P2 domains in mid-pink, the Bcr/RacGAP homology (BH) domains in dark pink, the SH2 domains in black and the p110-binding regions in red. 32–42-amino-acid extensions with 60% homology are shown as black-and-white chequered rectangles.

PI 3-kinases exist as heterodimers which consist of an adapter/regulatory subunit tightly associated with a catalytic subunit [23–26]. The catalytic subunits of the class 1a PI 3-kinases share significant sequence homology with the G-protein-regulated class 1b PI 3-kinase catalytic subunits, although there are clear differences in the mechanisms of regulation of these two enzyme classes. The class 1b PI 3-kinases associate with a distinct family of adapter subunits which are unrelated to the class 1a adapter subunits [27]. These are activated by binding of $\beta\gamma$ subunits of heterotrimeric G-proteins to specific sites in both the p101 adapter subunit and the catalytic subunit [27,28], and there is no evidence that they are activated by insulin. Class 2 PI 3-kinases are widely expressed, and these can be activated by insulin (P. R. Shepherd and R. Brown, unpublished work). However, it would seem unlikely that they will play a role in mediating any of the common insulin responses discussed below for two reasons: (i) they cannot use phosphatidylinositol 4,5-bisphosphate [$\text{PI}(4,5)\text{P}_2$] as a substrate and so are unable to produce PIP_3 [29,30], which is thought to be a key mediator in insulin signalling (see below) and (ii) they are relatively resistant to the PI 3-kinase inhibitors [29,30], while many insulin responses are very sensitive to these inhibitors (see below). Class 3 PI 3-kinases are also widely expressed, but these can only use phosphatidylinositol as a substrate and, as insulin does not increase cellular levels of phosphatidylinositol 3-phosphate (PI3P), it seems unlikely that these will play a role in insulin signalling [17,31].

Reagents for studying PI 3-kinases

The recent explosion in interest in the role of PI 3-kinases in cellular processes has been facilitated by the availability of a

range of reagents which have greatly simplified experimental investigations in this area. The most widely used of these are two specific and cell-permeable inhibitors of PI 3-kinase, namely wortmannin [32] and LY294002 [33]. Wortmannin has an IC_{50} in the low-nanomolar range for mammalian class 1a [32], class 1b [34] and class 3 [35] PI 3-kinases, although the IC_{50} for class 2 PI 3-kinases is greater [30,36]. Wortmannin's specificity exists over a two-orders-of-magnitude concentration range, although at concentrations greater than 100 nM, wortmannin will inhibit some isoforms of PI 4-kinase [37] and also phospholipase A_2 [38]. LY294002 is another highly specific inhibitor of class 1 PI 3-kinases, although class 2 PI 3-kinases are again relatively resistant to this inhibitor [36]. As with all inhibitors the possibility exists that wortmannin and LY294002 will be found to inhibit other enzymes, and therefore some caution must be taken in interpreting results based solely on the use of these reagents. A further problem is that these reagents inhibit all classes of PI 3-kinases to variable extents, so it is difficult to implicate class 1a PI 3-kinases in a particular pathway using only such compounds. However, in the case of class 1a PI 3-kinases a range of molecular reagents have been developed which provide more definitive evidence that this class of PI 3-kinase is necessary and/or sufficient for a certain process. Dominant negative forms of the class 1a adapter/regulatory subunits [39] and antisense vectors to p85 [40] both block signalling via class 1a PI 3-kinases. Additionally it has been demonstrated that $\text{PI}(3,4)\text{P}_2$ and PIP_3 levels in the cell can be increased [41,42], and various downstream pathways can be activated [41,43–45] by overexpression of wild-type or constitutively active forms of the class 1a PI 3-kinase catalytic subunit. Biologically active membrane-permeable forms of PIP_3 have also recently been developed [46].

Figure 2 Domain structure of class 1a PI 3-kinase adapter subunit isoforms and splice variants

The p85-binding domain is shown in red, the Ras-binding domains in dark pink, the PIK (phosphoinositide kinase homology domain) domains as black-and-white chequered ellipses, the kinase domains as mid-pink elongated ellipses and the C2 domain as a pale pink ellipse.

Domain structure of class 1a PI 3-kinases

Several isoforms of both the adapter/regulatory and catalytic subunits of class 1a PI 3-kinases exist, and further complexity is added by the occurrence of several splice variants of the adapter/regulatory subunit.

Three highly homologous isoforms of the class 1a catalytic subunit have been cloned, all of which have a molecular mass of approx. 110 kDa. These have been designated p110 α [25], p110 β [26] and p110 δ [47]. All contain the kinase domain at the C-terminus, with binding domains for p85 and Ras at the N-terminus. The α and β isoforms are the most likely to play a major role in insulin signalling, as these are widely expressed, whereas expression of the δ isoform is restricted to haematopoietic cells. Few studies have been undertaken to directly compare the functional properties of the α and β isoforms of p110, and what information is available suggests that they have very similar catalytic properties.

The class 1a PI 3-kinase adapter/regulatory subunits are encoded by at least three genes which generate highly homologous products (Figure 2). All contain two SH2 domains and an inter-SH2 domain containing sequences allowing the interaction of the adapter and catalytic subunits [23,48–51]. Two isoforms generate products of 85 kDa, these being termed p85 α and p85 β . Both of these contain domains N-terminal to the SH2 domains, these being an SH3 domain, a Bcr/Rac GTPase-activating protein (GAP) homology (BH) domain, as well as two proline-rich regions (P1 and P2), which are located either side of the BH domain [24]. Three splice variants of p85 α have been reported, including one form arising from insertion of eight amino acids in the inter-SH2 domain, which adds two potential serine phosphorylation sites [52]. The other two are truncated versions of p85 α with apparent molecular masses of 55 kDa, termed AS53 or p55 α [52,53] (hereafter referred to as p55 α) and 46 kDa, termed p50 α [53,54]. These lack the SH3, P1 and BH domains and probably arise from alternative transcription start sites which generate short novel sequences at the N-terminus (34 amino acids in the case of p55 α and six amino acids in the case of p50 α). p55 α is highly expressed in brain and muscle [52,53] and p50 α is highly expressed in brain, liver, muscle and kidney [53,54]. A third PI 3-kinase adapter subunit gene has recently been characterized termed p55^{PIK} [55] or p55 γ [56] (hereafter referred to as p55 γ). This produces a protein of 55 kDa which is highly homologous in sequence and has an identical domain structure

to the p55 α splice variant of p85 α [55,56]. The 32-amino-acid N-terminal regions of p55 α and p55 γ are closely related, but their function is currently unknown. Interestingly this domain contains a YXXM motif, which, if phosphorylated, could potentially form intramolecular (and therefore potentially autoregulatory) interactions or links to other SH2 domain-containing proteins. However, it is not clear whether this tyrosine residue is phosphorylated *in vivo*. The one study which investigated this issue found that insulin stimulated phosphorylation of p55 γ but not in the unique N-terminal region [55].

Function of class 1a PI 3-kinase adapter/regulatory subunit domains

The major role of the SH2 domains is to facilitate tyrosine kinase-dependent regulation of PI 3-kinase activity by increasing the catalytic activity of the p110 subunit [57,58] and by inducing recruitment of PI 3-kinase to signalling complexes adjacent to membranes where the enzyme's substrate is located [59–61]. An assessment of the sequence specificity of various SH2 domains indicates that both SH2 domains of p85 have high selectivity for binding phosphorylated YxxM sequences, and especially YMxM sequences, a specificity not shared by SH2 domains from other proteins [62]. However, there is a slight variance in the exact sequence preferences of the N- and C-SH2 domains [62,63] and the N- and C-SH2 domains of p85 have different inhibitory effects on insulin signalling when microinjected into oocytes [64]. The physiological relevance of these differences has not been fully addressed.

The *in vivo* roles of the SH3, proline-rich and BH domains of p85 are less well defined. SH3 domains interact with specific proline-rich sequences [65], and the proline-rich domains of p85 fit reasonably with the consensus sequence for the binding of the p85 α SH3 domain [66,67]. This suggests that intramolecular interactions between the p85 SH3 and proline-rich regions may occur. Such interactions could provide an autoregulatory mechanism such as has been demonstrated in a member of the Src family of tyrosine kinases [68]. It has also been reported that the p85 SH3 domain can interact *in vitro* with specific proline-rich regions of a number of other cellular proteins, including dynamin [69], p125^{FAK} [70] and the proto-oncogene *cbl* [71]. The proline-rich regions contained in p85 can also associate *in vitro* with SH3 domains of other proteins, including the small adapter protein

Grb-2 [72], α -actinin [73] and the SH3 domains of members of the Src family of tyrosine kinases [74–76].

If these various interactions between SH3 and proline-rich domains do indeed occur in intact cells, they could affect PI 3-kinase signalling pathways in two ways. First, such interactions might regulate the intrinsic activity of the PI 3-kinase catalytic subunit. It has been reported that the association of p85 with other proteins via its SH3 domain *in vitro* affects the catalytic activity, although it is difficult to determine whether similar processes act *in vivo* to regulate PI 3-kinase activity [70,74,75]. In addition, it is possible that the interactions of adapter subunits via SH3 domains may allow formation of specific tertiary signalling complexes, the exact composition of which would dictate the type of downstream response that could be generated. For example, it is claimed that the constitutive association between c-abl and p85 facilitates the tyrosine phosphorylation and down-regulation of PI 3-kinase activity following stress-induced activation of c-abl [76].

The BH domain is the least well understood domain in p85. The sequences of p85 α and p85 β differ most noticeably in the BH domain, suggesting that investigation of this region may shed some light on how these two isoforms differ in signalling potential. The BH domains of both p85 α and p85 β both differ from the Rac GAP domain in the prototypical Bcr at critical residues, suggesting that p85 is not a functional Rac GAP. However, the BH domain of p85 α is still able to bind to the GTP-loaded forms of the small GTPases Rac [77] and Cdc-42Hs [78], and it has been suggested that these interactions regulate activity of the PI 3-kinase catalytic subunit.

Role of individual adapter/regulatory subunit isoforms

It is not clear why so many variants of the adapter/regulatory subunit exist, although it is likely to be of importance in insulin signalling, as muscle and liver, two of the major insulin target tissues, express p85 α , p85 β , p55 α and p50 α [52–56]. The fact that all adapter/regulatory subunit variants retain the capability to recruit p110 to proteins containing tyrosine-phosphorylated YMxM motifs suggests a degree of functional redundancy. Indeed this is borne out by studies in mice which are homozygous for a targeted disruption of the p85 α gene such that expression of full-length p85 α is blocked, while the ability to express the truncated p85 α splice variant is retained [79]. These mice are viable, indicating that other PI 3-kinase adapter/regulatory subunits compensate for the loss of p85 α . Glucose metabolism in these animals in fact appears hyper-responsive to insulin, and it appears that up-regulation of the p50 α hypercompensates for the loss of p85 α in insulin-sensitive tissues [79]. Further knockout studies are in progress to address the role of individual variants of the adapter/regulatory subunit. The sequence differences between adapter/regulatory subunit isoforms, and particularly the major differences in domain structure between the full-length and truncated forms, suggest that functional differences will be found. One report comparing p85 α and p85 β found that only the latter bound to the proto-oncogene *abl*, although the functional implications of this are not clear [80]. Three studies have concluded that there are significant differences in the relative ability of individual splice variants to be recruited into insulin-induced signalling complexes, with the relative order of importance being p50 α > p85 α > p55 α [52,53,81]. Conflicting data exist on whether insulin stimulates p85 β , as it has been reported that insulin induces little or no stimulation of PI 3-kinase associated with p85 β in transfected Chinese-hamster ovary (CHO) cells [53,82], while insulin clearly stimulates recruitment of p85 β to signalling complexes in human muscle [81]. Similarly it has been reported

that insulin does not [53,81] or does [55] stimulate the recruitment of p55 γ into signalling complexes. There is clearly scope for much further work to define the properties and roles of individual adapter variants as well as the insulin-receptor substrate (IRS) proteins with which they interact.

Regulation of class 1a PI 3-kinases by insulin is largely mediated by IRS proteins

The first growth factor which was shown to activate PI 3-kinase was PDGF. In the case of the PDGF receptor, the ligand-induced receptor autophosphorylation results in recruitment of PI 3-kinase activity by direct binding of the p85 SH2 domains to tyrosine phosphorylated YMxM motifs in the kinase insert region of the intracellular portion of the receptor [49,83,84] (Scheme 1).

In contrast, insulin causes very little recruitment of PI 3-kinase activity directly to the insulin receptor, and the major mechanism of activating PI 3-kinase appears to be via the IRS proteins (Scheme 1). Four members of the IRS family have now been identified, namely IRS-1 [85], IRS-2 [86], IRS-3 [87] and IRS-4 [88]. IRS-1 and IRS-2 are widely distributed, although the relative levels are very variable between different mammalian tissues. IRS-3 expression has been demonstrated in adipose tissue, fibroblasts and liver cells [89–92], whereas IRS-4 has as yet been observed only in cultured cells originating from embryonic kidney [88].

All the IRS proteins have the same overall architecture, sharing highly homologous N-terminal pleckstrin homology (PH) and phosphotyrosine-binding domain (PTB) domains. The PTB domain binds directly to the juxtamembrane region of the insulin and IGF-I receptors at an NPEY motif, and this interaction accounts for the specificity of phosphorylation of IRS proteins by receptors possessing such a motif in the correct sequence context [93,94]. However, the PH domain is also important in mediating association with the receptor [95,96]. It is very likely that the PH domain allows interaction of IRS-1 with membrane phosphoinositides, which have been shown to bind to PH domains of other proteins [97,98]. Very little is known about the phosphoinositide-binding characteristics of the PH domains of IRS family members, although preliminary results indicate that the PH domain of IRS-1 does bind to PI(4,5)P₂ (G. Panayatou, personal communication).

The IRS proteins all contain multiple potential tyrosine phosphorylation sites, of which the majority are in YMxM or YxxM motifs. It has been demonstrated by unbiased analysis that tyrosines in YMxM motifs are the preferred substrates of the insulin-receptor tyrosine kinase [99,100]. Thus the IRS proteins are ideally structured for the insulin-dependent recruitment and activation of PI 3-kinase. In addition to its YxxM motifs, IRS-1 possesses other tyrosine phosphorylation sites to which a number of proteins can bind via their respective SH2 domains. These proteins include the tyrosine-specific phosphatase SHP2, the tyrosine kinase Fyn, and adapters such as Grb2, Nck and Crk [101]. Such proteins may secondarily influence binding of PI 3-kinase through direct effects on the tyrosine phosphorylation of IRS-1 and by steric or other interactions, quite apart from their independent signalling potential.

The IRS proteins are not phosphorylated by the epidermal-growth-factor (EGF)-receptor or PDGF-receptor tyrosine kinases, although IRS-1 and IRS-2 are also phosphorylated by tyrosine kinases of the JAK family associated with receptors for growth hormone, interleukin-4 and other cytokines [86,102–104]. It is likely that these different stimuli cause different patterns of

Scheme 1 Role of class 1a PI 3-kinase in insulin signalling

Red rectangles bounded by a double line indicate endpoint responses. Pale-pink boxes bounded by a single line indicate enzymes activated in response to insulin. Black boxes indicate enzymes inactivated in response to insulin. Further abbreviations: DAG, diacylglycerol; IP₃, inositol 1,4,5-triphosphate.

tyrosine phosphorylation of IRS proteins and that this dictates the nature of the signalling responses that are generated.

There is evidence for some degree of functional overlap between IRS-1 and IRS-2. In transgenic mice with a targeted disruption of the IRS-1 gene, insulin-stimulated PI 3-kinase activity was greatly diminished in muscle, where IRS-2 levels are low, but near-normal in liver, where IRS-2 is expressed at similar levels to IRS-1 [105,106]. The IRS-1 knockout mice are only mildly insulin-resistant, suggesting that IRS-2 is indeed able to substitute for IRS-1 in insulin signalling via PI 3-kinase to pathways regulating glucose metabolism. Clear evidence for overlapping functional roles has been obtained in fibroblast cell lines derived from IRS-1-deficient mice, where IGF-1-induced activation of PI 3-kinase and immediate early gene expression were restored to the same degree by retrovirus-mediated expression of IRS-1 or IRS-2 [107]. However, cell-cycle progression was reconstituted by IRS-1, but not IRS-2, in the IRS-1-deficient fibroblasts, indicating that IRS-1 and IRS-2 are not functionally interchangeable for all cellular responses. Further, recent studies on mice with targeted disruption of the IRS-2 gene indicate that IRS-1 cannot compensate for loss of IRS-2 as IRS-2-deficient mice developed overt diabetes, apparently reflecting a failure of

compensatory β -cell hyperplasia in the face of peripheral insulin resistance [108]. Therefore, overall the data suggest that the functional overlap between IRS-1 and IRS-2 is not total.

The roles of the recently cloned IRS-3 and -4 are as yet unclear. IRS-3 undergoes tyrosine phosphorylation and binds PI 3-kinase in response to insulin, and in adipocytes [109]. IRS-3 rather than IRS-2 was the major alternative pathway for PI 3-kinase activation in adipocytes from IRS-1-deficient mice, but this was unable to compensate fully for lack of IRS-1. Binding of IRS-4 to PI 3-kinase remains to be demonstrated, but this would certainly be expected, given that it possesses seven potential tyrosine phosphorylation sites in YxxM motifs.

There may of course be further, as yet undiscovered, members of the IRS family, or other related proteins which are able to mediate activation of PI 3-kinase. For instance, a Grb2-associated binding protein, Gab-1, has been identified which is phosphorylated in response to insulin and EGF and is widely expressed in mammalian tissues [110]. This protein lacks the PTB domain which is characteristic of the IRS family, but has a PH domain with some similarity to that of IRS-1 and multiple potential tyrosine phosphorylation sites, including three in YxxM motifs. Both EGF and insulin were shown to increase Gab-1-

associated PI 3-kinase activity. In 3T3-L1 fibroblasts, Gab-1 appeared to be the major binding partner for insulin-stimulated PI 3-kinase activity, in contrast with differentiated 3T3-L1 adipocytes, where IRS-1 fulfills this role. It therefore appears unlikely that Gab-1 is a major mediator of insulin signalling to PI 3-kinase in the major insulin target tissues.

The relationship between IRS proteins and PI 3-kinase has been most extensively investigated in the case of IRS-1. During insulin stimulation, IRS-1 undergoes multi-site tyrosine phosphorylation and binds PI 3-kinase [85,111,112], and the interaction between PI 3-kinase and IRS-1 is specifically mediated by the SH2 domains of p85 [113–116]. *In vitro*, the insulin receptor phosphorylates IRS-1 on four tyrosine residues in YMxM motifs (Tyr⁶⁰⁸, Tyr⁶²⁸, Tyr⁹³⁹ and Tyr⁹⁸⁷ in rat IRS-1) and one in a YxxM motif (Tyr⁶⁶⁰) [117]. The best evidence that these sites are important in the recruitment of PI 3-kinase comes from studies where deletion of these sites abrogated the ability of IRS-1 to bind to PI 3-kinase [118,119]. Conversely, a mutant form retaining only Tyr⁶⁰⁸, Tyr⁶²⁸ and Tyr⁶⁶⁸ in YMxM motifs as potential tyrosine phosphorylation sites, 15 other potential sites having been deleted, supported normal insulin stimulation of PI 3-kinase activity and cell growth when compared with wild-type IRS-1 [120].

IRS proteins play a dual role in insulin signalling via PI 3-kinase. As discussed in detail below, there is evidence that IRS-1 mediates targeting of PI 3-kinase to specific intracellular sites during insulin action, which may contribute to the specific ability of insulin and IGF-1 to stimulate responses such as translocation of the glucose transporter GLUT4. Secondly, a number of studies have demonstrated that binding of PI 3-kinase to IRS-1 directly stimulates its activity [58,114,116]. This can be mimicked by peptides containing a phosphorylated YMxM motif, although the potency and extent of activation is much greater when peptides containing two phosphorylated YMxM motifs are used [57,121,122]. This indicates that simultaneous occupancy of both SH2 domains of p85 is necessary for efficient recruitment and activation of PI 3-kinase. This is an appealing mechanism in physiological terms, as the affinity of the SH2 domains, even for the optimal phosphotyrosine motif, is relatively low, so two simultaneous engagements would favour formation of a stable complex and would in turn allow a greater deal of selectivity for the interaction of p85 with its targets [123].

Mechanisms attenuating the ability of IRS proteins to stimulate PI 3-kinase

The capacity of insulin to activate PI 3-kinase is inhibited by diverse agents, including phorbol esters, tumour necrosis factor- α , glucagon and okadaic acid [124–127], which are presumed to act by increasing the serine/threonine phosphorylation of IRS proteins. Relatively little is known about the protein kinases (PKs) which act on IRS proteins or their sites of phosphorylation. IRS-1 contains multiple consensus phosphorylation sites for a number of different protein-serine kinases [85,86] and is an effective substrate for casein kinase-2 [128], glycogen synthase kinase-3 (GSK3) [129] and mitogen-activated protein (MAP) kinase [130], at least *in vitro*. The p110 catalytic subunit of PI 3-kinase also has limited serine kinase activity towards IRS-1 *in vitro*, which could, in principle, participate in an autoregulatory loop [131–134]. Mutational analysis suggested that some or all of the serine residues (Ser⁶¹², Ser⁶³², Ser⁶⁶² and Ser⁷³¹) in IRS-1 participate in the negative modulation of insulin-stimulated PI 3-kinase activity induced by the phosphatase inhibitor okadaic acid [135]. These residues all lie in consensus PXSP motifs for

MAP kinase phosphorylation, in close proximity to YMxM tyrosine phosphorylation sites implicated in p85 binding. Ser⁶¹² was also identified as a site of phosphorylation induced by phorbol esters, apparently resulting from protein kinase C (PKC)-induced MAP kinase activation [130,136]. As MAP kinase is activated by insulin in parallel with PI 3-kinase, this could provide a basis for cross-talk between the two pathways, leading to feedback regulation of PI 3-kinase activity.

Several mechanisms may contribute to the negative modulation of PI 3-kinase activity by serine phosphorylation of IRS-1. It has been suggested that serine phosphorylation may alter the subcellular location of IRS-1 and hence impair the ability of insulin to phosphorylate it [137], although this remains to be formally tested. Serine phosphorylation may also directly impair the capacity of IRS-1 to undergo tyrosine phosphorylation at adjacent sites [130] or may convert IRS-1 into a more general inhibitor of the insulin-receptor tyrosine kinase [127,129]. Serine phosphorylation of IRS-1 also allows interaction with the 14-3-3 β protein, and this interaction decreases the catalytic activity of the PI 3-kinase associated with IRS-1 [138]. Interestingly, 14-3-3 β binds specifically to a consensus motif RSXS(P) [139], which is a potential site for phosphorylation by kinases, including PKCs and PKB [140]. Both IRS-1 and IRS-2 contain several such motifs, and it is known that insulin activates PKB and PKC- ζ in a PI 3-kinase-dependent manner. This suggests a potential feedback mechanism for regulating insulin signalling. However, it has yet to be shown whether PKB or PKC- ζ do indeed phosphorylate IRS-1 *in vivo*. It also remains to be determined whether similar mechanisms involving serine phosphorylation of IRS-1 contribute to pathophysiological states of insulin resistance *in vivo*.

Direct association of PI 3-kinase with receptors

The potential role of direct association with the insulin receptor and IGF-1 receptor in the recruitment and activation of PI 3-kinase has received some attention. The fraction of PI 3-kinase activity associated with the insulin and IGF-1 receptors following stimulation is at best small [141–143]. These observed associations may in part reflect the formation of ternary complexes in which phosphorylated IRS-1 binds simultaneously to the juxtamembrane region of the receptor and to SH2 domains of PI 3-kinase [144–146]. However, the insulin receptor and the IGF-1 receptor possess a conserved tyrosine autophosphorylation site in a YxxM motif, located towards the C-terminus of the β -subunit, and several studies have demonstrated direct binding of PI 3-kinase to this C-terminal site of autophosphorylated insulin and IGF-1 receptors [147–151]. However, it is not clear that occupancy of one SH2 domain of p85 by the single available YxxM phosphotyrosine motif on the receptor would result in full activation. It is conceivable that p85 might bind simultaneously to both β -subunits within the native receptor, or that it might bind to both receptor and IRS-1 *in vivo*. Indeed, it has been reported that such intramolecular bridging can occur, with the N-SH2 domain binding preferentially to the receptor and the C-SH2 domain to IRS-1 [152]. According to this model, p85 might function not only as a regulatory subunit of PI 3-kinase, but also as an adapter molecule facilitating other aspects of IRS function. Even if the fraction of PI 3-kinase which becomes associated directly with activated insulin or IGF-I receptors is small relative to that associated with IRS proteins, it is possible that this pool of activity might have a distinct function by virtue of its specific subcellular location.

One potential function of the direct binding of SH2-domain-containing proteins to receptor tyrosine kinases is their re-

cruitment for phosphorylation. Tyrosine phosphorylation of both the p85 and p110 subunits by activated PDGF receptors [153–155], of p85 by insulin receptors [156] and of the p55^{PIK} adapter subunit by the insulin receptor [55] have all been reported. It was suggested that phosphorylation of p85 by PDGF or insulin might contribute directly to increased PI 3-kinase activity [157,158], although the evidence was somewhat indirect. Moreover, the major sites on p85 phosphorylated *in vitro* by PDGF receptors [159] and insulin receptors [156] are different. The level of phosphorylation of p85 and p110 *in vivo* in response to PDGF is low [26,29], and most studies which have investigated the matter have failed to detect any phosphorylation of p85 in response to insulin [61,111,116,146,160]. Overall, it can be concluded that tyrosine phosphorylation of PI 3-kinase does not play a major role in insulin's activation of the enzyme, but the possibility that specific pools of activity are regulated in this way cannot be ruled out.

Role of Ras and PKC in PI 3-kinase activation

While the major means by which insulin activates PI 3-kinase is via association of the SH2 domains of p85 with tyrosine-phosphorylated IRS proteins, other mechanisms for regulating PI 3-kinase activity and function could also potentially be utilized by insulin. One such mechanism is via Ras. It has been demonstrated that activated GTP-loaded Ras interacts directly with PI 3-kinase [42,161]. This interaction involves the previously characterized effector domain of Ras and an N-terminal domain of the p110 catalytic subunit of PI 3-kinase [41]. Studies with dominant negative and oncogenic forms of Ras suggest that the interaction activates the PI 3-kinase [42,162,163] and *in vitro* reconstitution experiments indicate that this activation is synergistic with that induced by binding of tyrosine-phosphorylated peptides to the p85 subunit [41]. It is also conceivable that Ras–p110 interactions could play a role in localizing PI 3-kinase to specific sites on membranes, as Ras is a membrane-anchored protein and is almost exclusively present at the plasma membrane [164].

It is well established that insulin can activate Ras, through binding of Grb2–Sos complexes to phosphorylated SHC and/or IRS proteins [165]. Thus it has been proposed that activation of PI 3-kinase by Ras may provide an alternative effector system to the well-established Raf/MAP kinase kinase (MEK)/MAP kinase cascade, and this pathway may make a contribution to the overall activation of PI 3-kinase by insulin. However, this issue is far from straightforward, as other data suggest that PI 3-kinase can cause activation of Ras, which would make Ras an effector for PI 3-kinase [44]. The hydrolysis of GTP on Ras is regulated by RasGAP and activation of PI 3-kinase can reduce Ras GTPase activating protein ('RasGAP') activity in some cell types thus contributing to activation of Ras [166]. Further, GDP ↔ GTP exchange on Ras may also be modulated by PI 3-kinase, as the PIP₃ can interact with the PH domain of the Ras guanine-nucleotide exchange factor (GEF) Sos, potentially affecting the localization or activity of Sos [97]. The possibility that Ras may be an effector of PI 3-kinase is supported by the finding that inhibition of PI 3-kinase blocks insulin-stimulated Fos induction, an effect that can be overcome by the expression of activated Ras [167]. Further, inhibition of PI 3-kinase decreases the activation of the MAP kinase by insulin in some cells [168–170]. However, it is not clear whether PI 3-kinase is acting upstream or downstream of Ras in this situation.

A further possible mechanism for stimulating PI 3-kinase may involve the PKC system, as phorbol esters which activate PKC also stimulate an increase in cellular levels of PIP₃ [17,171]. However, the molecular basis of this is poorly understood.

Mechanisms by which activation of PI 3-kinase leads to activation of signal transduction pathways

Lipid products of PI 3-kinase as signalling molecules

The most obvious way that class 1a PI 3-kinases might signal is via their lipid products. PIP₃ is the major product of class 1a PI 3-kinases, and there are three major lines of evidence to suggest that this plays a direct role in signalling.

(a) The rise in PIP₃ levels correlates with activation of downstream responses. Levels of PIP₃ are very low in unstimulated cells and are increased within seconds of agonist stimulation in a range of cell types [16–19,22,31,172]. The rise in PIP₃ levels precedes the activation of signalling molecules thought to lie downstream of PI 3-kinase [18,19]. Further, pretreatment with the specific PI 3-kinase inhibitors wortmannin and LY-294002 blocks the agonist-stimulated accumulation of PIP₃ in parallel with their inhibitory effects on activation of downstream response [19,33,173,174]. These findings are consistent with PIP₃ acting as an early upstream element in signal-transduction cascades.

(b) The rise in PIP₃ is transient. The duration of the rise in PIP₃ levels depends on cell type and the agonist used to stimulate the cells, and can range from a matter of seconds up to several hours [16,18,22,31]. The transience of the increase in PIP₃ is consistent with a role as an agonist-regulated molecular on/off switch. Cellular PIP₃ levels are regulated by the balance between the rate of PIP₃ production, which relies on continued stimulation and PIP₃ degradation. The mechanism for the degradation of PIP₃ is specific and is also regulated. Unlike its precursor PI(4,5)P₂, PIP₃ is not subject to degradation by any known phospholipases. The main degradative route of PIP₃ appears to be to PI(3,4)P₂ via the action of a phosphatidylinositol 3,4,5-trisphosphate 5'-phosphatase (PIP₃–5'-phosphatase), a number of forms of which have been identified [175]. There is evidence that insulin stimulates PIP₃–5'-phosphatase activity [176], although the form stimulated by insulin is not known. In platelets a PIP₃–5'-phosphatase activity has been found that directly associates with p85, although this remains uncloned, and it is not clear if it is present in insulin-responsive tissues [177]. However, the SH₂-containing inositolide 5'-phosphatase ('SHIP') PIP₃–5'-phosphatases are likely candidates as an insulin-responsive PIP₃–5'-phosphatase, as they contain an SH2 domain which binds to tyrosine-phosphorylated SHC and insulin stimulates the tyrosine phosphorylation of SHC. SHIP1 is only expressed in haematopoietic cells [178], although the recently cloned SHIP2 is expressed in insulin-responsive tissues [179] and could potentially be activated via an SHC-dependent mechanism, but this remains to be tested.

Co-ordinated regulation of PI 3-kinase and PIP₃–5'-phosphatase potentially provides an elegant mechanism for temporal regulation of linked signalling pathways. In this model the rapid activation of PI 3-kinase by insulin causes elevation of cellular PIP₃ levels, which allows for activation of a certain subset of signalling pathways. The PIP₃–5'-phosphatase activation would down-regulate PIP₃ signals, but by increasing PI(3,4)P₂ it may also activate a different subset of signalling pathways.

(c) PIP₃ can directly modulate downstream events. Direct evidence for a functional role for PIP₃ has come from studies showing that addition of membrane-permeant forms of PIP₃ to cells can directly modulate downstream events [46]. Two mechanisms have been suggested whereby PIP₃ could modulate downstream events. One is by directly affecting the properties of cellular membranes. For example, it has been proposed that localized production of phosphoinositide products could cause membrane curvature and thus contribute to vesicle budding [180]. This may be particularly relevant in the case of PIP₃, owing

Table 1 Characteristics of proteins that bind PIP₃

Further abbreviations used: Gro, glycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; NP40, Nonidet P40; N.D., not determined.

Target protein	Protein function	Form of side-chain fatty acids present	Method of lipid presentation	K _m for binding	Binding via PH domain	Reference(s)
PDK-1	PK upstream of Akt/PKB	Synthetic stearoylarachidonyl, dilinoleoyl and dipalmitoyl.	PC/PS/PI (10:10:1) as sonicated vesicles	1–2 μ M	Yes	[192,193]
Akt-1/PKB	PK	Synthetic dipalmitoyl	PC/PS unilamellar vesicles		Yes	[193,194]
PKC- δ , - ϵ , - η	PKs	(a) Labelled phospholipid with purified PI 3-kinase (b) Synthetic dioctanoyl	(a) + PS, PE (b) None	(a) $\approx$ 0.2 μ M (b) $\approx$ 1 μ M	Contain no PH domain	[189]
PKC- ζ	PKs	Labelled phospholipid with purified PI 3-kinase	+ PS, PE	$\approx$ 50 mM	Binding domain not known	[190]
PKC- β 1, - ϵ , - η	PKs	Labelled phospholipid with purified PI 3-kinase	+ PS in Triton X-100 micelles	4–8 mol%	Contain no PH domain	[191]
PRK	PKC-related protein kinase	Labelled phospholipid with purified PI 3-kinase	+ PS in Triton X-100 micelles	4–8 mol%	Binding domain not known	[191]
PLC- γ	Phospholipase	Gro-PIP ₃	Not stated	2 μ M	Yes	[195]
Dynamin	Vesicle budding	Synthetic dipalmitoyl	PC/PI (9:1) as sonicated vesicles	3 μ M	Yes	[184]
Synaptotagmin	Synaptic-vesicle fusion	Synthetic dipalmitoyl	PC/PS liposomes	N.D.	No; via C2 domain	[181]
p85 α C-SH2 domain	Phosphotyrosine-motif-binding domain	Labelled purified PI using recombinant PI 3-kinase	PS ($\pm$ PC, PE, PA and cholesterol) sonicated vesicles + 0.02% NP40	N.D.	No; via SH2 domain.	[196]
Profilin	Regulates actin assembly	Synthetic dipalmitoyl and dioctanoyl	—	5.7 μ M	Binding domain not known	[197]
AP-2	Clathrin adapter, involved in endocytosis	Synthetic dioctanoyl	—	$\approx$ 0.3 μ M	Binding domain not known	[198]
AP3	Clathrin adapter, involved in endocytosis	Synthetic dioctanoyl	—	5–10 μ M	Binding domain not known	[199]
BTK	Bruton's tyrosine kinase	Synthetic dipalmitoyl	—	—	Yes	[98]
GRP-1	Arf GEF	Labelled brain phospholipid with recombinant p110	Used PS as carrier	3–8 μ M	Yes	[188]
Cytohesin-1	Arf GEF	Labelled brain phospholipid with recombinant p110	Used PS as carrier		Yes	[188]
SOS	Ras-GEF	(a) C ₁₆ PIP ₃ (b) C ₈ PIP ₃	(a) Micelles (b) Soluble	$\approx$ 8 μ M 0.3 μ M	Yes	[97]
Tiam-1	Rho GEF	(a) C ₁₆ PIP ₃ (b) C ₈ PIP ₃	(a) Micelles (b) Soluble	$\approx$ 3 μ M 0.4 μ M	Yes	[97]
Neurogranin	PKC substrate with homology to Arf GAP	Synthetic dipalmitoyl	PIP ₃ micelles	2 μ M	Yes	[200]
PIP ₃ -binding protein	Homology with Arf GAP	IP ₃ -ADP (PIP ₃ analogue)	None	N.D.	Yes	[185]
p42 _{p4}	Homology with Arf GAP	Gro-PIP ₃	—	0.9 nM	Yes	[186]
α -Centaurin	Homology with Arf GAP	Synthetic dipalmitoyl	—	120 nM	Yes	[187]

to its extremely high charge density and its ability to be produced at distinct cellular locations adjacent to activated signalling complexes. However, the biophysical effects of PIP₃ on vesicle budding and fusion events have not been formally tested.

The other possibility, which has received much more attention, is that PIP₃ specifically interacts with molecules in downstream signal-transduction pathways. Several mechanisms have been identified by which such interactions might mediate changes in activity of target proteins: (i) by acting in an allosteric manner they may alter the catalytic activity of targets, (ii) by changing conformation the interactions may expose regulatory sites for phosphorylation, and (iii) the interactions may regulate co-localization with other PIP₃-binding proteins to form specific signalling complexes at membranes. There is mounting evidence for such mechanisms, as PIP₃ is able to interact with, and in some cases activate, a range of molecules, including some well known components of signal-transduction cascades (see Table 1). However, the *in vivo* significance of many of these interactions remains in some doubt, and it is currently very difficult to make

predictions based on *in vitro* PIP₃ binding data. This is due to a lack of consistency in the methodology, and in particular to differences in the method of lipid presentation and in the type of lipid side chain in PIP₃ used in these studies (see Table 1). The exact buffer conditions are also likely to have profound impact on the pattern of binding observed as, for example, it has been demonstrated that increasing the concentration of calcium can shift the synaptotagmin C2 domain binding preference from PIP₃ to PI(4,5)P₂ [181]. The most appropriate form of PIP₃ to use in these experiments is *sn*-1-stearoyl-2-arachidonyl PIP₃, as this is the form in which it is found in cell membranes. The importance of this is highlighted by the studies of Alessi and colleagues [182], who have performed one of the few direct comparisons of the efficacy of different forms of PIP₃. As described below, they found that PKB was a far better substrate for 3-phosphoinositide-dependent PK-1 (PDK-1) when incubated with *sn*-1-stearoyl-2-arachidonyl PIP₃ compared with incubations in the presence of dipalmitoyl PIP₃. Therefore, physiologically relevant evaluations of protein-PIP₃ interactions will be aided by the availability of

synthetically produced *sn*-1-stearoyl-2-arachidonyl PIP₃, with the availability of the D and L enantiomers also being important to facilitate appropriate control experiments [182,183].

Another problem with much of the data regarding binding of PIP₃ to targets is that the selectivity of this interaction is often weak. If these interactions are to represent a mechanism by which PI 3-kinase may selectively activate a downstream signalling pathway, then there must be a high degree of specificity for binding PIP₃ or PI(3,4)P₂. Particularly important is the specificity relative to PI(4,5)P₂, the major substrate for PI 3-kinase, which must necessarily be present wherever PIP₃ is produced, and which is present at more than 100 times the level of PIP₃, even in stimulated cells. Thus, although dynamin GTPase activity was increased by dipalmitoyl PIP₃ with an EC₅₀ of 3 μ M, PI(4,5)P₂ caused a greater increase in activity with a lower EC₅₀ [184], suggesting that PIP₃ is unlikely to be the major stimulator of dynamin *in vivo*, even taking into account the form of PIP₃ used in these experiments. Specificity must also be demonstrated relative to inositol polyphosphates which are present at relatively high concentrations in the cytosol and have structures which might mimic the head groups of PIP₃ or PI(3,4)P₂.

One structural feature shared by many of the PIP₃-binding proteins is the presence of a PH domain. In many cases binding of PIP₃ to target proteins is via PH domains, although not all PH domains bind PIP₃ [97,98], and there is a great deal of variability in the relative preference of PH domains for other phosphoinositides. This suggests that a distinct subclass of PH domains exists with the ability to bind either PIP₃ or PI(3,4)P₂. Proteins containing PIP₃-binding PH domains include a range of molecules which affect the GTP loading status of small GTP proteins, including a molecule having homology with ARF GTPase-activating protein (GAP) [185–187], cytohesin and GRP-1, both of which show homology with ARF GEF [188], the Ras-GEF Sos [97] and the rho GEF TIAM-1 [97]. PIP₃-binding PH domains are also found in a range of kinases, including BTK [98], PKB and PDK-1 [182,183]. PIP₃ also binds to some isoforms of PKC [189–191], although these do not contain PH domains. The domain responsible for PIP₃ binding to PKCs is poorly characterized, and the specificity of PIP₃ binding compared with PI(4,5)P₂ does not seem to be as great as with the PH domains described above.

PK activity of p110 may play a role in signalling

Class 1a PI 3-kinases also possess a protein serine kinase activity, raising the prospect that this could play a role in growth-factor signalling. The PK activity of p110 α has been best characterized, but only a limited range of protein substrates have been identified. The major substrate appears to be Ser⁶⁰⁸ on the p85 α adapter/regulatory subunit and this phosphorylation decreases the lipid kinase activity of the complex [201,202]. However, it is not clear whether alteration in the serine phosphorylation of p85 plays a significant role in regulating PI 3-kinase activity *in vivo*, as in the only case where it has been investigated the level of serine phosphorylation of p85 did not change following PDGF stimulation [29]. IRS-1 can also be phosphorylated by p110 α , although the sites of phosphorylation and physiological importance of this reaction remain unknown [131,203].

Other domains of p85/p110 may facilitate formation of tertiary signalling complexes

As well as interacting with p110, full-length p85 α and p85 β are able to interact with a range of other molecules via their SH3, BH or proline-rich domains (as described in detail above). Therefore the growth-factor-induced recruitment of p85 to receptors or

Table 2 Effects of PI 3-kinase inhibitors on the cellular actions of insulin

Processes totally inhibited
Stimulation of:
GLUT4 and transferrin receptor translocation
Membrane ruffling
4EBP1 phosphorylation
Expression of hexokinase gene
Activation of:
Glycogen synthase
Acetyl-CoA carboxylase
p70 ^{s6k}
PKB
PKC- ζ
Jun kinase
PDE3b
Inhibition of:
HSL
GSK3
eEF2-kinase
Processes partially inhibited or inhibited in cell-type-specific manner
Activation of:
Ras
MAP kinase
Stimulation of:
Mitogenesis
Processes not inhibited
Activation of:
Pyruvate dehydrogenase
Insulin-receptor tyrosine kinase
GLUT4 endocytosis

docking proteins will also result in the recruitment of these additional proteins and, indeed, some of these may be critical to the exact nature of the response generated by growth factors. Further, it would be expected that the relative importance of these complexes would be attenuated in tissues where large amounts of the truncated forms of the adapter/regulatory subunit are expressed.

LINKS BETWEEN PI 3-KINASE AND DOWNSTREAM SIGNALLING

Protein kinase B

A great deal of evidence has accumulated to indicate that activation of PI 3-kinase is necessary and in some cases sufficient to trigger many insulin-stimulated signalling pathways (Table 2 and discussed below). This suggests that PI 3-kinase lies at the top of a signal-transduction cascade. However, it is known that many of the downstream signalling events are controlled by serine/threonine kinase cascades and the key question is: how does a lipid kinase translate its signal into a PK cascade? One answer to this lies in the PDK/PKB system. PKB, the cellular homologue of the transforming oncogene *v-akt*, is a PH domain containing serine/threonine kinase that is acutely activated by a range of growth factors, including EGF, PDGF, basic fibroblast growth factor, insulin and IGF-1 [18,204–208]. Three isoforms of PKB have been identified. PKB α is the major isoform activated by insulin in muscle and liver, the two major insulin target tissues [209]. Both PKB α and PKB β play a role in insulin signalling in adipocytes, but PKB γ is not activated by insulin in muscle, liver or adipocytes, although it is activated by insulin in most cell-culture models [209]. There is very strong evidence to indicate that activation of PI 3-kinase is both necessary and sufficient for the activation of all PKB isoforms by growth factors. The activation of PKB by growth factors is blocked by inhibitors of

PI 3-kinase [205–208], by expression of dominant negative forms of the PI 3-kinase adapter/regulatory subunit [206,208] and by expression of receptor mutants that lack the ability to stimulate PI 3-kinase [205,206]. Expression of constitutively active class Ia PI 3-kinase also results in the activation of PKB [45,210]. The magnitude and timing of activation of PKB also closely correlates with the magnitude and timing of increases in cellular PIP_3 levels [18,19].

A direct mechanism for PI 3-kinase-dependent activation of PKB was initially suggested by reports that PI3P and PI(3,4)P_2 could both bind to, and activate, PKB [205,211,212]. However, other groups have reported that while PIP_3 and PI(3,4)P_2 can bind to PKB they do not directly activate the kinase [182, 183,194,212]. It is now clear that the major means by which growth factors and insulin increase PKB activity is by phosphorylation on two sites (Thr^{308} and Ser^{473} in $\text{PKB}\alpha$) and in this form PKB is active independent of phosphoinositides [204]. The dependence on phosphoinositides for activation lies in the processes regulating these phosphorylation events. A kinase responsible for phosphorylating Thr^{308} of $\text{PKB}\alpha$ has been characterized and cloned and named PDK-1 [182,193,213]. This also phosphorylates the corresponding site of $\text{PKB}\beta$ (Thr^{309}) and $\text{PKB}\gamma$ (Thr^{305}) [209]. PDK-1 has a single PH domain which avidly binds PIP_3 . The observation that phosphorylation of Thr^{308} on PKB by PDK-1 was stimulated by PIP_3 was initially interpreted as an activation of PDK-1 by binding of PIP_3 to its PH domain [182,183]. However, the consensus that is now emerging indicates that PIP_3 binding does not greatly increase the intrinsic activity of PDK-1 towards PKB [192,193,213]. Further, PDK-1 is constitutively active as a kinase towards p70^{s6k} in the absence of PIP_3 [192,214] and a PKB construct lacking a PH domain is phosphorylated and activated by PDK-1 *in vitro* in a PIP_3 -independent manner [213]. Therefore the most likely mechanism is that PIP_3 binding induces a conformational change which makes Thr^{308} accessible to PDK-1. The other major function of PIP_3 binding in this system is likely to be in promoting co-localization of PDK-1 and PKB following growth-factor stimulation and thereby facilitating efficient phosphorylation of PKB. The kinase responsible for phosphorylating Ser^{473} is poorly characterized, but has been tentatively termed PDK-2 as it is also PI 3-kinase-dependent [182].

Glycogen synthase kinase

It is now apparent that PKB and PDK-1 represent the means by which PI 3-kinase can regulate several PK cascades (Scheme 1). One important downstream effector of PKB is GSK3. Insulin stimulation leads to inhibition of GSK3 isoforms by inducing the phosphorylation of $\text{GSK3}\alpha$ on Ser^{21} and $\text{GSK3}\beta$ on Ser^9 . Insulin stimulation of this phosphorylation is blocked by wortmannin, with a dose-dependency suggesting that PI 3-kinase activity is required for this process [169,170,207,215]. It has previously been reported that GSK3 isoforms are substrates for the p70^{s6k} [216], although this seems unlikely to occur *in vivo* as rapamycin, which potentially blocks activation of p70^{s6k} , does not block the insulin-induced inactivation of GSK3 in whole cells [170,217]. Both Ser^{21} of $\text{GSK3}\alpha$ and Ser^9 of $\text{GSK3}\beta$ are also direct substrates for PKB *in vitro* [218], and the temporal pattern of activation of PKB is consistent with a role in the *in vivo* phosphorylation of GSK3 [18]. This suggests that GSK3 lies immediately downstream of PKB in PI 3-kinase-dependent signalling cascades.

p70 ribosomal s6 protein kinase

There is also strong evidence indicating that activation of PI 3-kinase and subsequent activation of the PDK/PKB system is

involved in the activation of p70^{s6k} . The activity of p70^{s6k} is increased by expression of constitutively active class Ia PI 3-kinases [210,219], and activation of p70^{s6k} is blocked by dominant negative forms of the PI 3-kinase adapter/regulatory subunit [206] and by wortmannin and LY294002 [21,215,219]. Full activation of p70^{s6k} depends on a sequential phosphorylation of eight sites, culminating in phosphorylation of Thr^{252} [220]. PKB may play a role in the activation process as expression of constitutively active PKB also activates p70^{s6k} [206,221]. Importantly, similar results were found when a conditionally active form of PKB was expressed and activated, acutely indicating that it is not a consequence of long-term exposure of cells to elevated kinase activity [222]. No direct phosphorylation of p70^{s6k} by PKB has yet been demonstrated, although it has recently been established that PDK-1 directly phosphorylates p70^{s6k} at Thr^{252} [192,214] and thus contributes to activation. Another of the phosphorylation sites on p70^{s6k} , Thr^{389} , has homology with Ser^{473} on PKB, which suggests that the yet-to-be-cloned PDK-2 is responsible for phosphorylating both these sites. It seems that other PI 3-kinase-dependent processes must also be involved, as activation of p70^{s6k} is quite slow and there is a lag of several minutes between activation of PKB and the activation of p70^{s6k} [18,223]. It has been suggested that the PI 3-kinase/PKB-dependent activation of p70^{s6k} may be mediated via Rac [221]. The observation that growth-factor-stimulated phosphorylation and activation of p70^{s6k} is blocked by rapamycin suggests that the mammalian target of rapamycin (mTOR), the sole cellular target for rapamycin, may also be a component of the insulin signalling cascade [18,206]. Further, overexpression of PKB leads to 4E-BP1 phosphorylation, and this is blocked by rapamycin when wild-type mTOR is expressed, but not when a rapamycin-resistant form of mTOR is expressed. This suggests that PKB lies upstream of mTOR [224]. mTOR contains several consensus phosphorylation sites for PKB, although whether such a phosphorylation event occurs *in vivo* remains to be demonstrated. mTOR itself contains a domain with weak homology with the catalytic subunit of PI 3-kinase, which has no lipid kinase activity but has a PK activity that is inhibited by wortmannin and LY294002 [225,226]. While previous studies have reported that p70^{s6k} is not a substrate for mTOR [227], one recent report claims that Thr^{389} of p70^{s6k} is a substrate for mTOR [228], an observation that raises the possibility that mTOR and PDK-2 are one and the same thing. However, if mTOR was directly responsible for phosphorylation of p70^{s6k} , it would be expected that insulin would dramatically increase mTOR kinase activity, and while it has recently been reported that serum stimulation causes a small increase of mTOR kinase activity [228], this seems insufficient to explain the large increase in phosphorylation of downstream molecules such as p70^{s6k} . Therefore the exact architecture of the PI 3-kinase-dependent pathways regulating p70^{s6k} remain unresolved.

Protein kinase C

Evidence is emerging to suggest that PI 3-kinase also plays a crucial role in activation of certain isoforms of PKC. Inhibition of the PKC-dependent phosphorylation of pleckstrin in platelets by low-nanomolar concentrations of wortmannin provided the first suggestion that PI 3-kinase may be involved in PKC-dependent signal transduction [229]. More recently it has been established that introduction of active PI 3-kinase into cells can lead to activation of a range of atypical PKC isoforms [230,231]. These proteins do not contain PH domains, and the exact mechanism of this activation is not known, although it is clear that a number of atypical PKC isoforms bind PIP_3 with

reasonably high affinities and that this results in activation [189–191]. A role for PIP_3 in this process has been further bolstered by the recent finding that PKC- ζ can be activated by insulin, and this is blocked by inhibitors of PI 3-kinase and expression of dominant negative p85 [232,233]. As in the PDK/PKB system, PIP_3 may also facilitate PKC signalling by co-localizing the enzyme with substrate or altering conformation of substrate to facilitate phosphorylation. One example of this is phosphorylation of neurogranin, as PIP_3 binding to this protein makes it a better substrate for phosphorylation by PKC [200]. Phosphorylation of PKC isoforms is necessary for full activation, and two of the regulatory phosphorylation sites on PKCs could potentially be targets for phosphorylation by PDK-1 and PDK-2 [234]. This remains to be formally tested, but represents another possible mechanism by which activation of PI 3-kinase could affect PKC activity.

Jun kinase

The jun-N-terminal kinase (JNK) also lies downstream of PI 3-kinase, as demonstrated by the findings that PI 3-kinase activity is necessary [235] and sufficient [210,236] for the growth-factor-stimulated activation of JNK. No direct effects of PIP_3 on JNK have been described, and it is likely that the activation pathway in this case involves the small GTP-binding proteins Rac or cdc42 as these lie upstream of JNK [237] and the activation of Rac is PI 3-kinase-dependent [238].

Serine phosphatases

As well as activating kinases, insulin also regulates the activity of the serine protein phosphatases, and this regulation is likely to be of great importance in dictating the phosphorylation level and hence activation state of many of the downstream signalling molecules described above. Insulin activation of protein phosphatase-1 (PP1) is blocked by low doses of PI 3-kinase inhibitors but not by rapamycin [239], while insulin-induced inactivation of protein phosphatase-2A (PP2A) is blocked by both PI 3-kinase inhibitors and by rapamycin [240]. This indicates that insulin regulates both PP1 and PP2A via PI 3-kinase-dependent pathways, but that the regulatory mechanisms for these diverge downstream of PI 3-kinase.

EVIDENCE LINKING PI 3-KINASE TO ENDPOINT RESPONSES

Cell growth and proliferation

A number of findings in different experimental systems indicate that PI 3-kinase plays a critical role in growth-factor signalling to cell growth and proliferation. This was first suggested by studies using PDGF-receptor mutants unable to bind PI 3-kinase in parallel with add-back mutants, which showed that PI 3-kinase was a downstream mediator of the PDGF-receptor mitogenic signal [241]. Similar mechanisms appear to exist in the insulin/IGF-1 signalling system as the mitogenic effects of insulin and IGF-1 are attenuated by inhibitors of PI 3-kinase [21]. This is further supported by the finding that overexpression of constitutively active p110 is sufficient to induce DNA synthesis in 3T3-L1 adipocytes [43]. Insulin-stimulated DNA synthesis is also stimulated by microinjection of antibodies that stimulate PI 3-kinase activity [242] and blocked by microinjection of dominant negative forms of p85 α [243].

However, it has become evident that not all growth factors activate PI 3-kinase and that the involvement of this signalling pathway in the proliferative response is cell-type-specific. For example, antibodies to p110 α , which inhibit the activity of the

enzyme, also inhibit PDGF- and EGF-stimulated DNA synthesis but not that stimulated by lysophosphatidic acid and bombesin, when microinjected into Swiss 3T3 cells [155].

The potential role of PI 3-kinase in signalling to cell growth and proliferation has sparked interest in its role in cell transformation. This has been heightened by the recent identification of a transforming retrovirally encoded PI 3-kinase isolated from chicken haemangiosarcomas [244]. Interestingly, overexpression of a form of p85 lacking the C-SH2 domain also constitutively activates PI 3-kinase and induces cellular transformation [245]. Consistent with this, expression of dominant negative p85 reduced the ability of Ras, but not Src, to transform cells [246]. This highlights the need for tight regulation of PI 3-kinase activity in the physiological context.

Differentiation

Insulin and IGF-1 activate signalling pathways important in the differentiation processes of two major insulin target tissues, namely muscle and adipose tissue, and PI 3-kinase activity is necessary for these effects. IGF-1-stimulated myoblast differentiation is blocked by PI 3-kinase inhibitors and dominant negative forms of p85 [247]. Wortmannin also blocks differentiation of 3T3-L1 cells into adipocytes [248], and this process is stimulated by overexpression of constitutively active PKB, suggesting that a kinase cascade is downstream of PI 3-kinase in this process [249]. Rapamycin also blocks the differentiation of 3T3-L1 adipocytes, implying that p70^{s6k} and/or the mTOR are also involved [250]. One clue may lie in the finding that both PI 3-kinase inhibitors and rapamycin block the dephosphorylation of C/EBP α , a transcription factor essential for the differentiation of adipocytes [251].

Apoptosis

PI 3-kinase has been implicated in the regulation of cell survival by a number of growth factors. In PC12 cells, nerve growth factor prevents apoptosis induced by serum withdrawal, and the PI 3-kinase inhibitors wortmannin and LY294002 block this effect [252]. Additionally, PDGF prevents apoptosis and stimulates PI 3-kinase in PC12 cells expressing the wild-type PDGF receptor, but does not prevent apoptosis in cells expressing mutant PDGF receptor incapable of recruiting PI 3-kinase. The critical role of PI 3-kinase in anti-apoptotic signalling has also been shown in other cell types. Apoptosis induced in fibroblasts by overexpression of c-Myc was blocked by low concentrations of wortmannin and LY294002 or by co-expression of a constitutively activate mutant p110 [253]. The c-Myc-induced apoptosis was also blocked by constitutively expressed PKB, and this effect was not blocked by PI 3-kinase inhibitors, implying that PKB is involved in anti-apoptotic signalling downstream of PI 3-kinase [253]. Analogous studies by other groups have reached similar conclusions, demonstrating that activation of PI 3-kinase and PKB, but not the MAP kinase, p38/HOG1 and p70^{s6k} pathways, are involved in anti-apoptotic signalling by growth factors, including insulin/IGF-I [254–256]. PI 3-kinase-dependent activation of the PKB pathway also protects against apoptosis induced by cell detachment (anoikis) [257]. Consistent with this, the detachment of epithelial cells from a matrix led to a rapid decrease in the cellular levels of PI 3-kinase products and the activity of PKB, whereas re-attachment of cells had the opposite effects [257]. There is evidence that effects of PKB on cell survival are at least in part mediated by direct phosphorylation of BAD, a Bcl2 homologue, by PKB [258]. This phosphorylation facilitates the sequestration of BAD by 14-3-3 protein and prevents formation of BAD–Bcl2 dimers. It is currently not clear whether

additional molecules downstream of PI 3-kinase are involved in protecting cells from apoptosis.

Vesicle traffic and glucose-transporter translocation

The regulation of blood glucose levels is one of the most important physiological functions of insulin. A key step in this process is the unique ability of insulin to stimulate translocation of GLUT4 glucose transporters to the cell surface and thus increase the rate of glucose uptake into muscle and adipocytes [259]. The first evidence that PI 3-kinase was necessary for this process came from experiments showing that insulin stimulation of recruitment of the GLUT4 glucose transporter to the cell surface was completely abolished by low-nanomolar concentrations of wortmannin [260]. These findings have subsequently been confirmed by a number of groups using both wortmannin and LY294002 in a range of insulin-sensitive tissues, including isolated rat adipocytes [261], 3T3-L1 adipocytes [21,262], L6 myotubes [172,263] and isolated muscle [81,173,264,265]. These compounds block insulin's ability to stimulate the rate of exocytosis of GLUT4 glucose transporters, with little effect on the rate of endocytosis [262,264]. The effects of wortmannin on insulin-stimulated glucose transport can be overcome by addition of membrane-permeable forms of PIP_3 [46], providing further evidence that PI 3-kinase is involved. Specific evidence for the involvement of class Ia PI 3-kinases in this process was provided by studies with dominant negative forms of the p85 and p55^{PIK} adapter/regulatory subunits which block insulin stimulation of glucose transport and glucose-transporter translocation [39,266,267]. This has since been bolstered by the finding that constitutively active forms of p110 are able to induce GLUT4 translocation to the plasma membrane in a range of cell types, albeit to a lesser extent than insulin [43,268,269]. However, PI 3-kinase inhibitors also block insulin stimulation of the trafficking of transferrin receptors [270,271]. As transferrin receptors are not present on GLUT4 vesicles, this suggests that the PI 3-kinase is involved in regulating a step in exocytosis that is not restricted to movement of GLUT4 vesicles.

Insulin stimulates the recruitment of PI 3-kinase from the cytosol to a low-density fraction in the cell [60,61,272–274]. This fraction also contains the GLUT4 vesicles, which immediately led to speculation of a direct link between this location of PI 3-kinase and translocation of GLUT4 vesicles. Indeed one study has claimed to observe a direct recruitment of PI 3-kinase on to GLUT4 vesicles [274]. However, a more detailed evaluation of the components of the low-density fractions revealed that insulin is recruiting PI 3-kinase to IRS-1 associated with a low-density cytoskeletal structure that is distinct from, but which co-sediments with, microsomal membranes upon subcellular fractionation [137].

The elements downstream of PI 3-kinase in the insulin-stimulated signalling pathways regulating GLUT4 translocation are poorly understood. The involvement of a serine/threonine kinase downstream of PI 3-kinase is suggested by the finding that stimulation of GLUT4 translocation by inhibitors of serine phosphatases is not blocked by PI 3-kinase inhibitors [260,265]. One kinase downstream of PI 3-kinase which has been ruled out is the p70^{S6k}, as rapamycin has no effect on insulin-stimulated glucose uptake [270,275,276]. However, PKB may be a better candidate. Consistent with this, it has recently been claimed that insulin causes the recruitment of the β isoform of PKB to GLUT4-containing microsomal vesicles [277]. Further, constitutively active PKB results in up-regulation of glucose transport and redistribution of GLUT4 to the plasma membrane in a range of cell types [278–281]. However, the long-term activation of

such a pivotal kinase would be predicted to have multiple effects on the cell. A role for PKB could be more definitively ascribed using specific inhibitors of PKB or using dominant negative or conditionally active forms of PKB. No specific inhibitors have yet been described for PKB. Further, attempts to block GLUT4 translocation by expressing kinase-dead versions of PKB produce only a minor reduction in insulin's effects [280]. This failure to act in a dominant negative manner probably reflects the fact that it would be difficult to produce enough kinase-dead PKB to sequester all the PIP_3 produced. Efforts to produce a conditionally active form of PKB have so far shed little light on the situation, as in 3T3-L1 adipocytes this construct only produces a slow and weak activation of the PKB and this does not correlate well with stimulation of GLUT4 translocation [222]. Therefore it is not yet clear whether activation of PKB is sufficient to stimulate GLUT4 translocation. On top of this, the issue of whether activation of PKB is necessary for insulin stimulation of GLUT4 translocation is further clouded by the finding that overexpression of PKC ζ leads to a translocation of GLUT4 similar to that induced by PKB overexpression [233]. Activation of PKC ζ by insulin is also PI 3-kinase-dependent, suggesting that PKB may not be the only kinase involved in the PI 3-kinase-dependent pathways regulating GLUT4 translocation.

It appears that small GTP-binding proteins are involved in GLUT4 translocation downstream of PI 3-kinase. One line of evidence for this is the finding that guanosine 5'-[γ -thio]triphosphate can stimulate GLUT4 translocation and can largely overcome wortmannin's inhibitory effect on insulin-induced stimulation of this translocation [262,282]. Rab4 is a candidate for this role, as it has been implicated as a potential insulin-regulated switch involved in the release of GLUT4-containing vesicles from the microsomal membrane fraction thus allowing their translocation to the plasma membrane [265,283–285]. The involvement of PI 3-kinase in the regulation of Rab4 function is suggested by the findings that wortmannin potently blocks the insulin-induced redistribution of Rab4 from GLUT4-containing microsomal membranes to the cytosol and insulin stimulation of GTP exchange on Rab4 [286,287]. The mechanism by which this occurs is unknown. The finding that insulin's effects on Rab4 redistribution are mimicked by okadaic acid [265] suggests a PK is involved, although the nature of this kinase is unknown.

Glycogen synthesis

Another key element in insulin's ability to promote glucose uptake into cells is its ability rapidly to stimulate the storage of glucose as glycogen. Glycogen synthase is the rate-limiting step in glycogen synthesis, and the glucose-6-phosphate-dependent activity of this enzyme is acutely increased by dephosphorylation of at least four regulatory serine residues (reviewed in [288]). Both wortmannin and LY294002 block insulin stimulation of glycogen synthesis and activation of glycogen synthase, suggesting the involvement of PI 3-kinase in pathways regulating this process [18,166,207,215,265,275,289,290]. Indeed, the recent experimental evidence described above provides circumstantial evidence for a linear PI 3-kinase-dependent pathway from the insulin receptor to glycogen synthase. In this pathway PKB-induced phosphorylation and inactivation GSK3 β would lead to decreased phosphorylation, and consequently activation, of glycogen synthase. In support of this, overexpression of constitutively active mutants of PKB or GSK3 β leads to an increase in the glycogen synthase activity ratio in intact cells [281]. However, in these experiments the cells are exposed to the activated forms of these kinases in greater amounts and for much longer periods

than during normal insulin stimulation. Therefore, while this seems the most plausible pathway identified to date to explain insulin's effects on glycogen synthase, these experiments still need to be interpreted with care.

Indeed several lines of evidence suggest that more than one pathway exists by which insulin can activate glycogen synthase. First, in some cell types the concentrations of PI 3-kinase inhibitors required to completely block insulin's activation of glycogen synthase are three to five times higher than those required to block stimulation of glucose transport and PI 3-kinase activity ([166,290]; P. R. Shepherd, unpublished work). It is also noteworthy that dominant negative forms of p85 do not block insulin's stimulation of glycogen synthase in CHO-IR cells [290]. A further strong piece of evidence for an alternative pathway is that insulin stimulation of glycogen synthase in 3T3-L1 adipocytes was not mimicked by either overexpression of p110 [43] or overexpression of PKB [281]. This may reflect patterns of gene expression, as it has been reported that GSK3 expression is low in these cells [281,291,292]. Taken together this indicates that insulin can regulate glycogen synthase by pathways other than via the PKB–GSK3 axis. The nature of the alternative signalling pathway to glycogen synthase in 3T3-L1 cells is not clear, although stimulation of glycogen synthase in these cells still apparently requires PI 3-kinase, as the insulin-induced activation is completely blocked by wortmannin [275]. One pathway that has been suggested to play a role in insulin's ability to regulate glycogen synthase is the JNK kinase via GSK3 [295]. This is consistent with the studies described above, which show activation of JNK kinase by insulin requires PI 3-kinase activity. However, this possibility has not been rigorously tested.

A further pathway for regulating glycogen synthase activity must also exist, as rapamycin partially inhibits the activation of glycogen synthesis and glycogen synthase in muscle and 3T3-L1 adipocytes without inhibiting insulin's stimulation of glucose transport [18,207,275,293]. The inhibitory effects of rapamycin and wortmannin can be temporally separated with the rapid phase of glycogen synthase activation (within 5 min) suppressed by wortmannin, but not by rapamycin, while a later phase of glycogen synthase activation (up to 30 min) is blocked by both wortmannin and rapamycin [18]. Rapamycin's effects are potentially mediated via blocking activation of p70^{s6k}, although it is unclear how this could regulate the activity of glycogen synthase. One untested possibility is that the effect of rapamycin on the late phase of glycogen synthase activation involves inhibition of the effect of insulin to up-regulate translation of glycogen synthase mRNA [294]. The rapamycin-sensitive pathway may also involve PP2A. No direct mechanism for regulation of glycogen synthase has been described, but rapamycin-dependent pathways are involved in the regulation of PP2A [296], and insulin-induced decrease in PP2A activity is PI 3-kinase-dependent and blocked by rapamycin [240].

The activity of glycogen synthase is also regulated by PP1, the main enzyme involved in dephosphorylating the regulatory sites on glycogen synthase (reviewed in [288]). The importance of PP1 as a regulatory mechanism for glycogen synthase varies with cell type. For example, insulin stimulates PP1 activity in 3T3-L1 adipocytes, but not in 3T3-L1 fibroblasts [292]. The insulin-induced increase in PP1 activity is blocked by PI 3-kinase inhibitors, suggesting this represents an additional PI 3-kinase-dependent pathway for regulating glycogen synthase activity [239].

Other actions of insulin on glucose metabolism

In cardiac muscle, insulin can stimulate glycolysis by phos-

phorylating and activating phosphofructokinase-2 (PFK-2). This action is inhibited by wortmannin [297], albeit at an IC₅₀ higher than is required to block insulin stimulation of glucose transport. However, the finding that PKB phosphorylates and activates PFK-2 *in vitro* supports the idea that PI 3-kinase is involved in this pathway [298].

There is some evidence that insulin stimulates the activity of ATP citrate-lyase (E. D. Saggerson and M. R. Orford, unpublished work), which could in turn contribute to an increase in the rate at which glucose could be converted into fatty acids. GSK3 β is known to phosphorylate ATP citrate-lyase [291], and it has recently been reported that this phosphorylation decreases the activity of this enzyme [299], thus suggesting that insulin-dependent activation of PI 3-kinase lies at the top of this cascade.

Insulin also regulates glucose homeostasis by controlling gluconeogenesis and hepatic glucose output. One mechanism by which this is achieved is by attenuating glucose 6-phosphatase activity. Evidence has been presented for a novel mechanism by which insulin might regulate this enzyme via PI 3-kinase, as it has been demonstrated that PIP₃ acts as a competitive inhibitor of glucose 6-phosphatase [300].

Insulin can also affect glucose metabolism by affecting the expression of genes encoding key enzymes in metabolism. For example, phosphoenolpyruvate carboxykinase (PEPCK) controls a key step in gluconeogenesis, and insulin attenuation of PEPCK gene expression is a key event in switching the liver from gluconeogenic to glycolytic function. Inhibitors of PI 3-kinase block insulin's effect on PEPCK gene transcription in liver cells, suggesting that PI 3-kinase might be involved in this process [301]. Insulin also up-regulates the expression of hexokinase-2 in muscle cells, and this is blocked by wortmannin but, unlike insulin's effects on PEPCK expression, this is also blocked by rapamycin, suggesting that the p70^{s6k} and/or mTOR lie downstream of PI 3-kinase in this process [302]. Insulin-induced expression of glucose 6-phosphate dehydrogenase is also blocked by low doses of wortmannin and LY294002, indicating that activation of PI 3-kinase is also required for this process [303].

Lipid metabolism and antilipolysis

PI 3-kinase has been implicated as a necessary element in signal-transduction pathways by which insulin regulates both the synthesis and degradation of triacylglycerols. Inhibitors of PI 3-kinase block the antilipolytic effect of insulin by preventing inactivation of hormone-sensitive lipase (HSL) [261,304]. The mechanisms whereby PI 3-kinase activity is involved in this process have been partly elucidated. Regulation of HSL activity by insulin is dependent on phosphorylation and activation of phosphodiesterase 3b (PDE3b), which results in a decrease in levels of cAMP and in PKA-induced phosphorylation and activation of HSL. PI 3-kinase inhibitors block the insulin-stimulated phosphorylation of PDE3b [304], and two possible mechanisms for this have been suggested. A preliminary report provided evidence that PDE3b is a direct target for the PK activity of class 1 PI kinases [305]. Another study indicated PDE3b may be a target for activated PKB [306], although it should be noted that the major insulin-responsive phosphorylation site on PDE3b [307] does not lie in a PKB consensus motif.

Insulin also stimulates lipogenesis in part by stimulating *de novo* fatty acid synthesis. Activations of pyruvate dehydrogenase and acetyl-CoA carboxylase are key steps in this process. Activation of pyruvate dehydrogenase is remarkably one of the few insulin-stimulated processes that is clearly not blocked by PI 3-kinase inhibitors, although activation of acetyl-CoA carboxy-

lase is blocked by wortmannin, implying that PI 3-kinase activity is necessary for this process [215].

A further level at which PI 3-kinase may play a role in lipid metabolism is in the production and secretion of very-low-density lipoprotein by the liver, where it is observed that PI 3-kinase inhibitors counteract insulin's inhibitory effects on apolipoprotein B incorporation into very-low-density lipoprotein [308].

Protein synthesis

Growth-factor regulation of protein synthesis involves the coordinate regulation of many steps, including stimulation of amino acid uptake, gene transcription and mRNA translation. PI 3-kinase appears to be an upstream regulator of many of these processes. Inhibitors of PI 3-kinase block insulin stimulation of amino acid uptake, which will ultimately affect the ability to stimulate protein synthesis [172]. However, the acute effects of insulin and growth factors on protein synthesis are largely mediated by increases in the rate of translation of existing mRNAs (reviewed in detail in [309]). This is achieved by regulating the function of multiple components of the initiation and elongation complexes involved in mRNA translation. PI 3-kinase plays a role in this process at several levels. The eukaryotic initiation factor 2b ('eIF2b') is phosphorylated in response to insulin and this activation is mediated by a pathway involving PI 3-kinase, PKB and GSK3 [310]. An insulin-induced reduction in phosphorylation of eukaryotic elongation factor-2 (eEF2) is associated with increases in translation, and both rapamycin and wortmannin block this effect by blocking insulin's inhibitory effect on the kinase upstream of eEF-2 [311]. Insulin stimulates phosphorylation of eukaryotic initiation factor 4E (eIF-4E) binding protein-1 (4EBP-1 or PHAS-1), resulting in its dissociation from eIF-4E, thus relieving an inhibition of translation of a specific subset of mRNAs containing polypyrimidine tracts [312,313]. The phosphorylation of 4EBP-1 is blocked by both wortmannin and rapamycin, suggesting the involvement of PI 3-kinases and mTOR. However, it has recently been suggested that 4EBP-1 may be a direct target *in vivo* for the kinase activity of mTOR [314].

Cytoskeletal rearrangements

The rearrangement of actin to form lamellipodia and subsequent membrane ruffles is the most easily observable effect of insulin on the cytoskeleton in most cell types. There is strong evidence that activation of PI 3-kinase is necessary and sufficient for lamellipodia formation, as this process is blocked by wortmannin and dominant negative forms of p85 [315–317], and it has subsequently been established that activated PI 3-kinase can mimic growth-factor effects on lamellipodia formation [318]. Evidence strongly suggests that the small GTP-binding protein Rac is an intermediate in the PI 3-kinase-dependent formation of the lamellipodia [238,318].

Insulin, via PI 3-kinase, may also play some role in regulating microtubule assembly, as the γ -isoform of tubulin has been shown to interact with the inter-SH2 domain of p85 following insulin stimulation [319].

How does insulin achieve specificity via PI 3-kinase ?

The above studies provide strong evidence that activation of PI 3-kinase is both necessary and sufficient to set in motion signalling pathways that result in stimulation of many of the responses that are known to be stimulated by insulin. The major conundrum is

that many of these same responses are not stimulated by a range of other growth factors that are also known to stimulate PI 3-kinase. For example, both insulin and PDGF cause a similar large recruitment of PI 3-kinase into tyrosine-phosphorylated signalling complexes in 3T3-L1 adipocytes, but insulin stimulation of glucose transport and glycogen synthase greatly exceeds that by PDGF in these same cells [61,275,292,320]. These observations would seem to indicate that activation of PI 3-kinase is not in itself sufficient to elicit the full spectrum or magnitude of insulin-like effects, even when activation is at levels comparable with, or exceeding, those induced by insulin. However, several mechanisms may be envisaged to explain how both insulin and PDGF are able to activate PI 3-kinase, yet achieve different signalling outcomes.

One important consideration is that recruitment of PI 3-kinase activity into tyrosine-phosphorylated signalling complexes is the most widely used assay for activation of PI 3-kinase, but does not necessarily correlate with *in vivo* production of 3'-phosphoinositide products. In the limited number of studies that have directly compared changes in PIP_3 levels induced by insulin and other stimuli in insulin-responsive cell types, it has been found that insulin stimulates larger increases in PIP_3 levels than either PDGF [31], EGF [18] or phorbol esters [17]. In 3T3-L1 pre-adipocytes, which are less insulin-responsive than differentiated 3T3-L1 adipocytes, the reverse is true [31]. The degree of elevation in PIP_3 levels in insulin-sensitive cell types following insulin, EGF or PDGF stimulation also correlates well with the ability to regulate downstream targets such as PKB, GSK3, glycogen synthase and glucose transport [17,18,31,292]. This suggests the ability to generate PIP_3 is a limiting factor in signalling to these events.

The explanation for the apparent discrepancy between activation of PI 3-kinase and production of PIP_3 may be that different growth factors activate different subsets of downstream pathways, some of which may potentiate and some of which may antagonize, a particular endpoint response. One potential source of differential signalling is phospholipase C (PLC), as it has been demonstrated that activation of PLC by thrombin blocks insulin-stimulated PIP_3 production [20]. Further, stimulation of PIP_3 production by PDGF is greatly enhanced by deletion of the sites on the PDGF receptor that allow binding of PLC [163]. As $PI(4,5)P_2$ is the major substrate for both class 1a PI 3-kinases and PLC, they could potentially compete for substrate. Therefore the explanation as to why PDGF has very little effect on PIP_3 production in comparison with insulin in 3T3-L1 adipocytes [31] may lie with the fact that PDGF activates $PLC\gamma$, while insulin does not.

Another rationalization of the conflicting observations is that activation of PI 3-kinase at a critical subcellular location may be essential for the mediation of specific endpoint responses. Despite the importance of knowing the local levels of PIP_3 , it has not yet been possible to measure PIP_3 levels directly at different intracellular locations. However, it may be possible to infer location of PIP_3 accumulation by following the subcellular distribution of proteins containing PH domains known to specifically bind PIP_3 . Such studies have been performed in 3T3-L1 adipocytes using green fluorescent protein–Arf nucleotide binding site opener ('ARNO') chimera [321], and in HEK293 cells, immunocytochemical studies have been performed using epitope-tagged PKB [208]. Both studies show insulin causes a PI 3-kinase-dependent and PH-domain-dependent recruitment from the cytoplasm to the sites adjacent to the plasma membrane, with no evidence for recruitment to clearly definable intracellular vesicles. These findings are at odds with subcellular-fractionation studies. The latter find that the majority of anti-phosphotyrosine-

precipitable PI 3-kinase is found in low-density fractions following insulin stimulation [60,61,137,272,273,308,322]. PI 3-kinase enzyme activity and p85 levels are also increased in this fraction following insulin stimulation [60,61]. In contrast, PDGF-stimulated activity is almost exclusively associated with high-density fractions containing the plasma membranes [60,61,137]. The exact site of the insulin-stimulated pool of PI 3-kinase remains to be established, although it has been suggested that this could potentially be physically very close to, but distinct from, the plasma membrane [137]. This is an appealing mechanism, as it would reconcile the immunocytochemical and fractionation data.

An obvious basis for differential localization is the difference in the characteristics of the proteins to which the PI 3-kinase binds. While the PDGF receptor is largely located at the plasma membrane, IRS proteins are found at intracellular locations after insulin stimulation [137,322]. Indeed, insulin stimulation of PI 3-kinase activity in low-density cell fractions is accompanied by an increase in p85 protein and phosphorylated IRS-1 [60,61,137,272,273,322]. This could explain the specificity of insulin relative to PDGF, but not its specificity relative to cytokines, as these can also induce IRS-associated PI 3-kinase activity [323]. However, it is not yet clear whether cytokines and insulin induce phosphorylation of the same sites on IRS proteins or whether they induce IRS proteins to follow the same intracellular itinerary. The structural features of the phospho-IRS-1-p85 complex and the low-density compartments which are responsible for specific targeting are completely unclear. The capacity of constitutively active PI 3-kinase to mimic insulin action without specific targeting can be ascribed to the fact that overexpression and membrane association may result in a portion of the activity localizing non-specifically to the appropriate compartment for stimulation of GLUT4 vesicle translocation. Elucidation of the mechanism and function of specific subcellular targeting of PI 3-kinase in relation to different actions of insulin is an important area for further study.

Factors influencing insulin signalling via PI 3-kinase *in vivo*

An important issue governing the degree to which insulin can activate PI 3-kinase *in vivo* is the extent to which insulin stimulates pathways that may cause feedback inhibition of PI 3-kinase. Several such potential feedback mechanisms exist. As discussed above, the activation of PKB could potentially contribute to serine-phosphorylation events that decrease the activity of PI 3-kinase associated with IRS-1. Insulin has also been reported to activate PIP_3 5'-phosphatases [176], and such an activation could potentially counteract the actions of PI 3-kinase. It has also been observed that PIP_3 binds with reasonably high affinity to the SH2 domains of the class 1a PI 3-kinase adapter subunits, and it has been suggested that this could provide a mechanism for disengaging PI 3-kinase from signalling complexes [196]. However, the extent to which any of these feedback mechanisms actually restrict insulin's ability to maximally stimulate PI 3-kinase activity is not known.

A further issue which is only now beginning to be investigated is how insulin regulation of PI 3-kinase might function in the complex *in vivo* milieu, where cross-talk between different signalling systems under the influence of multiple agonists is likely to be important. One possibility is that insulin stimulation of PI 3-kinase-induced PIP_3 production could be decreased by substrate-competition mechanisms induced by simultaneous stimulation by other growth factors. Indeed, as described above, activation of PI 3-kinase by insulin and growth factors is reduced in the presence of agonists which activate PLC [20,163,195,324],

and this may involve competition for $\text{PI}(4,5)\text{P}_2$ substrate. Simultaneous stimulation of cells with more than one growth factor capable of recruiting PI 3-kinase may result in competition for p85, thus potentially affecting insulin's ability to stimulate the IRS associated PI 3-kinase pools. One study exists addressing this issue which found that insulin stimulation displaced the PI 3-kinase p85 subunit from activated PDGF receptor and resulted in its recruitment instead to IRS-1 and the insulin receptor [325], suggesting that *in vivo* the p85-binding sites on IRS-1 have a higher affinity for p85 SH2 domains than the sites on the PDGF receptor.

A further possibility is that that kinase cascades stimulated by agonists other than insulin could modulate insulin's ability to stimulate PI 3-kinase. The cAMP system is an obvious candidate and reports do exist to indicate that raised cAMP can interfere with growth-factor stimulation of PI 3-kinase [326,327], although this has not been directly measured in relation to insulin signalling. It has also recently been demonstrated that prior stimulation of cells with angiotensin-II results in a reduction of PI 3-kinase activity associated with IRS-1, while the amount of PI 3-kinase protein associated with IRS-1 remains unchanged [324]. It was speculated that this decrease in intrinsic activity of PI 3-kinase may be due to increased serine phosphorylation of Ser⁶⁰⁸ of p85, although this was not formally tested. The mechanism regulating PI 3-kinase *in vivo* is an important issue and obviously much remains to be done in this area.

PI 3-kinase and diabetes

Development of insulin resistance, reflecting impairment of insulin action at the cellular level, plays an important role in the aetiology of type-2 diabetes. Given the critical role of PI 3-kinase in regulating glucose metabolism, it is reasonable to suspect that alterations in expression or regulation of this enzyme may contribute to the development of insulin resistance. Indeed such alterations have been observed in insulin-resistant animal models. Insulin stimulation of PI 3-kinase was greatly decreased in the *ob/ob* insulin-resistant mouse model, but not in the insulinopenic streptozotocin-treated diabetic rat model [328]. This was in part due to down-regulation of IRS-1 and IRS-2, but also appears to be linked to alterations in expression of PI 3-kinase isoforms. Interestingly, in the *ob/ob* mice there was a differential alteration in expression of adapter/regulatory subunit isoforms, with the level of p85 α being decreased by 50% in liver, while the levels of its p50 α and p55 α splice variants were increased greatly [329]. Expression of all these isoforms was not different in muscle from these animals versus controls [329]. The expression of PI 3-kinase isoforms was also altered in the *fa/fa* rat, in which, like the *ob/ob* mouse, the obesity is caused by lack of functional leptin production. The pattern of PI 3-kinase expression is broadly similar between these two models, although one notable difference is that, in the livers of *fa/fa* rats, p55 α was greatly decreased [330]. Levels of the p110 catalytic subunits do not seem to be altered in these insulin-resistant animals [330]. The functional consequences of these alterations are not yet fully understood, but there are likely to be implications for insulin signalling via PI 3-kinase in these tissues. Decreased insulin stimulation of PI 3-kinase is also observed in muscle and liver of IRS-2 knockout mice, and it has been suggested this is a cause of the insulin resistance observed in these animals [108].

The situation in humans is less clear. It has been reported that levels of IRS-1 are markedly decreased (by at least 50%) in adipocytes from subjects with non-insulin-dependent diabetes, and in this situation IRS-2 became the major docking protein for PI 3-kinase [305]. It was noted in that study that IRS-2 required

a higher concentration of insulin than IRS-1 to achieve a given level of phosphorylation and PI 3-kinase activation, but this difference may have reflected the relative levels of the two proteins in adipocytes. Decreases in insulin stimulation of PI 3-kinase have been identified in muscle and adipose tissues from insulin-resistant and diabetic human subjects [331–333]. This appears to be due to defects both in the level of expression of PI 3-kinase isoforms and in upstream aspects of insulin signalling. Pathogenic mutations in class Ia PI 3-kinases could also potentially contribute to insulin resistance. To date only the p85 α gene has been scanned for such mutations, and in one of these studies a common Met³²⁶Ile polymorphism has been identified in a Danish population and is associated with insulin resistance in its homozygous form [334]. An Arg⁴⁰⁹Gln polymorphism in p85 α has also been identified which may also contribute to insulin resistance in a British family [335]. However, biochemical characterization of these p85 variants has not yet been performed.

CONCLUSIONS

In recent years a great deal of evidence has accumulated to implicate a large number of molecules in insulin signalling pathways (Scheme 1). Working forward from the receptor, attention has focused on tyrosine-phosphorylated substrates and their complexes. Working backwards from intracellular targets such as glycogen synthase, the regulatory role of serine/threonine phosphorylation became apparent. It is now clear that activation of class Ia PI 3-kinases by insulin plays a pivotal upstream role in insulin signalling pathways, acting as a switch to translate the receptor tyrosine-specific PK activity to regulatory serine/threonine-specific kinase cascades. In this respect, PI 3-kinase appears to play a much more important role in most of insulin's actions than the alternative switching mechanism involving Grb2/Sos/Ras and the Raf/MEK/MAP kinase pathway. Emerging data indicate that the products of class Ia PI 3-kinase act as both membrane anchors and allosteric regulators, serving to localize and activate downstream enzymes and their protein substrates. However, many details of PI 3-kinase function remain to be elucidated. The specific roles of different adapter and catalytic subunits and of the various IRS proteins in the activation and action of PI 3-kinase remain obscure. The significance of alternative modes of activation, by direct association with insulin/IGF receptors or via Ras, is also unclear. It seems likely that there are multiple immediate downstream effectors for PI 3-kinase, of which only the PDKs have yet been characterized in any detail. In particular, the mechanism of involvement of PI 3-kinase activity in insulin-stimulated glucose-transporter translocation is still poorly understood. The role of temporal and spatial factors, and of cross-talk with other signalling pathways, in determining the specificity of responses to PI 3-kinase has yet to be fully explored. Finally, the contribution of aberrant expression or regulation of PI 3-kinase to human disease, ranging from diabetes to cancer, must be further investigated, and it is an exciting possibility for the future that PI 3-kinase and its effectors will provide important targets for development of novel therapeutic agents.

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