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Topical Review

The Insulin Signaling Pathway

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Introduction

Insulin mediates a wide spectrum of biological responses including stimulation of glucose uptake, glycogen, lipid and protein synthesis, antilipolysis, activation of transcription of specific genes, and modulation of cellular growth and differentiation. Since the discovery of insulin 77 years ago, great progress has been made in understanding the diverse signaling pathways that emanate from the insulin receptor itself to generate the pleiotropic actions of the hormone. Many of the molecular actions of insulin involve protein phosphorylation and dephosphorylation. Although the insulin receptor is a *tyrosine* kinase that undergoes ligand-dependent activation, many of the resulting downstream changes in phosphorylation/dephosphorylation occur on *serine and threonine.* Here we review the multiple signaling cascades responsible for translating the binding of insulin to its receptor into endpoint biological responses, with specific focus on the molecular mechanisms mediating stimulation of glucose uptake, glycogen synthesis, and control of mRNA translation and protein synthesis.

A Web of Interacting Proteins

The Insulin Receptor, the IRS-Proteins, and SH2-Containing Molecules

The insulin receptor is an $\alpha 2/\beta 2$ tetramer. The α-subunit is located entirely at the extracellular face of the plasma membrane and contains the insulin binding site whereas the β-subunit is a transmembrane peptide (Van Obberghen et al., 1981). The intracellular portion of the β-subunit contains the insulin-regulated tyrosine protein kinase (Kasuga et al., 1983). Insulin binding activates the tyrosine kinase leading to autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit and the tyrosine phosphorylation of the IRS-proteins. The reader is referred to an in-depth review on the structure of the insulin receptor (White, 1997).

The insulin receptor substrate-1, IRS-1, was the first docking protein identified and serves as the prototype for this class of molecules (White, Maron & Kahn, 1985; Sun et al., 1991). Five related proteins have recently expanded this family, including IRS-2 (Sun et al., 1995), IRS-3 (Lavan, Lane & Lienhard, 1997*b;* Smith-Hall et al., 1997), IRS-4 (Lavan et al., 1997*a*), Gab1 (Holgado-Madruga et al., 1996), and $p62^{dok}$ (Yamanashi & Baltimore, 1997). All the IRS-proteins have the same overall architecture: an NH_2-terminal pleckstrin homology (PH) and/or a phosphotyrosine-binding (PTB) domains; multiple tyrosine residues that create SH2-protein binding sites; proline-rich regions to engage SH3 or WW domains; and serine/threonine-rich regions (White, 1998). IRS coupling to the insulin receptor is provided by the PTB domain. This domain binds specifically but weakly to the phosphorylated NPXY motif in the juxtamembrane region of the insulin receptor (Kaburagi et al., 1995). A role for the PH domain in IRS-protein binding to the insulin receptor has also been proposed (Myers, Jr. et al., 1995).

IRS-1 and IRS-2 are widely distributed but their relative levels vary among different mammalian tissues. IRS-3 expression has been demonstrated in adipose tissue, fibroblasts and liver cells (Hosomi et al., 1994; Milarski et al., 1995; Zhang-Sun et al., 1996; Smith-Hall et al., 1997) whereas IRS-4 has so far only been observed

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in cultured cells derived from embryonic kidney (Lavan et al., 1997*a*). Gab1, Grb2-associated binding protein, is widely expressed in mammalian tissues and is phosphorylated on multiple tyrosines in response to insulin (Holgado-Madruga et al., 1996).

Tyrosine-phosphorylated IRS-proteins generate downstream signals by the direct binding to the SH2 domains of various signaling proteins. Several enzymes and adaptor proteins have been identified which associate with IRS-1 and IRS-2, including phosphatidylinositol 3-kinase (PI 3-kinase), phosphotyrosine phosphatase SHP2, Grb2, Nck, and Crk (White, 1998). Although there is evidence that some degree of functional overlap occurs between IRS-1 and IRS-2, these two molecules are not functionally interchangeable for all cellular responses. For example, cell lines derived from IRS-1-deficient embryos exhibit three classes of responses: (i) those that do not require IRS-1 (mitogen-activated protein kinase, MAPK, activation), (ii) those that are impaired by IRS-1 deficiency and can be reconstituted by either IRS-1 or IRS-2 (PI 3-kinase activation and immediate early gene induction), and (iii) those that are impaired by IRS-1 deficiency, can be reconstituted by IRS-1, but are not fully reconstituted by IRS-2 (mitogenesis) (Bruning et al., 1997). Additionally, in muscles derived from IRS-1-deficient mice, the responses to insulin-induced PI 3-kinase activation, glucose transport, p70 S6 kinase, mRNA translation and protein synthesis are significantly impaired (Yamauchi et al., 1996).

IRS-3 undergoes tyrosine phosphorylation and binds PI 3-kinase in response to insulin, and in adipocytes IRS-3, rather than IRS-2, is the major alternative pathway for PI 3-kinase activation in adipocytes from IRS-1-deficient mice (Smith-Hall et al., 1997). Binding of IRS-4 to PI 3-kinase remains to be demonstrated, but it is expected given that it has several potential tyrosine phosphorylation sites. $p62^{dok}$ is unique among the IRS-proteins in that it does not bind the PI 3-kinase (Yamanashi & Baltimore, 1997).

The products of the *shc* gene are also substrates of the insulin receptor (Kovacina & Roth, 1993). The proteins encoded by this gene, with molecular masses of 46, 52, and 66 kDa, also contain SH2 domains and are tyrosine phosphorylated in response to insulin (Kovacina & Roth, 1993). Shc and IRS-proteins compete for the same binding site in the juxtamembrane domain of the insulin receptor (Kaburagi et al., 1995; Isakoff et al., 1996).

Phosphatidylinositol 3-Kinase, Protein Kinase B, p70 Ribosomal S6 Kinase, and Atypical Protein Kinase Cs

PI 3-Kinase

One of the earliest steps in the insulin signaling pathway is the activation of PI 3-kinase. PI 3-kinase catalyzes the phosphorylation of phosphoinositides at the D3 position of the inositol ring. Current evidence indicates that class 1a PI 3-kinases account for the bulk of the increase in PI 3-kinase activity in response to insulin, for a review *see* (Shepherd, Withers & Siddle, 1998). The class 1a PI 3-kinases exist as heterodimers which consist of an adaptor 85 kDa subunit tightly associated with a catalytic 110 kDa subunit (Hu et al., 1993; Dhand et al., 1994). The class 1b PI 3-kinases are regulated by G-protein-coupled receptors and there is no evidence that they are regulated by insulin (Stephens et al., 1994; Stoyanov et al., 1995). The catalytic subunit of class 1b PI 3-kinases is homologous to the catalytic subunit of class 1a PI 3-kinases, however, the class 1b PI 3-kinases associate with adaptor subunits distinct from the 85 kDa class 1a PI 3-kinases adaptor subunits (Stephens et al., 1997). The preferred substrates for class 1 PI 3-kinases in vitro are phosphatidylinositol [PI], phosphatidylinositol 4-phosphate [PI4-P], and phosphatidylinositol 4,5-biphosphate [$PI(4,5)P_2$] (Domin & Waterfield, 1997). However, in the cell they appear to preferentially phosphorylate $PI(4,5)P_2$ (Domin & Waterfield, 1997). Indeed, $PI(3,4,5)P_3$ is the major product formed by PI 3-kinases activated by insulin and many other growth factors (Nave, Siddle & Shepherd, 1996*b*). Production of lipid products by class 1 PI 3-kinases is inhibited by wortmannin (Ui et al., 1995) and LY294002 (Vlahos et al., 1994). Class 2 PI 3-kinases can be activated by insulin (Shepherd et al., 1998) but they are resistant to wortmannin (Virbasius, Guilherme & Czech, 1996; Domin et al., 1997). Additionally, class 2 PI 3-kinases cannot use $PI(4,5)P_2$ as substrate and therefore cannot generate $PI(3,4,5)P_3$ (Domin et al., 1997). Class 3 PI 3-kinases only use phosphatidylinositol as a substrate (Shepherd et al., 1998). Hence, it is unlikely that class 2 or class 3 PI3-kinases play a role in insulin signaling.

Several isoforms and splice variants of both the adaptor and catalytic subunits of class 1a PI 3-kinases exist. Three highly homologous isoforms of the class 1a catalytic subunit have been cloned. These are p110α, p110β, and p110δ, with the α and β isoforms playing a major role in insulin signaling (Vanhaesebroeck et al., 1997*a*, *b*). The class 1a PI 3-kinases adaptor subunits are encoded by at least three genes. Two isoforms generate products of 85 kDa, these being designated p85α and p85β (Dhand et al., 1994). The third PI 3-kinase adaptor subunit gene has been termed $p55^{PIK}$ or p55γ because it encodes a protein of 55 kDa (Pons et al., 1995). Further complexity is added by the occurrence of several splice variants of the adaptor subunit. Indeed, three splice variants of p85α have been reported, namely p85α1, p50α, and p55α (Antonetti, Algenstaedt & Kahn, 1996).

Class 1a PI 3-kinases play a major role in many insulin-regulated responses including stimulation of glucose uptake (Clarke et al., 1994; Okada et al., 1994; Tsakiridis et al., 1995*a;* Yeh et al., 1995), general and

growth-specific protein synthesis (Mendez et al., 1997), membrane ruffling (Kotani et al., 1994; Tsakiridis, Vranic & Klip, 1995*b*), and cell growth and proliferation (Jhun et al., 1994; McIlroy et al., 1997). Class 1a PI 3-kinases also participate in regulating expression of key genes in metabolism. For example, insulin attenuation of phosphoenolpyruvate carboxykinase (PEPCK) (Agati, Yeagley & Quinn, 1998) and glucose-6-phosphatase (Dickens et al., 1998) gene expression is PI 3-kinase-dependent. Additionally, induction of hexokinase II (Osawa et al., 1996), glucose-6-phosphate dehydrogenase (Wagle et al., 1998), and GLUT4 gene expression (Valverde et al., 1999) requires PI 3-kinase. Activation of class 1a PI 3-kinases by insulin is achieved through binding of SH2-domains of adaptor subunits to phosphorylated YMXM motifs in IRS-proteins (Giorgetti et al., 1993). Several enzymes appear to carry the signal initiated by PI 3-kinase activation to its final destination. PKB, p70 S6 kinase, and atypical PKCs are thought to be downstream of PI 3-kinase and have been implicated in various insulin responses (*see below*).

PKB

Protein kinase B, PKB, is the cellular homologue of the transforming oncogene v-Akt (Belacossa et al., 1991). The catalytic domain of PKB is most similar to that of protein kinase A (PKA) and protein kinase C (PKC), findings that gave rise to its three names: PKB (between PKA and PKC), RAC (related to A and C kinase), and cAkt (Coffer & Woodgett, 1991). PKB is a PH domain-containing serine/threonine kinase (Kohn, Kovacina & Roth, 1995). Three isoforms of PKB have been identified. PKBα is the major isoform activated by insulin in muscle, hepatocytes and adipocytes (Walker et al., 1998). PKBβ is activated is adipocytes (Walker et al., 1998). PKBγ is not activated by insulin in muscle, hepatocytes, or adipocytes, but is activated by insulin in cell culture models (Walker et al., 1998). The activation of class 1a PI 3-kinase has been shown to be both necessary and sufficient for the activation of PKB. Inhibitors of PI 3-kinase block the activation of PKB (Burgering & Coffer, 1995; Alessi et al., 1996). Moreover, expression of dominant negative forms of the p85 adaptor subunit of class 1a PI 3-kinase (Burgering & Coffer, 1995), or expression of tyrosine kinase receptors that lack the ability to bind PI 3-kinase block the stimulation of PKB (Burgering & Coffer, 1995; Franke et al., 1995). Expression of a constitutively active PI 3-kinase independently promotes PKB activation (Didichenko et al., 1996; Klippel et al., 1996).

Earlier reports have suggested that phosphoinositol lipids bind PKB resulting in its activation (Frech et al., 1996; Franke et al., 1997; Klippel et al., 1997). Other reports, however, have demonstrated that binding of $PI(3,4,5)P_3$ or $PI(3,4)P_2$ is not the direct means for PKB activation (James et al., 1996; Alessi et al., 1997; Stokoe et al., 1997). It is now accepted that the primary mechanism for the activation of PKB by insulin is phosphorylation on two sites (Thr 308 and Ser473 in PKBα). Phosphorylation at Thr308 (PKBα), Thr309 (PKBβ) and Thr305 (PKBγ) is achieved by the recently cloned PDK1 (phosphoinositide-dependent protein kinase) (Walker et al., 1998). The kinase responsible for Ser473 phosphorylation has not been cloned yet, but has been tentatively named PDK2 (Alessi & Cohen, 1998). Taken together, the accepted mechanism for PKB activation involves interaction with $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ through its PH domain. This interaction with phosphoinositol lipids recruits PKB to the plasma membrane and results in conformational changes in PKB so that Thr and Ser become accessible to phosphorylation by PDK1 and PDK2, respectively (Alessi et al., 1996; Kohn, Takeuchi & Roth, 1996*b;* Andjelkovic et al., 1997). One of the well-characterized substrates of PKB is glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995).

p70 S6 Kinase

The p70 ribosomal S6 kinase is a serine/threonine kinase. Full activation of p70 S6 kinase requires phosphorylation of at least eight sites (Pullen & Thomas, 1997). There is mounting evidence that activation of PI 3-kinase and the subsequent activation of PKB participate in the initial stimulation of p70 S6 kinase. Inhibitors of PI 3-kinase block the early p70 S6 kinase activation (Cheatham et al., 1994; Chung et al., 1994). Further, expression of constitutively active class 1a PI 3-kinases increase basal p70 S6 kinase activity (Weng et al., 1995; Klippel et al., 1996). PKB may also be involved in the activation process since expression of constitutively active PKB renders p70 S6 kinase active (Burgering & Coffer, 1995), and a mutant PKB in which Thr308 and Ser473 are replaced by alanine (PKB-AA) inhibits the insulin activation of p70 S6 kinase by ~75% in CHO cells and ~30% in 3T3-L1 adipocytes (Kitamura et al., 1998). However, no direct phosphorylation of p70 S6 kinase by PKB has been demonstrated. Interestingly, PDK1 directly phosphorylates p70 S6 kinase at Thr252 (Alessi et al., 1998) and Thr229 (Pullen et al., 1998) contributing to its activation. Moreover, coexpression of p70 S6 kinase with PDK1 results in strong activation of the S6 kinase in vivo (Alessi et al., 1998). PI 3-kinase-independent processes must also be involved in p70 S6 kinase activation. Indeed, insulin is still capable of activating p70 S6 kinase in CHO cells overexpressing dominant negative class 1a PI 3-kinase adaptor subunits (Hara et al., 1995). In L6 muscle cells, p70 S6 kinase can be activated by insulin while PI 3-kinase and PKB are inhibited by wortmannin, albeit with a delayed response (Somwar et al., 1998). Therefore, insulin appears to use an early PI 3-kinase-dependent and a delayed PI 3-kinase-independent pathways leading to p70 S6 kinase activation.

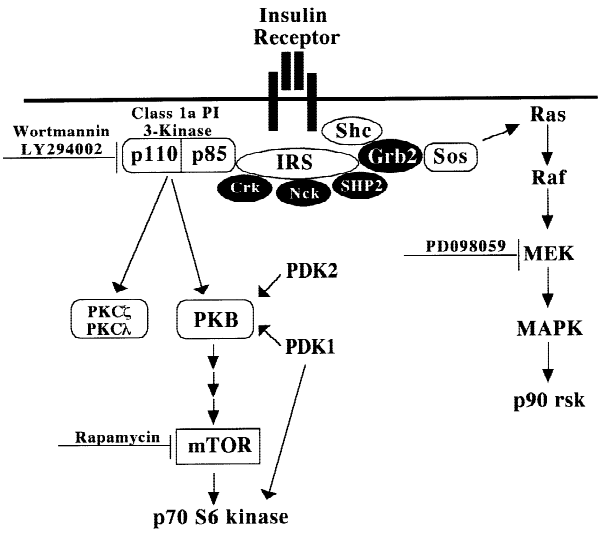

Fig. 1. The insulin signaling pathway. The insulin receptor is a tyrosine kinase that undergoes autophosphorylation which increases the kinase activity of the receptor. The activated receptor phosphorylates IRS-proteins and Shc resulting in the activation of the Grb2/Sos and Ras/Raf/MEK/MAPK pathway. Phosphorylated IRS-proteins activate PI 3-kinase and its downstream targets, namely, PKB, mTOR, p70 S6 kinase and atypical PKCs (PKCζ and PKCλ). The sites inhibited by wortmannin, LY294002, rapamycin, and PD098059 are indicated. *See* text for details.

The activation of p70 S6 kinase is also prevented by rapamycin suggesting that mTOR (mammalian target of rapamycin) is one of the pathways leading to this enzyme (Sehgal, 1995). Recent reports have demonstrated that PKB lies upstream of mTOR since activating PKB mimics insulin by increasing the kinase activity of mTOR (Scott et al., 1998), and dominant negative mutants of PKB block the insulin-mediated phosphorylation of 4E-BP1 (Gingras et al., 1998), a substrate of mTOR (Brunn et al., 1997). Thus, the activation of p70 S6 kinase can be accessed by a variety of routes (*see* Fig. 1).

PKGζ and PKCλ

It is now apparent that PI 3-kinase can contribute to the activation of PKCζ and PKCλ, atypical PKC enzymes which are not activated by diacylglycerol or phorbol ester. The insulin-stimulated activation of PKCζ is blocked by PI 3-kinase inhibitors (Bandyopadhyay et al., 1997*a;* Standaert et al., 1997). Furthermore, PI(3,4,5)P_3 and PI(3,4)P_2 directly stimulate PKCζ enzyme activity in vitro to levels similar to those observed by insulin in vivo (Standaert et al., 1997). Additionally, activation of PKCζ is lost in cells that express IRS-1 mutants lacking PI 3-kinase-binding sites, and it is fully restored when those sites are reexpressed (Mendez et al., 1997). Two recent reports have also identified PDK1 as the kinase that phosphorylates and activates PKCζ in the PI 3-kinase signalling pathway, both in vitro and in human embryonic kidney 293 cells (Chou et al., 1998; Le Good et al., 1998). However, the fact that ectopic expression of constitutively activated PKCζ does not stimulate p70 S6 kinase indicates that PI 3-kinase/PDK1 represent a bifurcation in the insulin signaling pathway, one branch leading to PKCζ and one leading to PKB → mTOR → p70 S6 kinase (Mendez et al., 1997) (*see* Fig. 1).

The insulin-stimulated activation of PKCλ is also PI 3-kinase-dependent. Indeed, endogenous or transfected PKCλ in 3T3-L1 adipocytes or CHO cells have now been shown to be activated by insulin in a manner sensitive to wortmannin and to a dominant negative mutant of class 1a PI 3-kinase (Kotani et al., 1998). However, the insulin-elicited signals that pass through PI 3-kinase subsequently diverse into a PKB pathway and a PKCλ pathway (*see* Fig. 1), since a mutant PKB (PKB-AA, discussed above) does not inhibit the insulin-induced activation of PKCλ (Kotani et al., 1998).

The MAP Kinase Cascade

As mentioned above, following insulin stimulation, IRS-proteins and Shc bind to the SH2-domains in several small adaptor proteins such as Grb2 (Skolnik et al., 1993*a*). Flanking its SH2-domain, Grb2 contains two SH3-domains that associate constitutively with proline-rich regions in mSos, a guanine nucleotide exchange factor that stimulates GDP/GTP exchange on Ras (Skolnik et al., 1993*a,b*). Activated Ras recruits the serine/threonine kinase Raf to the plasma membrane via a direct interaction with its effector region (Pronk & Bos, 1994). Raf activation results in the activation of MEK by phosphorylation on two serine residues (Dent et al., 1992). MEK is a dual specificity kinase that activates MAPK (mitogen-activated protein kinase) by both tyrosine and threonine phosphorylation (Crews, Alessandrini & Erikson, 1992). Several isoforms of MEK and MAPK have been identified (Cobb & Goldsmith, 1995). MAPK activation results in the phosphorylation of p90 ribosomal S6 kinase (p90 rsk), phospholipase A_2 (PLA_2), and many transcription factors including the ternary complex factor $p62^{TCF}$ (Fingar & Birnbaum, 1994*a;* Gille, Sharrocks & Shaw, 1992). Although the MAPK pathway is a well-characterized insulin signaling cascade, it is not very sensitive to insulin and, as discussed below, is not involved in many of the important metabolic responses of the hormone. The MAPK pathway may interact with the PI 3-kinase pathway since activated GTP-loaded Ras can interact directly with PI 3-kinase (Rodriguez-Viciana et al., 1994). However, it is still unclear whether PI 3-kinase acts upstream or downstream of Ras since both possibilities have been proposed (Hu et al., 1995; Rodriguez-Viciana et al., 1996; Marte et al., 1997).

Tyrosine Phosphatases and Serine/Threonine Phosphatases

Insulin can simultaneously stimulate the phosphorylation of some proteins and the dephosphorylation of others. Although a great deal of effort has concentrated on understanding the kinases responsible for phosphorylation, little is known about phosphatases. One tyrosine phosphatase, PTPase 1B, has emerged as a possible candidate for a negative regulator of insulin action because of its ability to dephosphorylate the insulin receptor in vitro (Tonks, Diltz & Fischer, 1988). Additionally, microinjection of PTPase 1B into *Xenopus* oocytes blocks insulin-stimulated maturation (Cicirelli et al., 1990). However, little is known about the role of PTPase 1B in the metabolic actions of insulin in vivo.

Another tyrosine phosphatase that may play an important role in insulin action is the SH2-containing phosphatase SHP2 (also known as Syp or SH-PTP2). This phosphatase associates with tyrosine-phosphorylated IRS-proteins via its SH2 domains (Kuhne et al., 1993). SHP2 can diminish the tyrosine phosphorylation of IRS-1, providing a mechanism to attenuate certain signals (Arrandale et al., 1996). However, it was also suggested that SHP2 plays a positive role in insulin signaling since catalytically inactive mutants of SHP2 inhibit insulin-stimulated MAPK and *c-fos* transcription in intact cells (Milarski & Saltiel, 1994; Yamauchi et al., 1994).

Protein phosphatase (PP)-1 is a serine/threonine phosphatase activated by insulin and is primarily responsible for the regulation of glycogen metabolism (*see below*). Activation of PP-1 has been suggested to be downstream of p90 rsk (Dent et al., 1990). A summary of the insulin signaling pathway is shown in Fig. 1.

Signaling Pathways Leading to Specific Endpoint Responses

Stimulation of Glucose Uptake

Insulin-stimulated glucose transport can be accounted for primarily by translocation of glucose transporters, mainly GLUT4, from intracellular pools to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Slot et al., 1991*a*,*b;* Tsakiridis, Vranic & Klip, 1994; Guma et al., 1995). The transporters cycle to and from the cell surface in the basal state. Insulin promotes their rate of exocytosis and diminishes their rate of endocytosis (Czech & Buxton, 1993; Yang & Holman, 1993; Yang et al., 1996). Many advances have been made in elucidating the intermediate signals which lead to glucose transport stimulation and GLUT4 translocation. Clearly, the tyrosine kinase activity of the receptor is essential for glucose transport since human insulin receptors mutated in the ATP-binding site of the tyrosine kinase domain are defective in stimulation of glucose transport (Quon et al., 1994*b*). IRS-1 is also utilized in initiating GLUT4 translocation since antisense ablation of IRS-1 in rat adipocytes results in a shift in the insulin dose response curve to the right (a reduction of insulin sensitivity), although the maximal level of stimulation of transport activity is unaltered (Quon et al., 1994*a*). The existence of an IRS-1-independent pathway leading to GLUT4 translocation and glucose transport has also been suggested, however, since microinjection of anti-IRS-1 antibodies (Moris et al., 1996), or a decrease in IRS-1-associated PI 3-kinase activity in 3T3-L1 adipocytes (Staubs et al., 1998) were without effect on GLUT4 translocation or glucose transport. Furthermore, transgenic mice lacking IRS-1 retain a significant capacity to regulate blood glucose levels in response to insulin (Araki et al., 1994; Tamemoto et al., 1994). In addition, overexpression of IRS-2 in rat adipocytes stimulates the translocation of GLUT4 in the absence of insulin (Zhou et al., 1997). A direct role of other members of the IRS-family has not been fully evaluated. These results suggest that one or more members of the IRS-family are in the pathway leading to stimulation of glucose uptake.

A key player in insulin's ability to promote glucose uptake into cells is PI 3-kinase. Wortmannin and LY294002, two structurally unrelated inhibitors of PI 3-kinases, inhibit insulin action on glucose transport in a wide range of cell lines (Cheatham et al., 1994; Clarke et al., 1994; Okada et al., 1994; Tsakiridis et al., 1995*a;* Yeh et al., 1995). Wortmannin has been shown to block insulin's ability to stimulate the rate of exocytosis of GLUT4 with little effect on the rate of endocytosis (Yang et al., 1996). The effect of insulin on GLUT4 and glucose transport is also abolished in cells expressing dominant negative mutants of p85α (Hara et al., 1994) or in cells microinjected with mutant p85α constructs that fail to bind to the p110 catalytic subunit (Kotani et al., 1995); supporting the notion that class 1a PI 3-kinases are required. Furthermore, overexpression of constitutively active forms of PI 3-kinase elevate glucose uptake and GLUT4 translocation in adipocytes, albeit not to the same extent as insulin (Katagiri et al., 1996; Tanti et al., 1996). Although necessary, activation of PI 3-kinase may not be sufficient to stimulate glucose uptake since several growth factors activate PI 3-kinase to a degree similar to that achieved by insulin but fail to stimulate glucose transport (Isakoff et al., 1995; Wiese et al., 1995). Hence, mechanisms additional to *activation* of PI 3-kinase are necessary. It was recently reported that the cellular *localization* of PI 3-kinase catalytic activity is affected differently by insulin and other growth factors. Insulin decreases the content of PI 3-Kinase in the cytosol and increases it in intracellular membranes (Nave et al., 1996*a;* Ricort et al., 1996; Yang et al., 1996). More

specifically, PI 3-kinase activity has been shown to increase in intracellular compartments containing GLUT4 (also called GLUT4 vesicles) (Heller-Harrison et al., 1996), an event that requires intact actin filaments (Wang et al., 1998*b*).

The elements downstream of PI 3-kinase regulating GLUT4 translocation and glucose uptake are starting to be unveiled. An involvement of PKB has been suggested based on the following observations: (i) Overexpression of wild-type or constitutively active PKBα results in upregulation of glucose transport and redistribution of GLUT4 to the plasma membrane in muscle and fat cells (Kohn et al., 1996*a;* Tanti et al., 1997; Cong et al., 1997; Hajduch et al., 1998; Ueki et al., 1998), and (ii) insulin increases the association of PKBβ with GLUT4-containing vesicles (Calera et al., 1998). However, a more definitive role of PKB can only be ascribed using specific inhibitors of PKB or using dominant negative forms. No specific inhibitors of PKB have been described yet. Attempts to express a kinase-inactive mutant of PKB that has a point mutation in the ATP-binding domain, resulted in only a minor reduction (20%) in insulin-stimulated GLUT4 translocation in rat adipose cells (Cong et al., 1997). A mutant PKBα (PKBα-AA) in which the phosphorylation sites (Thr308 and Ser473) targeted by insulin are replaced by alanine did not affect insulin-stimulated glucose uptake in either CHO cells or 3T3-L1 adipocytes (Kitamura et al., 1998). In contrast, a kinase-dead and unphosphorylatable PKBα transiently transfected into L6 muscle cells prevented GLUT4 translocation (Wang et al., 1998*a*). Therefore, it is possible that different cell types rely on PKB to different extents for the stimulation of glucose uptake.

Atypical PKCs are other elements downstream of PI 3-kinase that have been suggested to play a role in stimulation of glucose uptake. Insulin increases the enzymatic activity of PKCζ in L6 muscle cells and 3T3-L1 fibroblasts and adipocytes (Bandyopadhyay et al., 1997*a,b;* Standaert et al., 1997). Moreover, expression of wild-type PKCζ increases GLUT4 translocation and elevates basal and insulin-stimulated glucose transport (Bandyopadhyay et al., 1997*b;* Standaert et al., 1997). Consistent with this observation, overexpression of a dominant negative PKCζ mutant leads to partial inhibition of GLUT4 recruitment to the plasma membrane and a decrease in glucose uptake in adipocytes and L6 muscle cells (Bandyopadhyay et al., 1997*a,b;* Standaert et al., 1997). Additionally, overexpression of kinase-deficient mutants of PKCλ reduces insulin-induced glucose uptake by ~50–60% and inhibits translocation of GLUT4 to the plasma membrane (Kotani et al., 1998). In contrast, a PKCλ mutant that lacks the pseudosubstrate domain, when expressed in quiescent 3T3-L1 adipocytes results in GLUT4 translocation and stimulation of glucose uptake to an extent similar to that achieved by insulin (Kotani et al., 1998). Hence, atypical PKCs appear to participate in the insulin-elicited signals to stimulation of glucose uptake.

In contrast to the possible functions of PKB and atypical PKCs, p70 S6 kinase is not required for the insulin-stimulated glucose uptake or GLUT4 translocation as rapamycin has no effect on these responses (Fingar et al., 1993). Nonetheless, the mTOR/p70 S6 kinase-rapamycin sensitive route mediates some of the long-term effects of insulin such as the synthesis of the GLUT1 glucose transporter in both L6 (Taha et al., 1995, 1997) and 3T3-L1 cells (C. Taha and A. Klip, *unpublished observations*).

It is also unlikely that the MAPK pathway is involved in the rapid GLUT4 translocation or glucose transport stimulation based on the following observations: (i) In 3T3-L1 adipocytes, microinjection of activated Ras does not stimulate GLUT4 translocation, and agents that antagonize Ras fail to block this event (Haussdorff, Frangioni & Birnbaum, 1994), (ii) expression of a constitutively active form of Raf does not duplicate insulin stimulation of GLUT4 translocation (Fingar & Birnbaum, 1994*b*), and (iii) treatment with the MEK inhibitor, PD098059, does not inhibit insulin stimulation of glucose transport (Lazar et al., 1995). An exception to these observations has been reported for cardiomyocytes in which microinjection of activated Ras increases glucose transport (Manchester et al., 1994). However, it is generally accepted that the MAPK pathway has no role in the rapid stimulation of glucose transport in muscle and fat cells. The MAPK pathway does, however, mediate the ability of insulin to elevate the synthesis of GLUT3 glucose transporter in L6 muscle cells following prolonged insulin treatment (Taha et al., 1995, 1997).

Finally, participation of the tyrosine phosphatase SHP2 in GLUT4 translocation has also been ruled out considering that microinjection of SHP2 SH2-domains, or antibodies to SHP2, into 3T3-L1 cells blocks insulin-induced mitogenesis but not insulin-stimulated GLUT4 translocation (Hausdorff et al., 1995). A summary of the pathways leading to GLUT4 translocation and the biosynthesis of GLUT1 and GLUT3 is shown in Fig. 2.

Stimulation of Glycogen Synthesis

Skeletal muscle is the major site of insulin-stimulated glucose uptake, and most of the glucose that enters human muscle fibers in response to insulin in deposited as glycogen. Insulin causes stable activation of glycogen synthase by promoting dephosphorylation of multiple sites in the enzyme (Lawrence, Jr. & Roach, 1997). To this day, however, debate continues over the molecular mechanism(s) by which insulin activates glycogen synthase.

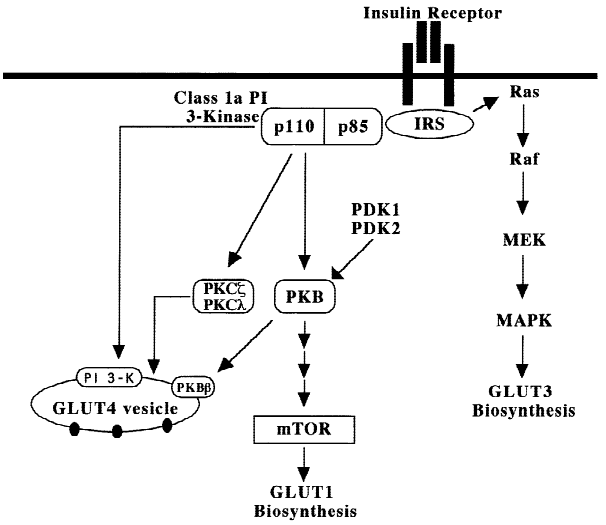

Fig. 2. Signaling pathways leading to stimulation of glucose uptake. PI 3-kinase and its downstream targets PKC, PKCζ, and PKCλ play a key role in the stimulation of GLUT4-containing vesicles from an intracellular pool to the plasma membrane. The mTOR/p70 S6 kinase pathway does not participate in GLUT4 translocation, but is involved in GLUT1 biosynthesis. The Ras/Raf/MEK/MAPK pathway is not needed for GLUT4 translocation but participates in GLUT3 biosynthesis.

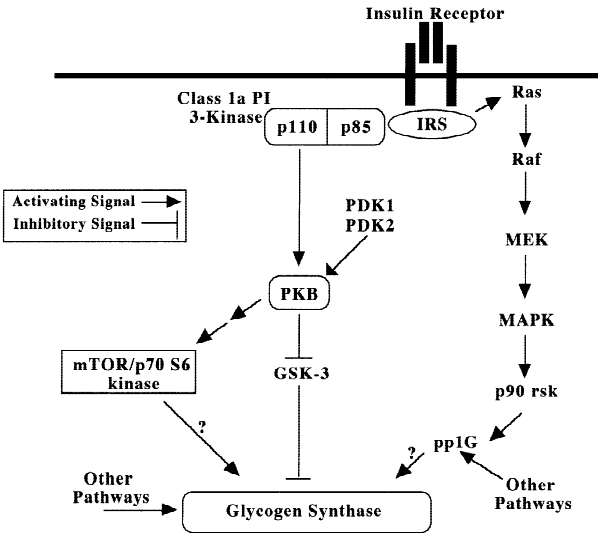

Fig. 3. Signaling pathways leading to stimulation of glycogen synthase and glycogen synthesis. Insulin dephosphorylates and thereby activates glycogen synthase. Dephosphorylation of glycogen synthase results from activation of PP1G and inhibition of GSK-3. Although p90 rsk phosphorylates and activates PP1G in vitro, it is unlikely that the MAPK pathway mediates insulin's ability to activate glycogen synthase. Inactivation of GSK-3 is mediated via PI 3-kinase and PKB. The role of mTOR/p70 S6 kinase appears to be cell type-specific.

The muscle isoform of glycogen synthase has elaborate regulatory properties. It is subject to phosphorylation at nine or more sites by many protein kinases (Roach, 1991). Two sites, sites 2 and 2a, are very close to the NH2-terminus. At least seven more sites (sites 3a, 3b, 3c, 4, 5, 1a, and 1b) are located in the COOH-terminus of the molecule (Lawrence, Jr. & Roach, 1997). In rat skeletal muscle, insulin causes a decrease in the phosphorylation state of sites 3a, 3b, and 3c and site 2 (and/or 2a) (Lawrence, Jr. et al., 1983; Smith & Lawrence, Jr., 1985; Skurat, Wang & Roach, 1994; Skurat & Roach, 1995), resulting in increased catalytic activity. The phosphatase thought to be responsible for dephosphorylation of glycogen synthase is PP1G, the glycogen-bound form of protein phosphatase 1. PP1G itself contains several phosphorylation sites and insulin has been reported to augment the phosphorylation and activation of PP1G (Dent et al., 199). p90 Ribosomal S6 kinase 2 (p90 rsk2) was subsequently identified as the kinase phosphorylating PP1G, thus linking the MAPK pathway to glycogen synthase activation (Dent et al., 1990). Although this model gained widespread acceptance, recent evidence argues against it. For example, incubating 3T3-L1 adipocytes, L6 muscle cells, or rat diaphragm with the MEK inhibitor PD098059 blocked the activation of MAPK and p90rsk2 without blocking the insulin-stimulated activation of glycogen synthase (Lazar et al., 1995; Azpiazu et al., 1996).

The dephosphorylation and activation of glycogen synthase need not involve the direct activation of a phosphatase but could result from inhibition of a kinase. The enzyme GSK-3 (glycogen synthase kinase-3) has been one of the best-studied kinases for glycogen synthase regulation. Insulin phosphorylates and thereby inactivates GSK-3 in several cell types (Welsh & Proud, 1993; Cross et al., 1994; Welsh et al., 1994). There is evidence that the inhibitor effect of insulin on GSK-3 is mediated by PI 3-kinase and its downstream targets PKB and mTOR/p70 S6 kinase as follows: (i) both wortmannin and LY294002 block insulin stimulation of glycogen synthesis and activation of glycogen synthase (Cross et al., 1994; Welsh et al., 1994; Shepherd, Nave & Siddle, 1995; Yamamoto-Honda et al., 1995), (ii) expression of constitutively active PKB induces glycogen synthesis in L6 muscle cells, but not in 3T3-L1 cells (Ueki et al., 1998), and (iii) rapamycin, a potent inhibitor of mTOR/p70 S6 kinase, partially inhibits the activation of glycogen synthesis and glycogen synthase in 3T3-L1 adipocytes (Shepherd et al., 1995). However, rapamycin does not antagonize glycogen synthase activation or GSK-3 inactivation in hepatocytes (Peak et al., 1998), rat skeletal muscle and adipocytes (Cross et al., 1997), or L6 muscle cells (Cross et al., 1994). Therefore, despite long-standing recognition that insulin activates glycogen synthase by promoting its dephosphorylation, the underlying mechanisms at the molecular level are still being debated. A summary of the pathways involved in the regulation of glycogen synthesis is shown in Fig. 3.

Stimulation of Protein Translation and Protein Synthesis

Insulin plays an important role in the overall regulation of protein synthesis. Some of the effects of the hormone involve changes in mRNAs abundance, but insulin also has important effects on the translation process itself. Indeed, several initiation and elongation factors are regulated by the hormone, often as a consequence of changes in their states of phosphorylation.

Translational initiation begins with the recognition of the mRNA cap structure by the mRNA cap-binding protein eIF-4E (eukaryotic initiation factor-4E). eIF-4E, together with eIF-4A (an RNA helicase) and eIF-4G (a bridge between eIF-4E and eIF-4A), form the eIF-4F initiation complex (Tahara, Morgan & Shatkin, 1981; Edery et al., 1983). In mammals, the eIF-4E-binding proteins (4E-BPs) comprise a family of three members termed 4E-BP1 (Lin et al., 1994; Pause et al., 1994), 4E-BP2 (Lin & Lawrence, Jr., 1996), and the recently cloned 4E-BP3 (Poulin et al., 1998). 4E-BPs compete with eIF-4G for interaction with eIF-4E, interfering in this way with the formation of the eIF-4F initiation complex and thereby inhibiting cap-dependent translation (Haghighat et al., 1995). Upon insulin stimulation of rat adipose tissue, 4E-BP1 (also termed PHAS-I: phosphorylated heat- and acid-stable protein) becomes phosphorylated and dissociates from eIF-4E, thereby alleviating translational inhibition (Lin et al., 1994; Pause et al., 1994). This phenomenon also occurs in 3T3-L1 adipocytes (Lin et al., 1995; Lin & Lawrence, Jr., 1996), rat skeletal muscle (Kimball et al., 1997), and L6 myoblasts (Kimball, Horetsky & Jefferson, 1998).

The major signal transduction pathway leading to insulin-induced phosphorylation of 4E-BPs is the PI 3-kinase $\rightarrow$ PKB $\rightarrow$ mTOR pathway. This is based on the following observations: (i) 4E-BP1 phosphorylation is inhibited by wortmannin and is eliminated in cells containing IRS-1 that lacks PI 3-kinase-binding sites (Mendez et al., 1996), (ii) phosphorylation of 4E-BP1 is stimulated in cells overexpressing an activated PKB and inhibited by a dominant negative mutant of PKB (Gingras et al., 1998), (iii) rapamycin abolishes 4E-BP1 phosphorylation by insulin and results in suppression of cap-dependent translation (Beretta et al., 1996; Lin & Lawrence, Jr., 1996; Fadden, Haystead & Lawrence, Jr., 1997), and (iv) mTOR functions a 4E-BP1 kinase both in vitro and in vivo, phosphorylating 4E-BP1 at serine and threonine residues identical to those phosphorylated in insulin-stimulated adipocytes (Brunn et al., 1997).

As p70 S6 kinase lies downstream of mTOR, it is conceivable that insulin increases 4E-BP1 phosphorylation via the PI 3-kinase/PKB/mTOR $\rightarrow$ p70 S6 kinase pathway. However, this possibility is excluded based on the observation that expression of an active, but rapamcyin-resistant, p70 S6 kinase could not protect 4E-BP1 from dephosphorylation upon treatment with rapamycin (Hara et al., 1997; von Manteuffel et al., 1997). Furthermore, mTOR, but not p70 S6 kinase, directly phosphorylates 4E-BP1 in vitro (Haystead et al., 1994; Brunn et al., 1997). Hence, the two mTOR targets, p70 S6 kinase and 4E-BP1, are regulated in parallel rather than in sequence. Although MAPK can phosphorylate 4E-BP1 in vitro, recent in vivo evidence has ruled out a role of the MAPK cascade in this phenomenon (Lin et al., 1995; von Manteuffel et al., 1996).

Insulin also activates mRNA translation by increasing the phosphorylation of eIF-4E itself (Flynn & Proud, 1996*a*) which is believed to increase the affinity of eIF-4E to the mRNA cap structure (Minich et al., 1994). eIF-4E is phosphorylated in vivo on a single site now known to be Ser209 (Flynn & Proud, 1995; Joshi et al., 1995). The insulin signaling pathway(s) resulting in eIF-4E phosphorylation remain unresolved. Flynn and Proud (1996*b*) have shown that the MEK inhibitor PD098059 blocks insulin's effect on eIF-4E phosphorylation in CHO cells overexpressing the insulin receptor. The newly characterized MAPK-interacting protein 1, also known as MAPK signal interacting kinase 1 (MNK1) might be the candidate mediating eIF-4E phosphorylation in response to insulin (Waskiewicz et al., 1997). In contrast, Mendez et al. (1996) reported that phosphorylation of eIF-4E by insulin depends on PI 3-kinase but not MAPK. Hence, the insulin-stimulated pathways leading to eIF-4E phosphorylation remain debated.

Regulation of eIF-2B represents another key step in the regulation of initiation of mRNA translation. eIF-2B is a guanine nucleotide exchange factor for eIF2 as it promotes the release of the tightly bound GDP and allows eIF2 to bind GTP (Proud & Denton, 1997). eIF2, which binds the initiator Met-tRNA to the 40S ribosomal subunit, is required for every initiation event (Proud & Denton, 1997). eIF-2B is activated by insulin (Welsh & Proud, 1992) and is a substrate for GSK-3 in vitro (Welsh & Proud, 1993). PI 3-kinase appears to be required for the activation of eIF-2B since both wortmannin and dominant negative mutants of the p85 regulatory subunit of class 1a PI 3-kinase prevent eIF-2B activation (Welsh et al., 1997). That study also showed that the in vivo activation of eIF-2B by insulin correlates with the in vivo inactivation of GSK-3, but is independent of mTOR/p70 S6 kinase or the MAPK pathways (Welsh et al., 1997).

Translational control at the initiation stage has been well described for mRNAs which contain an oligopyrimidine tract at their transcriptional start (5′TOP), most notably mRNAs encoding components of the translational apparatus itself (e.g., ribosomal proteins and elongation factors) (Jefferies et al., 1994, 1997). Regulation of translation by the mTOR-4E-BP1-eIF-4E pathway for other specific mRNAs has so far only been described for

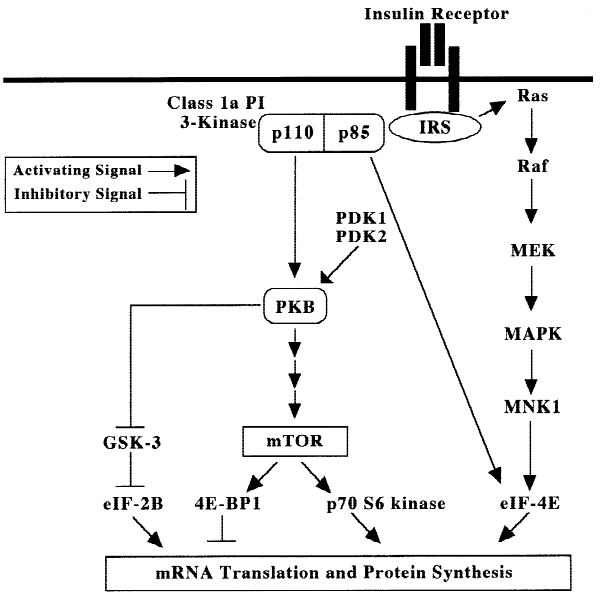

Fig. 4. Signaling pathways leading to stimulation of mRNA translation and protein synthesis. PI 3-kinase, PKB, and mTOR participate in the insulin-stimulated 4E-BP1 phosphorylation. mTOR is the kinase that directly phosphorylates 4E-BP1 both in vitro and in vivo. Phosphorylation of eIF-4E is proposed to be downstream of MNK1 and PI 3-kinase. GSK-3 phosphorylates and inactivates eIF-2B. Insulin activates eIF-2B through activation of PI 3-kinase and inhibition of GSK-3.

mRNAs encoding growth-related proteins such as ornithine decarboxylase (Manzella et al., 1991), cyclin D1 (Rosenwald et al., 1993), and P23 (Bommer et al., 1994). Interestingly, we have recently observed that insulin upregulates GLUT1 glucose transporter translation in 3T3-L1 adipocytes via the mTOR-rapamycin-sensitive pathway, and the in vitro translation of GLUT1 protein is inhibited by 4E-BP1 (C. Taha, N. Sonenberg and A. Klip, *unpublished observations*).

Peptide-chain elongation is also regulated by insulin. In mammalian cells, elongation requires two elongation factors, eEF1 and eEF2 (Proud & Denton, 1997). Addition of insulin to serum-deprived 3T3-L1 adipocytes stimulates the overall elongation rate, as assessed by ribosomal transit time, and also increases phosphorylation of eEF1 (Chang & Traugh, 1997). Stimulation of elongation requires a fall in eEF2 phosphorylation. In CHO cells expressing the insulin receptor, insulin reduces ribosomal transit time by about one-half, indicative of a doubling of the rate of elongation, which is accompanied by a very rapid dephosphorylation of eEF2 (Redpath, Foulstone & Proud, 1996). This effect is eliminated by both rapamycin and wortmannin suggesting a participation of the PI 3-kinase → mTOR pathway in insulin action at the stage of elongation (Redpath et al., 1996). A summary of the pathways mediating insulin's action on protein translation is shown in Fig. 4.

Summary and Conclusions

In recent years, great progress has been made in elucidating the tangled web of the insulin signaling pathway. Activating the tyrosine kinase activity of the insulin receptor results in tyrosine phosphorylation of various adaptor molecules, in particular the IRS-proteins. Tyrosine kinase activity of the receptor is then translated into downstream activation of serine/threonine cascades. Activation of class 1a PI 3-kinases and its downstream targets PKB, mTOR, and atypical PKCs play a much more important role in most of insulin's metabolic actions than the Grb2/SOS/Ras and the Raf/MEK/MAPK pathway. However, many details of the insulin signaling pathway remain to be elucidated. The contribution of *temporal* and *spatial* parameters of signaling, and the cross-talk with other signaling pathways are also poorly understood. New insights into the therapy of diabetes and other states of altered insulin action will no doubt stem from emerging concepts of the insulin signaling pathway.

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