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REGULATING GLUT4 VESICLE DYNAMICS BY PHOSPHOINOSITIDE KINASES AND PHOSPHOINOSITIDE PHOSPHATASES

Assia Shisheva

Department of Physiology, Wayne State University School of Medicine, Detroit, MI 48201

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1. ABSTRACT

Phosphorylated derivatives of phosphatydylinositol (PtdIns), collectively called phosphoinositides (PIs), have been recognized as versatile second messengers and modulators of lipid membrane composition in all eukaryotes. Over the last several years, PIs emerged as key membrane-localized signals for regulating a myriad of cellular processes, including insulin-induced membrane receptor signaling, GLUT4 membrane trafficking and the accompanying actin cytoskeletal rearrangement. PIs are synthesized from PtdIns by the action of kinases, specific for one of the 3 hydroxyls at positions D-3, D-4 and D-5 in the inositol head group and are degraded/turned over by the also position-specific action of phosphoinositide phosphatases. Work over the last several years has clearly implicated the products of PI 3-kinase activity, PtdIns 3,4,5-P_3 and PtdIns 3,4-P_2, as key elements in the proximal insulin receptor signaling circuit that regulates GLUT4 membrane dynamics. Emerging evidence has accumulated to suggest the role for the products of PI 4-kinases and PI 5-kinases in this process, likely at more distal steps. Here I review our current understanding of the role for PIs and the enzymes involved in their turnover in the regulation of GLUT4 membrane dynamics in response to insulin, endothelin-1 and hyperosmotic shock.

2. INTRODUCTION

GLUT4 is the mammalian insulin-regulated glucose transporter, whose expression is restricted to adipose tissue and striated muscle (reviewed recently in

Refs. 1-7). GLUT4 is an integral membrane protein and, although it continuously recycles, is almost entirely sequestered within the cell under resting conditions. This is due to the slow recycling rate of GLUT4-containing membranes (further referred to as GLUT4 vesicles), which is almost 5-fold slower than that of transferrin receptors. One of the most important metabolic effects of insulin is its ability to increase the rate of glucose uptake by increasing the number of GLUT4 glucose transporters on the adipose and muscle cell surface. This effect is associated with a dramatic insulin-regulated increase in GLUT4 vesicle exocytosis as well as a modest decrease of GLUT4 vesicle endocytosis (8-11). The net cell surface increase of GLUT4 molecules is responsible for more than 20-fold stimulation of glucose transport. This "translocation model" was put forward more than two decades ago by Cushman and Warzdala, 1980, and Suzuki & Kono, 1980 (12, 13) and was supported by numerous immunoelectron microscopy and biochemical data that followed from different laboratories (1, 4). This effect of insulin is essential in maintaining glucose homeostasis in humans and impaired insulin action contributes to pathogenesis of Type 2 diabetes (14). GLUT4 is certainly a key element in this homeostasis since muscle- or adipose tissue-specific knockout of *Glut4* gene in mice leads to profound insulin resistance (15, 16).

Numerous studies have focused on the sorting signals within the GLUT4 molecule that allow intracellular sequestration at basal states, without which insulin would not be able to acutely increase glucose uptake (17). This issue however remains unresolved and, in the light of recently published data (18), it appears still controversial. Elucidation of the intracellular sorting mechanisms is further complicated by the fact that the intracellular pool of GLUT4 is not a homogeneous population of vesicles. Electron microscopy and biochemical studies in primary and cultured adipocytes have detected GLUT4 in multiple membrane subcompartments, including clathrin-coated pits, peripheral small vesicles, and perinuclear tubulovesicular elements in the region of the trans-Golgi network (1, 4 and the references therein). It is now well accepted that intracellular GLUT4 resides in at least two distinct, but inter-related vesicular pools, and they both respond to insulin (4, 5). One of the pools is associated with the endosomal recycling system and undergoes continuous recycling through the endocytic pathway. This pool also contains other recycling proteins such as GLUT1, transferrin receptors, and insulin-like growth factor (IGF) II/manose-6-P receptors (MPR), whose membrane dynamics are also influenced by insulin (19-23). A significant proportion of GLUT4, however, resides in a compartment, referred to as GLUT4 storage vesicles (GSV) (4, 5) that lacks endosomal markers but contains IRAP (insulin-responsive aminopeptidase; 24). The GSV pool is thought to serve as a reservoir of GLUT4 that is more efficiently mobilized than the recycling endosomal pool upon insulin challenge. Recent data from cell-free reconstitution studies point to a population of pre-formed small vesicles as a major form of GLUT4 storage, whose mobilization to the plasma membrane accounts for the initial acute effect of insulin on glucose transport in adipose cells (25).

Over the last few years it became clear that insulin is not the only stimulus able to move GLUT4 vesicles to the adipose/muscle cell surface. Muscle exercise, hyperosmotic shock, and agonists that activate heterotrimeric G proteins such as endothelin-1 and bradykinin are also triggers of GLUT4 vesicle exocytosis (3). As will be discussed further in detail, data are now accumulating to suggest that these agonists use distinct signaling pathways that control the dynamics of separate subpopulations of GLUT4 vesicles. Consistent with this hypothesis, muscle contraction or hyperosmotic shock in a L6 cell line, had an effect additive to that of insulin (26, 27). In line with this idea are recent findings that activated forms of Akt/PKB, a serine/threonine kinase downstream of PI 3-Ks, or insulin-dependent activation of the newly discovered CAP/TC10 pathway primarily recruit GLUT4 vesicles from GSVs, but not from the endosomal recycling pool (28, 29). Conversely, muscle contraction mobilizes GLUT4 vesicles from the endosomal pool (26).

It should be emphasized that insulin or any other agonist that stimulates GLUT4 vesicle dynamics relays signals that affect not only the proximal receptor signaling but also more distal membrane events of vesicle externalization such as vesicle budding, fission, motility and fusion. Thus, the activation of GLUT4 vesicle translocation appears to be a complex multi-step process that is most likely under the control of different signaling pathways and control mechanisms, all of which have to act in a strictly precise and coordinated manner for the optimal response on the cell surface. The exact molecular mechanisms and signaling cascades regulating these distinct steps have not yet been completely elucidated and are under intensive investigation. Studies over the recent years have identified the PI 3-kinase activation and 3-phosphoinositide production as proximal insulin signaling events that are absolutely necessary for GLUT4 vesicle translocation and glucose transport increase. More recent studies have shown that other phosphoinositide kinases may also play a role in this insulin's effect at proximal or more distal steps. Below I review the different PIs, the pathways of their biosynthesis, and the role which the phosphoinositides and the enzymes implicated in their metabolism play in GLUT4 vesicle translocation regulated by insulin or other agonists such as endothelin-1 and hyperosmotic shock.

3. PHOSPHOINOSITIDES

3.1. Family members

The biological versatility of PtdIns is derived from its ability to serve as a substrate of reversible phosphorylation. Out of the 5 free hydroxyls in the inositol head group of PtdIns, those at positions D-3, D-4, and D-5 are found to be phosphorylated intracellularly, separately or in all possible combinations resulting in seven distinct species identified in eukaryotic cells (Figure 1). Phosphorylation at positions D-2 and D-6 is most likely sterically hindered; hence such phosphorylated derivatives have not been found intracellularly thus far. PIs are localized on membranes and their synthesis is catalyzed by phosphoinositide kinases with broader or more restricted

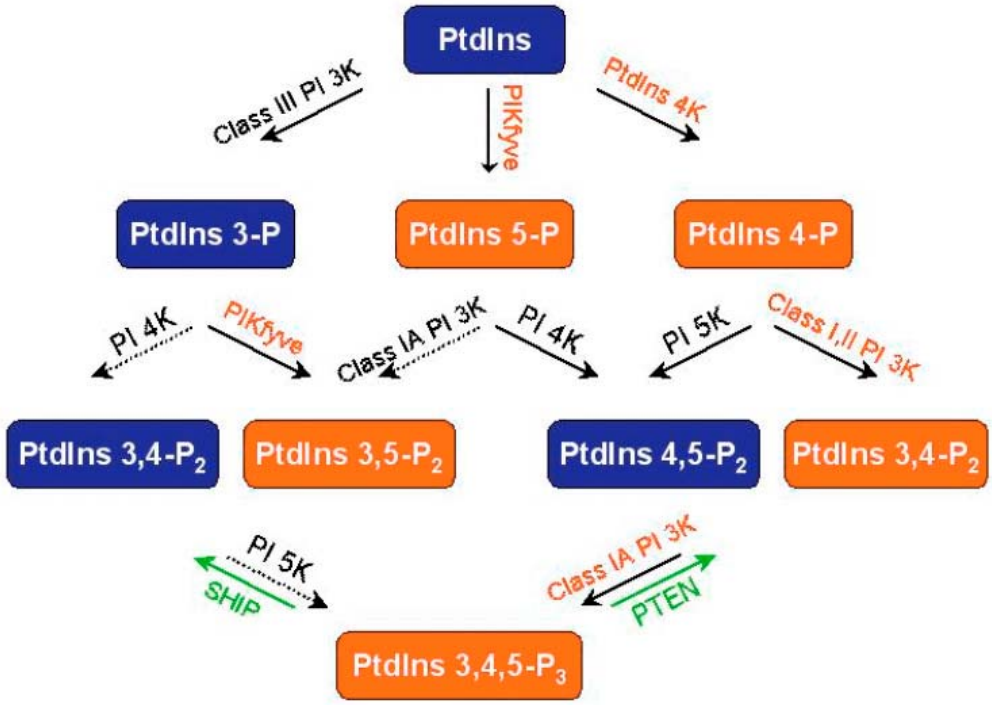

Figure 1. Pathways of phosphoinositide synthesis. Arrows show currently identified major routes of intracellular PI synthesis. Dashed lines indicate *in vitro* synthetic routes still not confirmed *in vivo*. The enzymes and products implicated in GLUT4 vesicle translocation are depicted in red. Only the enzymes dephosphorylating PtdIns 3,4,5-P_3 are mentioned (green); for a complete list of PI phosphatases see Refs. 33,34.

substrate specificity. In addition to the kinases (Ks) the biological activity of PIs can be spatially controlled by regulating the enzymes involved in their degradation and turnover. Accumulating evidence indicates that almost each of the PIs serves not only as an intermediate in the synthesis of the higher phosphorylated PIs but also as a regulatory molecule in its own right.

3.2. Enzymology

Below I discuss the identification, structure, activities, roles and regulations of those PI kinases and PI phosphatases, whose actions have been implicated in the mechanism of GLUT4 vesicle translocation and glucose uptake in adipose or muscle cells (Figure 2). The classification used herein is based on a position specificity within the inositol head group substrate, rather than on the sequence homology among enzymes. Recent reviews on generic PI metabolism are available (30-34).

3.2.1. 3-Kinases

Based on chromatographic purification, PI kinase activities were initially classified into 3 types, type I to III (35, 36). Type I was subsequently identified as PI 3-K (37), while the type II and III were found to be PtdIns 4-Ks (see below). Since the initial discovery that PI 3-K activity is associated with two viral oncoproteins, the polyoma middle T antigen and pp60Src, it became clear that it is a critical signal affecting many aspects of cell function (30, 38). Multiple PI 3-K isoforms have been cloned and are now divided into three classes, *i.e.*, I, II and III. Here I focus on class IA, whose pivotal role in insulin-regulated GLUT4 vesicle translocation and glucose transport is understood in more detail (for reviews on class IB, II and III PI 3-Ks, see Refs. 30, 31).

3.2.1.1. Class IA PI 3-kinases

Class IA PI 3-Ks are heterodimers composed of a p110 catalytic subunit and a p85 (p55, p50) regulatory subunit. Three catalytic isoforms, p110alpha, p110beta and p110delta encoded by three separate genes have been identified (39-41). Several isoforms of the regulatory subunit derived from 3 distinct genes are known: p85alpha and p85beta represent the full-length versions, whereas p55gamma is a shorter version (42-44). Shorter forms of the regulatory subunits, p55alpha and p50alpha are identified that represent splice variants derived from the gene encoding p85alpha (*Pik3r1)* (45, 46).

In vitro, class IA PI 3-K can phosphorylate PtdIns, PtdIns 4-P and PtdIns 4,5-P_2 on the D-3 position. However, it is largely accepted that PtdIns 3,4,5-P_3 and, likely, PtdIns 3,4-P_2 are the intracellular products, as they both are transiently induced following cell stimulations (30). The principal mechanism of class IA PI 3-K activation involves binding of the p85 SH2 domains to tyrosine phosphorylated receptors and other signaling molecules (30). New experimental results with transgenic animal models indicate that optimal signaling through class IA PI 3-K depends on a critical molecular balance between the regulatory and catalytic subunits. In wild-type cells, p85

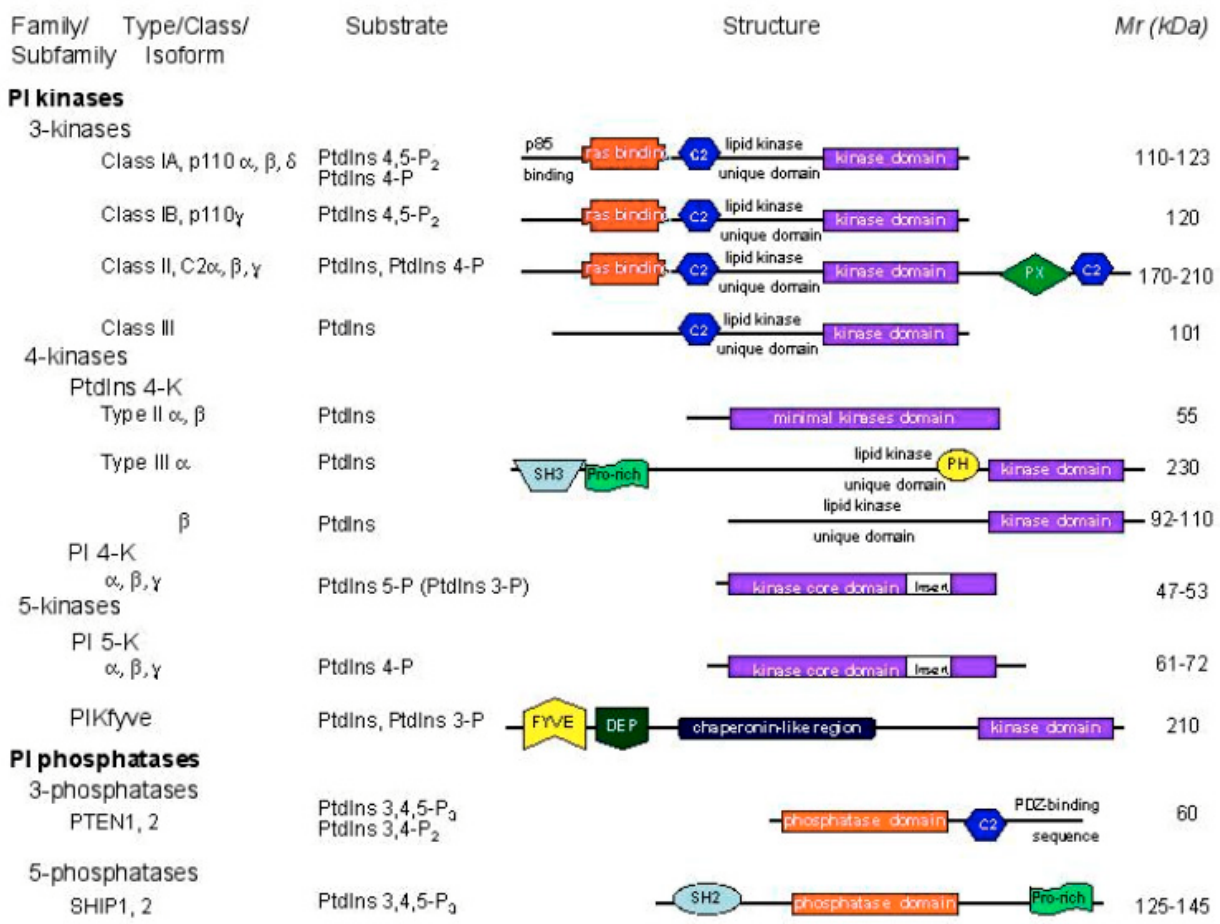

Figure 2. Schematic diagram of the domain structure of mammalian phosphoinositide kinases and phosphatases. Presented are the substrates, confirmed to be used in the context of live cells; the probable *in vivo* substrates are given in parenthesis. For the *in vitro* substrates used by each enzyme, see the text. Denoted is the apparent molecular weight (*Mr*) of the enzymes determined by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis, rather than the deduced molecular weight. The regulatory subunits of Class IA and Class IB PI 3-kinases are not presented (see the text). If the isoforms within the subfamily have similar structures only the structure of the alpha isoform is presented. Insulin is shown to activate Class II PI 3-kinase (247) but the significance of this activation to GLUT4 vesicles translocation is not clear at present. The list of the phosphatases is incomplete and depicts only those for which evidence has been presented to implicate their function in GLUT4 vesicle dynamics and glucose homeostasis. See text for further details.

is more abundant than p110 and the free p85 form inhibits signaling via the p85-p110-dimer by competing for binding to phosphorylated receptors and IRS (Insulin Receptor Substrate) proteins (47). This notion is further supported by the results demonstrating up-regulated PtdIns 3,4,5-P_3 production and enhanced IGF1/insulin signaling in cells derived from knockout embryos with heterozygous disruption of *Pik3r1* (47).

A full-length version of the regulatory subunits p85alpha and p85beta contain an SH3 domain, a bcr homology (BH) domain, flanked by two proline-rich domains, two SH2 domains and an inter SH2 domain, containing the p110 binding region. Each of the p110 catalytic subunits contain an N-terminal region that interacts with the regulatory subunits, a Ras-binding domain, a PIK domain homologous to a region found in other phosphoinositide kinases and a C-terminal catalytic domain (Figure 2). Analysis of genomic sequencing databases has revealed a singe type of class IA catalytic/regulatory heterodimer in *Drosophila melanogaster* and *Caenorhabditis elegans* (48, 49) but not in yeast.

3.2.2. 4-Kinases

3.2.2.1. PtdIns 4-kinases

PtdIns 4-K activities have long been described in several tissues and classified as type II and type III based on different catalytic properties (30, 50, 51). Mammalian type II PtdIns 4-K is a 55 kDa integral membrane protein believed to account for most of the PtdIns 4-K activity in cells. Two isoforms, alpha and beta, were recently identified by molecular cloning. They display a high degree of homology with each other but share no significant

similarity to other known lipid and protein kinases (52-54). They both are found associated with several endomembrane compartments where they likely regulate secretion or endosomal membrane trafficking in mammalian cells (30, 32, 54, 55). Two isoforms for type III PtdIns 4-Ks are also now cloned in mammalian cells: a larger 220 kDa alpha form and a smaller 110 kDa beta form (56-58). A splice variant of type IIIalpha has also been reported (59). Type IIIalpha and beta isoforms appear to display different localization, with the alpha form associated with endoplasmic reticulum, whereas the beta, with Golgi membranes (60). Consistent with this distribution, the activity of type IIIbeta but not that of type IIIalpha is required to create and maintain the structural integrity of the Golgi complex, most likely through a regulation by members of the ARF GTPase subfamily (61, 62). The yeast orthologs of type IIIalpha and type IIIbeta, Stt4 and Pik1, respectively, also appear to display a non-redundant function where the activity of Stt4 is implicated in actin rearrangements through RhoGTPase, whereas that of Pik1 is required for a normal secretory pathway at the Golgi (63).

Both type II and type III PtdIns 4-Ks use primarily PtdIns as a substrate and have no detectable activity for phosphorylated PIs. Herein, we refer to this subfamily as PtdIns 4-K and note that it should be distinguished from the PI 4-Ks that use PtdIns 5-P or PtdIns 3-P (see below).

3.2.2.2. PI 4-kinases

A subclass of PI 4-Kinases were recognized some time ago by their ability to generate PtdIns 4,5-P_2 (64, 65). It was initially thought that these enzymes do that through phosphorylating position D-5 in PtdIns 4-P and were called type II PIP 5-Ks. It was clarified later that a wrong specificity has been assigned for these enzymes and, that in fact, they phosphorylate PtdIns 5-P at the D-4 rather than the D-5 hydroxyl to make PtdIns 4,5-P_2 (66); hence they are PI 4-Ks. In mammalian cells three isoforms of PI 4-Ks encoded by distinct genes have been characterized, *i.e.,* alpha, beta and gamma (64, 65, 67, 68). The three isoforms display different intracellular localization patterns and are found in endoplasmic reticulum, cytosol, nucleus and actin cytoskeleton, but not at the plasma membrane (68, 69). The intracellular role and regulation of PI 4-Ks are still not understood, nor is the exact meaning of this alternative pathway for PtdIns 4,5-P_2 production. PtdIns 5-P substrate used by these enzymes is produced, at least in part, by PIKfyve enzyme (70, and see below) and it is likely, therefore, that the functions of these two enzymes are closely connected. However, this has not yet been addressed and needs to be determined in future studies. In any case, two studies implicate the PI 4-K activities in TNFalpha-mediated signaling (67) and thrombin-stimulated aggregation of platelets (71). In a cellular context and *in vitro*, all PI 4-Ks seem to produce PtdIns 4,5-P_2 through phosphorylation of PtdIns 5-P (66). *In vitro*, PtdIns 3-P has also been found to be a substrate, although weak, for these enzymes and it needs to be determined whether PtdIns 3,4-P_2 is produced through this pathway *in vivo.*

PI 4-Ks display a conserved kinase core domain separated by an insert domain (Figure 2). PI 4-Kbeta is the only PI kinase for which the three-dimensional structure has been resolved (72). The molecule is a dimer that forms a large flat surface thought to serve as a membrane interaction interface. A disordered loop of 20-25 amino acids termed "activation loop" has been identified and recent studies with chimeric molecules, containing reciprocal swaps with the PI 5-K-activation loops, demonstrated that this region determines substrate and signaling specificity (73, 74). Orthologs of PI 4-Ks are not found in yeast but are present in *Drosophila melanogaster* and *Caenorhabditis elegans* (73).

3.2.3. 5-Kinases

3.2.3.1. PtdIns(4)P 5-kinases

As mentioned above, animals use two different routes for PtdIns 4,5-P_2 synthesis, with the major route being phosphorylation of PtdIns 4-P at position D-5 by PI 5-Ks, also known as type I PIP kinases or PtdIns(4)P 5-kinases. Three isoforms of PI 5-Ks encoded by three distinct genes have been identified, *i.e.,* alpha, beta and gamma (75-77). Like PI 4-Ks, the PI 5-K molecules display a central kinase core domain separated by an insert domain (Figure 2). Sequence similarity between the three isoforms is restricted to the central kinase core, whereas the N-terminal and C-terminal regions are isotype-specific. PI 5-Ks play important roles in a wide variety of cellular responses, and their function appears to not be redundant with that of the other PtdIns 4,5-P_2 producing enzymes, the PI 4-Kinases. Thus, it is the PI 5-Ks, and PtdIns 4,5-P_2 produced by their activities that regulate actin cytoskeleton reorganization (77-79), most likely downstream of Rho or Rac protein family members (80-82). Consistent with this function are the data documenting interactions of PtdIns 4,5-P_2 with numerous cytoskeletal proteins, including profilin, gelsolin, vinculin, ERM (Ezrin/Radixin/Moesin) proteins, N-WASP (Wiskott-Aldrich Syndrom Protein), GMC proteins (GAP43/MARCKS/CAP23) and others (83-86), but it is still unclear which of these numerous proteins are the essential PtdIns 4,5-P_2 effectors in cytoskeletal rearrangement. Since yeast strains deficient in the PI 5-K ortholog Mss4 display defective actin filament formation that is rescued upon overexpression of either PI 5-K or a Rho-type GTPase (87, 88) there is little doubt that RhoGTPases, through PI 5-K and PtdIns 4,5-P_2 production, mediate actin cytoskeleton assembly *in vivo*. It is still unclear, however, whether *in vivo*, PI 5-Ks and Rho or RacGTPases utilize direct or indirect mechanisms of interaction since the data are conflicting (81, 82, 89).

A major step toward understanding the role of PI 5-Ks was the recent discovery that PI 5-Ks are recruited to active sites of cytoskeletal assembly at membrane ruffles or to Golgi membranes, and that the members of the ARF subfamily of GTPases mediate both a recruitment and activation (61, 62, 89). In some cases phospholipase D-catalyzed production of phosphatidic acid has been shown to promote the PI 5-K activation mechanism (89, 90). Together, these studies are consistent with the concept that PI 5-Ks are key effectors for multiple GTPase-mediated pathways related to membrane trafficking and cytoskeletal

rearrangement but the coordination among these pathways is currently only a matter of speculations.

As mentioned above, the *in vivo* substrate utilized by PI 5-Ks is PtdIns 4-P. *In vitro*, these enzymes could phosphorylate D-5 position in PtdIns or in any other PI (91, 92). However, studies in yeast did not confirm the broader substrate specificity determined *in vitro* (93). Therefore, further work in a mammalian cellular context is needed to determine whether or not these enzymes are responsible for alternative PtdIns 5-P, PtdIns 3,5-P_2 or PtdIns 3,4,5-P_3 intracellular production.

3.2.3.2. PIKfyve

PIKfyve (PhosphoInositide Kinase for five position containing a Fyve finger) was recently identified through a screen of fat/muscle enriched transcripts (94-96). Mouse PIKfyve is encoded by a single-copy gene transcribed in at least two closely sized splice variants, a long and a short form, which differ by the presence or absence of a 11-amino-acid peptide segment (94). Analysis of human genome and expressed sequence tags databases indicate that the single human PIKfyve gene is located on locus 2q33-q35 and can encode only the short form of the protein (70). PIKfyve harbors several functional domains including a FYVE finger domain (named after Fab1p, YOTB, Vac1p and EEA1), a DEP domain (named after Dishevelled, Egl-10 and Pleckstrin), a chaperonin-like region and a catalytic region at the C-terminus (Figure 2). The catalytic domain of PIKfyve displays sequence similarities with the catalytic domains in PI 4-Ks and PI 5-Ks but not with that in PI 3-Ks and PtdIns 4-Ks. PIKfyve is mainly cytosolic, with a small fraction in a membrane-bound form that appears to be associated partly with the membranes of the late endocytic compartment (97). It has been directly demonstrated that the FYVE finger domain in PIKfyve binds specifically to liposomes containing PtdIns 3-P, and since the PIKfyve membrane-association appears sensitive to cell treatment with wortmannin, it is conceivable that intracellular PIKfyve, at least in part, uses the FYVE finger - PtdIns 3-P interaction mechanism for membrane attachment (98). A subfraction of PIKfyve localized to cytoskeletal structures has also been detected (97). PIKfyve intracellular localization is likely regulated by extracellular stimuli, since biochemical studies show a recruitment of a fraction of the cytosolic PIKfyve to membranes upon acute insulin treatment without consequences on the enzymatic activity (97). A concomitant insulin-dependent Ser/Thr phosphorylation of PIKfyve may play a role in the membrane tethering mechanism (97). At present, no experimental evidence is available for the nature of the insulin-regulated PIKfyve membrane target, or the identity of the Ser/Thr kinase. One essential cellular function of PIKfyve (and its products) is associated with the maintenance of endomembrane homeostasis, since expression of kinase-deficient PIKfyve point mutants in several epithelial cell types induces a dramatic cytoplasmic vacuolation and alteration of cell morphology (99). PIKfyve orthologs are found in all eukaryotes, with Fab1p known as the structural and functional counterpart in yeast (70).

In vitro PIKfyve was found to phosphorylate the D-5 position in PtdIns and PtdIns 3-P to make PtdIns 5-P and PtdIns 3,5-P_2, respectively (100). Studies in different mammalian cell types confirm both PtdIns 5-P and PtdIns 3,5-P_2 production due to PIKfyve (99, 101) implicating PIKfyve as a main pathway for intracellular biosynthesis of these PIs.

3.2.4. 3-Phosphatases

3.2.4.1. PTEN

PTEN (Phosphatase and tensin homolog deleted in chromosome ten)/*MMAC* (Mutated in Multiple Advanced Cancers) was identified several years back simultaneously by two groups (102, 103) as a candidate tumor suppressor gene located on chromosome 10q23. A third group identified the same gene in a search for novel dual-specificity phosphatases and named it TEP-1 (TGFbeta-regulated and epithelial cells enriched phosphatases) (104). Since then, it has become clear that mutations in PTEN are associated with many human tumors, but at particularly high frequency in endometrial tumors and glioblastomas (105, 106). There are several genes in the human genome very similar to *PTEN*, and at least some of them express functional proteins, likely in a tissue-specific manner (107, 108).

Although PTEN is homologous to dual-specificity protein tyrosine phosphatases (PTP) studies have demonstrated that PTEN dephosphorylates preferentially phospholipids over proteins and peptides (109, 110). It removes efficiently the D-3 phosphate from PtdIns 3,4,5-P_3 and PtdIns 3,4-P_2, indicating the PTEN *in vivo* function is a 3-PI-phosphatase. *In vitro*, PTEN is shown to also dephosphorylate position D-3 in PtdIns 3,5-P_2 (111) and Ins 1,3,4,5-P_4, the soluble head-group of PtdIns 3,4,5-P_3 (110). However, in a cellular context PTEN is believed to act exclusively on PtdIns 3,4,5-P_3 and PtdIns 3,4-P_2 substrates since human cell lines, lacking the *PTEN* gene or expressing a phosphatase-deficient mutant of PTEN, appear to have higher levels of both PtdIns 3,4,5-P_3 and PtdIns 3,4-P_2 (112, 113). This and other evidence (108) define PTEN as a functional antagonist of class IA PI 3-Ks.

PTEN crystal structure has been recently resolved (114). These studies revealed that, in addition to the N-terminal phosphatase domain with homology to both the PTP family and the non-phosphatases tensin and auxilin, the enzyme exhibits a C-terminal C2-domain similar to that observed in Ca2+-independent PKCs (102, 103, 114). At the very C-terminus of the molecule is positioned a consensus PDZ domain-binding sequence (Figure 2). Several interacting PDZ domain-containing protein partners have been identified (107, 108), but the impact of these interactions to the primary function of PTEN in antagonizing the PI 3-K pathway is still unclear. PTEN homologous sequences are found in yeast (OMIM) as well as in *Drosophila melanogaster* and *Caenorhabditis elegans* (107).

3.2.5. 5-Phosphatases

3.2.5.1. SHIP1 and SHIP2

SHIP1 (SH2-containing Inositol 5-Phosphatase) has been cloned simultaneously by several laboratories and its expression, shown to be restricted to hematopoietic

cells and lung (115-119). Human SHIP1 is encoded by locus 2q36-q37, in which the occasional overt defects exhibit only sporadic association with tumor formation (120). SHIP1 is phosphorylated on tyrosine in response to multiple cytokines and B cell receptor engagement (121). Another isoform, closely related to SHIP1 (38% overall identity) has been identified and called SHIP2 (122-124). SHIP2 exhibits a broader tissue distribution and is found abundant in insulin target tissue including skeletal muscle and fat cells (122-124). Human SHIP2 is encoded by locus 11q23.

Both SHIP1 and SHIP2, when overexpressed in different cell systems, including 3T3-L1 adipocytes display the ability to hydrolyze PtdIns 3,4,5-P_3 to PtdIns 3,4-P_2 (112, 125). Unlike SHIP2, SHIP1 could also use, at least *in vitro*, Ins 1,3,4,5-P_4 (123). The principal mechanism of the enzyme activation involves binding through the SH2 domains to tyrosine phosphorylated receptors and other signaling molecules (121). In addition to the SH2 domain at the N-terminus, both SHIP1 and SHIP2 proteins bear an inositol-5-phosphatase domain in the middle of the molecule, an NPXY site for proteins, containing the phosphotyrosine binding domain, and a proline-rich motif at the C-terminus (Figure 2). Analysis of genomic sequencing databases has revealed no close SHIP orthologs in nonmammalian organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (121), indicating that SHIP proteins are a late evolutionary acquisition.

3.3. Abundance and distribution

PtdIns, the precursor of all PIs, is the most abundant inositol lipid in mammalian cells, with levels exceeding ~10-fold that of PtdIns 4-P and PtdIns 4,5-P_2. PtdIns 4-P and PtdIns 4,5-P_2 occur in cells roughly at equal levels and are the most abundant among the singly and doubly phosphorylated PIs, respectively, constituting ~90% of the phosphorylated PtdIns. PtdIns 3-P represents 2-5% of the monophosphorylated PIs in different cell types. The levels of PtdIns 5-P vary from undetectable in many cells to ~12 % of the monophosporylated PIs in 3T3-L1 adipocytes, a cell line that exhibits the highest PtdIns 5-P levels among the mammalian cells tested thus far (99, 101). The levels of the higher 3-phosphorylated species, *i.e.,* PtdIns 3,4-P_2, PtdIns 3,5-P_2 and PtdIns 3,4,5-P_3, are typically low or undetectable in nonstimulated cells.

Until recently, conclusions about the intracellular distribution and the acute dynamics in the levels of different PIs have been inferred from the localization of the enzymes responsible for their synthesis. Recent discovery of protein modules that bind selectively PIs and development of green fluorescent protein (GFP)-PI reporter constructs as well as the generation of relatively specific anti-PI antibodies, have provided new information on the distribution and dynamics of the phosphoinositide pools. These studies revealed that PIs are distributed non-uniformly within cells in spatially segregated pools. Thus, PtdIns 3-P, visualized by the FYVE finger domain, a specific binding module for PtdIns 3-P, was found predominantly on endosomal membranes (126). Spatially segregated PtdIns 4,5-P_2 pools visualized at the cell surface by using the phospholipase Cdelta1 or gamma2 PH (Pleckstrin Homology) domain probes that specifically bind PtdIns 4,5-P_2, have been associated with key functions such as phagocytosis, exocytosis, or agonist-stimulated ruffling activity (89, 127, 128). Specific PtdIns 4,5-P_2 antibodies detected PtdIns 4,5-P_2 in raft-like clusters dependent upon membrane cholesterol (129). These data are consistent with the biochemical detection of PtdIns 4,5-P_2 (130) and type II PtdIns 4-K in detergent insoluble, cholesterol-rich membranes (53). However, recent on-section labeling with the phospholipase Cdelta1 PH domain probe visualized PtdIns 4,5-P_2, in addition to the plasma membrane, at several intracellular locations, such as Golgi, endosomes, endoplasmic reticulum electron dense structures within the nucleus (131). These results indicate that PtdIns 4,5-P_2 has a broader intracellular distribution than initially anticipated. PtdIns 3,4,5-P_3 has been also visualized by specific probes comprised of the PH domains of ARNO (ADP-ribosylation factor nucleotide binding site opener) and GRP1 (General Receptor for 3-Phosphoinositides). Dynamic concentration changes in PtdIns 3,4,5-P_3 on the plasma membrane associated with acute insulin action in 3T3-L1 adipocytes and in other cell types have been possible to be directly detected (132-135). A recent study that uses specific anti-PtdIns 3,4,5-P_3 antibodies argues against the plasma membrane as the sole site of stimulated PtdIns 3,4,5-P_3 synthesis, visualizing distinct cytoplasmic PtdIns 3,4,5-P_3 pools in fibroblasts and neutrophils (136). PtdIns 3,4-P_2 was also found on the plasma membrane upon cell stimulation with growth factors or oxidative stresses, but not with insulin (134). Although the technology used in the studies discussed above may have some downsides related to the reagents' specificity or inability to interact with membrane PIs that are not always readily accessible (137), we have definitely learned a great deal about the functional dynamics of PI levels. Examination of the PtdIns 5-P or PtdIns 3,5-P_2 intracellular dynamics by selective reporter probes awaits a better characterization of modules and antibodies that specifically bind those lipids.

4. GLUT4 VESICLE TRANSLOCATION IN RESPONSE TO INSULIN RECEPTOR STIMULATION

4.1. Signaling pathway

As discussed above, the insulin-induced translocation of GLUT4 vesicles to the plasma membrane and the subsequent glucose entry is a complex multi-step process, which is a focus of intensive investigation. A critical early event in insulin-induced signaling that occurs following insulin binding is autophosphorylation and activation of insulin receptor (IR) tyrosine kinase (138). Activated IR, in turn, phosphorylates tyrosyl residues in a number of intermediate substrates. According to currently existing models, two subsets of phosphorylated substrates appear relevant to insulin action on GLUT4 vesicle translocation: the IRS proteins (139) and APS (Adaptor containing PH and SH2 domains)/c-Cbl (140-142). The first subset of substrates initiates a signaling arm that depends on class IA PI 3-Ks, whereas the second subset initiates a PI 3-kinase-independent signaling pathway. Activation of the PI 3-kinase-dependent pathway is

achieved upon tyrosine phosphorylation of IRS proteins that recruit and activate class IA PI 3-kinase through binding to the SH2 domain of the p85 regulatory subunit. Activation of this pathway increases cell surface production of PtdIns 3,4,5-P_2 (132, 135), and perhaps PtdIns 3,4-P_2 that, in turn, recruit and activate downstream targets including serine/threonine kinase PDK1 (Phosphoinositide-Dependent Kinase). Activated PDK1 in turn phosphorylates and activates Akt and members of the atypical PKC subfamily (143-146). Activation of the serine/threonine kinases Akt, PKCzeta and PKClambda appears to be a key event leading to GLUT4 vesicle translocation and enhanced glucose transport but their downstream targets remain enigmatic.

The essential role of the class IA PI 3-kinase-dependent pathway in insulin's effect on GLUT4 vesicle translocation and glucose entry is supported by work in many laboratories, and is discussed below in detail. Initial skepticism for accepting this pathway as being sufficient for insulin-regulated GLUT4 vesicle translocation came from data demonstrating that PDGF stimulation in 3T3-L1 adipocytes (147), integrin cross-linking in rat adipocytes (148) and interleukin-4 activation in the L6 cell line (149), while increasing the activity of class IA PI 3-Ks, were unable to induce GLUT4 vesicle translocation and to increase glucose uptake. While this paradox, at least in the case of PDGF receptor stimulation, seems to be resolved (being associated with insufficient PtdIns 3,4,5-P_3 production on the cell surface, Ref. 132) more recent studies raise additional mysteries. For example, exogenously introduced cell-permeable derivatives of the PI 3-K product PtdIns 3,4,5-P_3 were found unable to stimulate glucose transport yet they did restore insulin's effect on glucose uptake in 3T3-L1 adipocytes treated with the PI 3-kinase inhibitor wortmannin (150). Moreover, over the last few years it became clear that besides insulin, various other stimuli such as hypoxia, physical exercise (151) hyperosmotic shock (152), endothelin-1 (153-156) and other agonists (3) can induce the translocation of GLUT4 vesicles on the adipose/muscle cell surface and increase glucose transport without activating class IA PI 3-Ks, further lending support for a PI 3-kinase-independent pathway underlying these events.

Recently a series of experiments have begun to unravel the PI 3-kinase-independent arm in insulin signaling to GLUT4 vesicles. According to this model, activated IR phosphorylates the protooncogene *c-cbl* and the adaptor protein APS. The phosphorylation of c-Cbl requires an adaptor protein CAP, which is associated with Cbl via one of its three SH3 domains (29). The complex is recruited to lipid raft microdomains, large invaginations of the plasma membrane, enriched in caveolin and cholesterol, that appear to be crucial in initiating the PI 3-kinase-independent pathway (157). The CAP-Cbl pathway has been proposed to control the activity of G3C, a guanylnucleotide exchange factor, which is capable of activating TC10 (29, 158). TC10 is a small GTPase of the Rho family, closely related to Cdc42 (159), that appears to be a constitutive resident of plasma membrane lipid rafts. GTP-loaded activated TC10, by a mechanism that involves, at least in part, cytoskeleton rearrangement (see below), appears to be a necessary element for insulin-induced GLUT4 vesicle translocation and stimulation of glucose transport. This pathway seems to operate selectively in insulin-responsive adipose/muscle cells since insulin-stimulated cCbl phosphorylation does not occur in fibroblastic lines (141) and the TC10 expression is primarily restricted to fat and muscle cells (159, 160). Experimental evidence is now provided that activation of both PI 3-kinase-dependent and PI 3-kinase-independent pathways are necessary for the optimal GLUT4 vesicle translocation and increase of glucose transport in response to insulin (157).

4.2. Role for Class IA PI 3-kinases

Class IA PI 3-Ks and their PtdIns 3,4,5-P_3/PtdIns 3,4-P_2 products are central in insulin signaling of GLUT4 vesicle translocation. Their role has been supported by several lines of experimental evidence. Thus, a pharmacological inhibition of class IA PI 3-kinase activity results in an abrogation of insulin-regulated GLUT4 translocation and glucose transport (161, 162). Likewise, disrupted interactions between the regulatory and catalytic subunits of PI 3-Ks results in inhibition of insulin effect on GLUT4 vesicle translocation and glucose transport (163, 164). The ability of constitutively active forms of class IA PI 3-kinase to mimic the above effects of insulin (165-167) further reinforces the critical role of these enzymes. Downstream targets of PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 relevant in mediating insulin's effect on GLUT4 vesicle translocation include serine/threonine kinase PDK1 and Akt. They are directly activated upon binding to PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 (Table 1) and, in the case of Akt, through PDK1-dependent phosphorylation (143-146).

While activation of PI 3-kinase and 3-phopshoinositol lipid synthesis appear unequivocally to be a necessary event for insulin signaling of GLUT4 translocation, an important question is the intracellular site of this event. A common logic would implicate GLUT4 vesicles themselves as a key target but this did not turn out to be the case. Indeed, upon insulin stimulation of 3T3-L1 adipocytes or rat skeletal muscle, little (168) or no PI 3-kinase protein or activity (169, 170) were found associated with purified GLUT4 vesicles. Delivery of activated PI 3-kinase molecules to GLUT4 vesicles in this cell type has been shown to only partially stimulate glucose entry and GLUT4 vesicle translocation (171), further reinforcing the notion that GLUT4 vesicles themselves are, most likely, not the place of class IA PI 3-K action. Instead, an insulin-dependent increase in PI 3-kinase activity in 3T3-L1 adipocytes was reported to be associated with a detergent-insoluble fraction visualized as bundles and a filamentous network by electron microscopy (169). Similar association of class IA PI 3-kinase with the detergent insoluble fraction was observed in L6 myotubes (172). Furthermore, fluorescence microscopy studies demonstrate an acute insulin-dependent concentration of GFP-based PtdIns 3,4,5-P_3-binding reporter proteins at the plasma membrane, consistent with the concept that PtdIns 3,4,5-P_3 generation at the cell surface is the key site of the PI 3-K activation related to GLUT4 vesicle translocation (132). More studies

Table 1. Proteins that synthesize, metabolize or bind phosphoinositides, implicated in GLUT4 vesicle dynamics

Protein	Domain	Binding specificity	Protein function	Role related to GLUT4	References
IRS-1	PH	PtdIns 3,4,5-P_3	Recruits class IA PI 3-Ks	Proximal signaling (Ins)[1]	138,139,
IRS-2	PH	PtdIns 3,4-P_2			229,230
IRS-3	PH	PtdIns 3-P			
Class IA PI 3-Ks			Synthesizes PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3	Proximal signaling (Ins)	161-167
SHIP2			Dephosphorylates PtdIns 3,4,5-P_3 to PtdIns 3,4-P_2	Proximal signaling (Ins)	125,203,206
Akt	PH	PtdIns 3,4-P_2 PtdIns 3,4,5-P_3	Ser/Thr kinase downstream of class IA PI 3-Ks	Proximal signaling (Ins)	231,232
PDK1	PH	PtdIns 3,4-P_2 PtdIns 3,4,5-P_3	Ser/Thr kinase downstream of class IA PI 3-Ks	Proximal signaling (Ins)	145,231
PIKfyve	FYVE	PtdIns 3-P	Synthesizes PtdIns 5-P and PtdIns 3,5-P_2	Proximal signaling (Ins) Vesicle exocytosis?	98,181
PtdIns4-K typeIIIbeta			Synthesizes PtdIns 4-P	GVS sorting or exocytosis (Ins)	178
Adaptor complex	Lys-rich	PtdIns 3,4,5-P_3	Formation of clathrin-coated buds	Coating reaction	233-235
Dynamin	PH	PtdIns 4,5-P_2	GTPase, fission of clathrin-coated pits	Endocytosis	236-239
Phospholipase D	PH[2] PX	PtdIns 4,5-P_2 ?	Phospholipid hydrolysis	Exocytosis? (Ins)	240-244
ARF6	PH	PtdIns 4,5-P_2	GTPase, activates PI 5-K	F-actin remodeling (ET-1)	155,156,245
N-WASP	Lys-rich[2]	PtdIns 4,5-P_2	Regulates the assembly of actin monomers into filaments	F-actin remodeling (Ins)	191,192,196, 246

[1] In parenthesis is indicated the agonist for which the effect in stimulating GLUT4 vesicle translocation is determined. Ins, insulin; ET-1, endothelin-1; [2] The PtdIns 4,5-P_2 binding domain in phospholipase D (240) and in N-WASP (246) are still not identified.

will be necessary to reconcile the activation of PI 3-kinase within the actin filaments with the generated PtdIns 3,4,5-P_3 at the plasma membrane but it is likely that the place of PI 3-kinase-associated actin filaments is in close proximity to the cell surface. A recent report using dual-color wave microscopy to simultaneously measure PtdIns 3,4,5-P_3 production and GLUT4 vesicle insertion in 3T3-L1 adipocytes indicates that not only the cell surface PtdIns 3,4,5-P_3 synthesis but also the amplitude of the PtdIns 3,4,5-P_3 signals are essential in the insulin-triggered GLUT4 vesicle translocation (173), indicating that the events at the cell surface are even more complex.

Given the crucial role of class IA PI 3-Ks not only for insulin action on GLUT4 vesicle translocation but also for many other cellular events, it was no surprise that elimination of p110alpha or p110beta genes caused embryonic lethality (174, 175). However, the findings that mice lacking either *Pik3r1* gene (encoding the three splice variants, p85alpha, p50alpha and p55alpha) or *Pik3r2* gene (encoding p85beta) exhibit improved, rather than decreased, insulin sensitivity, marked by reduced insulin and glucose levels or improved glucose tolerance (176, 177), were rather unexpected. These results are consistent with the hypothesis that in addition to their roles in recruiting the p110 catalytic subunits, both p85alpha and p85beta negatively regulate insulin signaling. Thus, the molecular balance between the catalytic and regulatory subunits appears critical for insulin signaling through PI 3-K pathway.

4.3. Role for PtdIns 4-kinase

While abundant experimental evidence supports the role of activated Class IA PI 3-kinase and the generated 3-phosphoinositol lipids in insulin-regulated GLUT4 vesicle translocation, only few studies address the role of other inositol lipid kinases and their products in this event. Recent data implicate PtdIns 4-K type IIIbeta as a negative regulator of insulin-induced GLUT4 vesicle translocation in 3T3-L1 adipocytes (178). In this study, expression of both PtdIns 4-K type IIIbeta or NCS-1, a recently characterized activator of PtdIns 4-K type IIIbeta in mammalian cells (179), inhibited the insulin effect on GLUT4 vesicle translocation under conditions of functional proximal insulin signaling through both PI 3-kinase-dependent and PI 3-kinase-independent pathways (178). Intriguingly, NCS-1 expression was found to also arrest the insulin-dependent IRAP translocation, but not that of MPR and GLUT1, implying that events at the GSV but not at the endosomal recycling compartment, are sensitive to PtdIns 4-K (178). Remarkably, unlike the PI 3-kinase activity, the PtdIns 4-K activity is abundant in GLUT4 vesicles isolated from rat adipocytes and skeletal muscle but it is not regulated by insulin (170, 180). Further studies will be required to determine the step in GLUT4 membrane trafficking that is negatively affected by PtdIns 4-K type IIIbeta as well as the nature of PtdIns 4-K activities that are present in GLUT4 vesicles.

4.4. Role for PIKfyve

A kinase-deficient dominant-negative mutant of PIKfyve was recently shown to negatively regulate insulin stimulated GLUT4 vesicle translocation in 3T3-L1 adipocytes (181). The observation that cytosolic populations of endogenous PIKfyve are recruited to adipocyte internal membranes and phosphorylated in a manner stimulated by acute insulin action in this cell type (97) further reinforces a plausible role of localized PtdIns 5-P and/or PtdIns 3,5-P_2 production in the hormonal effect on GLUT4 vesicle translocation. These results are supported by pharmacological data, demonstrating that the inhibition of PIKfyve enzymatic activity by low doses of curcumin, coincides with a dramatic arrest of GLUT4 vesicle translocation in response to insulin stimulation of 3T3-L1 adipocytes (181). However, as in the case of class IA PI 3-kinases (169), the site of PIKfyve basal residence or insulin-regulated recruitment is not GLUT4 vesicles themselves as evidenced by results from morphological and biochemical studies (97). Rather, PIKfyve was found to co-fractionate with structures that contain class IA PI 3-Ks and other signaling molecules, but not GLUT4 and IRAP (97). These data suggest a plausible role for concerted activities of PIKfyve and class IA PI 3-Ks, most likely for PtdIns 3,5-P_2 production. This concept is further underscored by the results documenting co-immunoprecipitation of the two enzymes and the ability of

acute insulin action in 3T3-L1 adipocytes, but not that of PDGF, to selectively activate class IA PI 3-Ks in the PIKfyve immunoprecipitates (182). It is possible that PtdIns 3,5-P_2 production is needed for an optimal Akt activation since Akt phosphorylation at amino acid 473 seen at maximally activating insulin concentrations is largely augmented in the presence of overexpressed PIKfyve in CHO cells producing high levels of the insulin receptor (181). It is also quite possible that PIKfyve, and its lipid products, act at steps distal to the insulin receptor signaling, consistent with the observations demonstrating PIKfyve-catalized PtdIns 3,5-P_2 production to be essential in maintaining endomembrane morphology and homeostasis (99, 181). While the exact molecular mechanism of the role for PIKfyve and its lipid products PtdIns 3,5-P_2 and/or PtdIns 5-P relevant to insulin action on GLUT4 vesicle translocation will require further studies, it is likely that the PIKfyve function is needed at several steps in both the proximal insulin receptor signaling and more distal endomembrane events.

One intriguing observation from several independent studies in different laboratories is the similar morphological changes in the form of multiple large vacuolar structures that are induced upon expression of either kinase-deficient dominant-negative PIKfyve mutants or PtdIns 4-K type IIIbeta activator NCS-1 in COS cells (99, 179, 181). Intriguingly, in 3T3-L1 adipocytes such morphological defects are not apparent (178, 181). These results are consistent with the hypothesis that accumulation of PtdIns 4-P pool or lack of PtdIns 3,5-P_2 result in an activation or inactivation of a common step, the outcome of which in 3T3-L1 adipocytes is the arrest of GLUT4 vesicle translocation in response to insulin. Further studies designed to define the exact function of these PIs in the context of GLUT4 membrane trafficking will be interesting.

4.5. Role for PI 5-kinases

It is rather surprising that while PI 5-K-dependent PtdIns 4,5-P_2 production has been implicated in almost every step of endocytosis and regulated secretion, including vesicle budding, fission, motility and fusion (83-86), studies implicating localized PtdIns 4,5-P_2 production in the action of insulin on GLUT4 vesicle translocation are lacking. Experimental results in other trafficking systems give support for two mechanisms that underlie a PtdIns 4,5-P_2 role: a dynamic and localized *de novo* biogenesis or mobilization of the available PtdIns 4,5-P_2 pool for functional interaction with other proteins. Although direct data for the role of PI 5-Ks and PtdIns 4,5-P_2 in insulin action on GLUT4 vesicle translocation are currently unavailable, below I discuss potential steps that may rely on PtdIns 4,5-P_2 *de novo* synthesis and interactions with downstream protein effectors.

Studies in several laboratories have implicated the mobilization of actin filament assembly in insulin-regulated GLUT4 vesicle translocation onto the adipose/muscle cell surface (172, 183-189). It has recently become apparent that insulin can relay two types of signals to modulate actin rearrangements: one dependent on PI 3-kinase activity, while another appears to be PI 3-kinase independent (190-192). The PI 3-kinase-dependent signal is associated with actin filament remodeling known as membrane ruffling. Because the PI 3-kinase-dependent actin rearrangement could be selectively influenced by specific agents that are ineffective in insulin activation of GLUT4 vesicle translocation (193), it seems reasonable to suggest that the insulin-induced PI 3-kinase-dependent actin remodeling is unrelated to GLUT4 vesicle redistribution, although more work will be needed to support this contention. The PI 3-kinase-independent signal, however, implicates the function of N-WASP in the activation of cortical F-actin formation in 3T3-L1 adipocytes that appears to promote GLUT4 vesicle responsiveness to insulin (190-192). The mechanism involves N-WASP recruitment to TC10 at the cortical region of insulin-stimulated 3T3-L1 adipocytes. Evidence has been recently provided to suggest that TC10 regulates differentially two distinct compartmentalized actin populations, and the balance between these two opposite effects is suggested to be critical in the regulation of GLUT4 vesicle translocation (194). Under these conditions, a stimulation of actin comet tailing of GLUT4 vesicles was observed in both isolated vesicles or upon insulin stimulation of intact 3T3-L1 adipocytes (190). It is now established from other transport systems that PtdIns 4,5-P_2 is a key cofactor in signaling of the actin filament assembly related to vesicle motility mechanism involving comet tail propulsion (85, 86, 195). PtdIns 4,5-P_2 accumulates in membrane rafts and promotes a local recruitment and activation of specific intermediates at the cell surface. Members of the WASP family of proteins are now identified as such intermediate elements that are recruited to membrane PtdIns 4,5-P_2, and are activated in a coordinated action with the GTP-loaded Cdc42 or other adaptor proteins (196-198; Table 1). Activated N-WASP, in turn, increases the activity of Arp2/3 (Actin related proteins) protein complex that nucleates the polymerization of the new actin filaments to create a branching network with free fast-growing barbed filament ends oriented in the opposite direction to vesicle movement. Studies are now needed to identify whether PtdIns 4,5-P_2 has a role in the N-WASP/TC10 control of cortical or perinuclear actin polymerization related to GLUT4 vesicle trafficking in adipocytes and muscle cells.

It should be emphasized that in most cells, the total levels of PtdIns 4,5-P_2 do not change dramatically in response to agonist stimulation. This holds true for 3T3-L1 adipocytes, where levels of PtdIns 4,5-P_2 remained largely unaltered by acute insulin action (D. Sbrissa & A. Shisheva, unpublished data). Several points should be made relative to this issue. First, as discussed above, in insulin-sensitive cells a portion of PtdIns 4,5-P_2 is rapidly consumed for PtdIns 3,4,5-P_3 conversion by the insulin-activated PI 3-Ks. Second, acute insulin action reportedly stimulates phospholipase C activity in adipocytes (199, 200) that would rapidly degrade PtdIns 4,5-P_2. Thus, even in a scenario whereby insulin affects *de novo* synthesis of PtdIns 4,5-P_2 by activating PI 5-Ks, this may not be readily detectable by quantifying the total PtdIns 4,5-P_2 levels. Apparently, approaches detecting localized PtdIns 4,5-P_2

production should be more informative but currently such data related to the acute insulin action, are unavailable.

4.6. Role for PTEN

The function of PTEN as a 3-phosphatase on PtdIns 3,4,5-P_3, and likely PtdIns 3,4-P_2 substrates, together with the indisputable role of class IA PI 3-K products in insulin-induced increase of glucose uptake suggest that PTEN will antagonize insulin-regulated GLUT4 vesicle dynamics and glucose metabolism. This has been directly demonstrated in two reports using 3T3-L1 adipocytes, transduced with recombinant PTEN adenovirus (113, 201). Under these conditions insulin's ability to activate glucose transport and to redistribute GLUT4 vesicles onto the cell surface were abrogated without affecting the *in vitro* PI 3-kinase activity (113, 201). Consistent with this inhibitory PTEN's role, microinjection of anti-PTEN antibodies has been shown to increase GLUT4 plasma membrane accumulation in both basal and insulin-stimulated cells. This increase was abrogated in cells pretreated with the PI 3-kinase inhibitor wortmannin (201). Combined results from these studies suggest that PTEN negatively regulates insulin action through antagonizing the PI 3-kinase signaling pathway to GLUT4 vesicle translocation. This conclusion is further supported by the reported decrease or increase of insulin-induced intracellular PtdIns 3,4-P_2 or PtdIns 3,4,5-P_3 production upon 3T3-L1 adipocyte transduction with PTEN wild-type or phosphatase-deficient mutants, respectively (113).

A number of different *Pten* knockout mice have been generated and in all cases homozygous mutant embryos die during gestation at E6.5-9.5, displaying common features such as increased cell proliferation and abnormal pattern formation (202). Heterozygous *Pten*+/- mice develop neoplasia in multiple organ systems, but not hypoglycemia, with half of the mice dying within a year. These findings are consistent with the hypothesis that PtdIns 3,4,5-P_3 and PtdIns 3,4-P_2 levels regulated by PTEN are related to tumorogenesis activity rather than to glucose homeostasis.

4.7. Role for SHIP1 and SHIP2

The role of PtdIns 3,4,5-P_3 in insulin-induced GLUT4 vesicle dynamics has been further supported by the results from studies in 3T3-L1 adipocytes expressing SHIP1 or SHIP2 (125, 203). Both SHIP proteins were found to inhibit insulin-regulated GLUT4 vesicle translocation and glucose transport stimulation concomitant with a decreased PtdIns 3,4,5-P_3 production and arrested activation of the PtdIns 3,4,5-P_3-downstream targets Akt and PKClambda (125, 203) Thus, in cell systems both SHIP1 and SHIP2, acting as 5-phosphatases appear to be negative regulators of insulin effect on GLUT4 vesicle dynamics via the PI 3-kinase-dependent pathway.

Examination of knockout animal models, however, reveals substantial differences. Thus, *Ship1*-/- mice develop normally and are fertile but display a decreased life span due to myeloid cell infiltration (204, 205). Remarkably, SHIP2 appears to be a potent negative regulator of insulin signaling and insulin sensitivity *in vivo*. Mice lacking SHIP2 exhibit increased sensitivity to insulin, characterized by severe neonatal hypoglycemia, deregulated expression of the genes involved in gluconeogenesis, and perinatal death (206). Adult mice that are heterozygous for the SHIP2 mutation have increased glucose tolerance and insulin sensitivity in skeletal muscle evidenced by an increased recruitment of GLUT4 on the plasma membrane and increased glycogen synthesis (206). Although the PI levels in these animals have not been examined, these results would suggest that PtdIns 3,4,5-P_3 levels regulated by SHIP2 are related to glucose homeostasis. Thus, SHIP2 appears to be a major negative regulator of insulin action on glucose metabolism *in vivo*, implying that its inhibition may provide a novel therapeutic target for improving insulin sensitivity in type 2 diabetes.

5. GLUT4 VESICLE TRANSLOCATION IN RESPONSE TO HETEROTRIMERIC G-PROTEIN-COUPLED RECEPTOR STIMULATION

Agonists that activate the heterotrimeric G-protein Gq/G11 alpha subunit, as well as expression of constitutively active forms of Gq/G11 were shown to increase glucose transport and GLUT4 vesicle translocation in adipocytes (153, 154, 207-210). Although the results in these studies slightly differ, primarily in the role for PI 3-K activation, it is suggested that Gq/G11 is also a necessary factor in the regulation of GLUT4 vesicle translocation and glucose transport by insulin (209, 210) but it is still unclear how insulin activates Gq/G11 alpha subunit. An important role of Gi2 alpha subunit in glucose disposal at the level of GLUT4 vesicles has also been elucidated. Transgenic mice harboring an adipose/muscle tissue-specific expression of a constitutively activated GTPase-deficient mutant form of Gi2 alpha showed increased glucose tolerance and their adipocytes displayed enhanced plasma-membrane-accumulated GLUT4 or glucose transport in both the absence and presence of insulin (211, 212).

5.1. Endothelin-1 signaling pathway and role for PI kinases

Among the list of G-protein Gq/G11 a□phα subunit agonists, the signaling pathway to GLUT4 vesicle translocation induced by endothelin-1, a 21 amino acid-polypeptide hormone produced mainly in cardiac myocytes and vascular endothelial cells (213), is understood in somewhat more detail. Endothelin-1 receptors couple to Gq/G11, which are abundant in 3T3-L1 adipocytes (153-155). Endothelin-1 downstream signaling transmission is not completely clear but most of the studies show it to be PI 3-kinase-independent (153-156). It involves tyrosine phosphorylation mediated in part by a proline-rich tyrosine kinase PYK2 and a beta-arrestin 1-dependent recruitment of a Src family kinase (214, 215). Remarkably, similarly to insulin, endothelin-1 appears to mobilize cortical F-actin in cultured adipocytes, but unlike the insulin effect, this seems to be largely mediated by ARF6 (155, 156). Thus, the available experimental evidence is consistent with a model whereby the initial steps of regulation of GLUT4 vesicle translocation by insulin and endothelin-1 differ. However, the two patways converge at the step that mobilizes cortical actin filaments.

A well established signaling pathway for the Gq/G11 alfa subunit is activation of phospholipase Cbeta, hydrolysis of PtdIns 4,5-P_2, and a subsequent activation of protein kinase C (216). However, inhibition of phospholipase C and protein kinase C failed to block endothelin-1 stimulated glucose transport (153), further reinforcing the role of ARF6 as an intermediate, required downstream in endothelin-1 signaling to regulate cortical F-actin and GLUT4 vesicle translocation (155, 156). While a downstream target of activated ARF6 in response to endothelin-1 remains unknown, studies in other trafficking systems have identified ARF6 as a direct activator of PI 5-Kalpha in EGF signaling to regulate actin polymerization and membrane ruffling in HeLa cells (89). Elevated plasma membrane PtdIns 4,5-P_2 production in response to EGF reportedly also requires phosphatidic acid, thus supporting the role for both ARF6 and phospholipase D in activating PI 5-Kalpha (89). A more recent study (217) also implicates the ARF6-dependent regulation on PI 5-Kalpha activity and PtdIns 4,5-P_2 turnover in the control of trafficking through the plasma membrane and nonclathrin derived endosomal compartment. Together, these results suggest a potential role for PI 5-Ks as a downstream effector of activated ARF6 in endothelin-1 signaling to GLUT4 vesicle dynamics, a hypothesis that needs to be tested in future studies.

While endothelin-1 may use the ARF6-dependent pathway for F-actin assembly related to GLUT4 vesicle translocation and increased glucose uptake, the insulin signaling modulates these processes independently of ARF6 (132, 155, 156, 218). As discussed above (see Section 4.5.) acute insulin also increases cortical actin polymerization in the context of GLUT4 vesicle translocation but this likely involves TC10. Together, these data point to at least two separate PI 3-kinase-independent pathways controlling actin assembly relevant to GLUT4 vesicle translocation: one, initiated by Gq/G11 a□pha subunit activation and mediated by ARF6 GTPase, and another, initiated by IR activation and mediated by TC10 GTPase. Although no experimental evidence is currently available, it is likely that both pathways converge at the level of PI 5-K activation and localized PtdIns 4,5-P_2 production, a hypothesis that certainly deserves consideration and appropriate experimentation.

6. GLUT4 VESICLE TRANSLOCATION IN RESPONE TO HYPEROSMOTIC SHOCK

6.1. Signaling pathway and role for PI kinases

Early studies in 1960 and 1970 demonstrated that stress stimuli such as hyperosmolarity had potent insulin-like effects on glucose metabolism, including activation of glucose transport in adipocytes and skeletal muscle (219-221). More recently, it has been shown that in 3T3-L1 adipocytes the hyperosmotic-dependent increase of glucose transport is due to GLUT4 vesicle translocation to the cell surface, comprising 50% of the effect elicited at maximally activating insulin concentrations (152, 222). A similar subcellular redistribution of GLUT4 vesicles in response to hyperosmotic shock has been established in L6 myoblasts stably expressing GLUT4 (27). The proximal signaling pathway involved in the GLUT4 vesicles' response to hyperosmolarity is not very clear but seems to involve tyrosine phosphorylation of Gab-1 (Grb2-Associated Binder-1) protein, a member of the IRS protein family (223, 224). Other tyrosine phosphorylated proteins have also been implicated but the activation of IR tyrosine kinase seems not to be the initial triggering mechanism (152, 224, 225). Rather, the dramatic tyrosine phosphorylation of Gab-1 in response to hyperosmotic shock has been attributed to Src kinase family members (224). The importance of Gab-1 in the hyperosmotic shock-induced signaling to GLUT4 vesicle translocation is underscored by the findings that microinjected anti-Gab-1 antibodies inhibit this process (224).

In contrast to insulin, but like endothelin-1, hyperosmolatity-induced stimulation of GLUT4 vesicle translocation and glucose transport in both adipocytes and L6 myoblasts is reportedly insensitive (27, 152, 222) or only partially sensitive (224) to inhibition of PI 3-K activity by wortmannin. Consistent with the lack of PI 3-kinase activation, hyperosmotic shock was found to be without any effect on the phosphorylation and activation of the PI 3-K downstream effector Akt (152, 226, 227). In one study, however, evidence is presented that class IA PI 3-K activity associates with Gab-1 upon hyperosmotic shock (224) but the significance of this interaction for signaling to GLUT4 is still unclear at present.

Experimental evidence has been recently presented to implicate the activation of TC10 pathway and F-actin rearrangement in osmotic shock-activated GLUT4 vesicle translocation (228). Pharmacological disruptions of cortical actin integrity resulted in inhibition of hyperosmotic shock-stimulated glucose transport, further supporting the role of actin rearrangement in this process (228). Importantly, osmotic shock-dependent activation of actin rearrangement and increased amounts of cortical actin appear to be largely wortmannin resistant (224, 228). Thus, as in the case with insulin and endothelin-1, regulation of cortical actin remodeling that proceeds in a PI 3-K-independent mode appears necessary for GLUT4 vesicle translocation in response to osmotic shock. Noteworthy, a recent study utilizing metabolic labeling of inositol phospholipids in 3T3-L1 fibroblasts documents a substantial increase of total PtdIns 4,5-P_2 levels subsequent to 15 min cell treatment with sorbitol (101). The nature of activated PtdIns 4,5-P_2-producing enzymes under these conditions remains to be identified as well as whether this effect takes place in 3T3-L1 adipocytes. Demonstrating plausible effects of insulin, hyperosmotic shock and endothelin-1 to activate the PtdIns 4,5-P_2 producing enzymes in insulin-sensitive cells will be important, as will further insights into the role of TC10 or Arf6 for this activation in the context of GLUT4 vesicle translocation.

7. CONCLUSIONS AND PERSPECTIVES

The cellular biology underlying the activation of GLUT4 vesicle dynamics and glucose transport is extremely complex, indicating that we still have a long way to go before we have the complete picture. Since the initial

discovery that inhibition of PI 3-kinase activity arrests GLUT4 vesicle translocation and glucose transport in response to insulin, our understanding for the PIs' role and their metabolism expanded both in importance and complexity. It now seems clear that 3-phosphoinositides and the enzymes involved in their turnover play a central role in the action of insulin to move GLUT4 vesicles to the adipose/muscle cell surface. Other PI kinases have also proven to be essential for these processes. While we know now that the activity of several proteins relevant to GLUT4 vesicle translocation can be modified by the products of PI 3-kinase, we will certainly witness in the near future the identification of others, whose activity is altered by products of other PI kinases. This may not only influence the proximal signaling circuit but also more distal events of GLUT4 vesicle budding, fission and motility. Of particular interest is the localized PtdIns 4,5-P_2 production at specific sites which may serve as membrane platforms for regulation of targeted activities related to PI 3-kinase-independent signaling pathways. These events are likely to underlie the mechanism of F-actin remodeling seen on the way of GLUT4 vesicle relocation to the cell surface induced not only by insulin but also by a number of other agonists. The development of increasingly improved tools to monitor PI dynamics, combined with the ability to observe live cells with sophisticated optics in a temporal and spatial manner will certainly help to further illuminate the road of GLUT4 vesicle delivery to the plasma membrane.

8. AKNOWLEDGEMENT

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Send correspondence to: Dr. Assia Shisheva, Department of Physiology, Wayne State University School of Medicine, 5374 Scott Hall, 540 E. Canfield Ave., Detroit, MI 48201-1928, Tel: 313-577-5674, Fax: 313-577-5494, E-mail: ashishev@med.wayne.edu