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Review

Insulin Signaling in Microdomains of the Plasma Membrane

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Although the effects of insulin on glucose and lipid metabolism are well documented, gaps remain in our understanding of the precise molecular mechanisms of signal transduction. Recent evidence suggests that compartmentalization of signaling molecules and metabolic enzymes may explain the unique cellular effects of the hormone. Signal initiation from the insulin receptor is restricted in part to caveolae microdomains of the plasma membrane. A fraction of the insulin receptor directly interacts with caveolin, thus directing the protein to caveolae. Following its activation by insulin, the receptor recruits a series of adapter proteins, resulting in the activation of the G protein TC10, which also resides in caveolae. TC10 can influence a number of cellular processes, including changes in the actin cytoskeleton, recruitment of effector including the adapter protein CIP4, and assembly of the exocyst complex. These events play crucial roles in the trafficking, docking and fusion of vesicles containing the insulin-responsive glucose transporter Glut4 at the plasma membrane.

Key words: adaptor, caveolae, G proteins, glucose transport, phosphorylation

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Insulin promotes the synthesis and storage of carbohydrates, lipids and proteins, and inhibits their degradation and release back into the circulation. Insulin action is characterized by a diverse variety of effects at the cellular level, including changes in vesicle trafficking, stimulation of protein kinases and phosphatases, promotion of cellular growth and differentiation, and activation or in some cases, repression of transcription. This complexity suggests that insulin action must involve multiple signaling pathways that diverge at or near the activation of its tyrosine kinase receptor. In fact, it is likely that even individual effects of the hormone require multiple signaling inputs. Evidence is emerging that the coordination of these pathways might be governed by their intracellular compartmentalization. We will consider how spatial aspects of signal transduction play a critical role in determining the specificity of insulin action, with particular attention to the role of caveolae and lipid rafts.

Divergent Signaling Pathways Are Initiated by Insulin Receptor Substrates

The insulin receptor is a tyrosine kinase that catalyzes the phosphorylation of several intracellular substrates, including the insulin receptor substrate (IRS) proteins (1): GAB-1 (2), Shc (3), APS (4), $p60^{DOK}$ (5), and c-Cbl (6). Each of these substrates recruits a distinct subset of signaling proteins containing Src homology 2 (SH2) domains, which specifically interact with sequences surrounding the phosphotyrosine. Moreover, each of these substrates may be compartmentalized to distinct locations in the cell, due to sequences that direct interactions with proteins or lipids.

Most attention in the field of insulin receptor substrates has focused on the IRS family of proteins. Mice lacking the IRS-1 protein are insulin resistant, but do not develop diabetes unless crossed to mice in which another signaling molecule has been disrupted (7–9). Animals lacking IRS-2 exhibit both impaired glucose tolerance and diabetes (10), which appears to result from a defect in insulin secretion as well as insulin resistance, presumably due to decreased beta cell proliferation in the face of increased demand for insulin. The tyrosine phosphorylation of IRS family members generates docking sites for a number of SH2-containing proteins (1). Among these, the predominant partner seems to be the p85 regulatory subunit of the type 1 A phosphatidylinositol (PI) 3-kinase. Inhibition of the p110 PI 3-kinase catalytic activity with pharmacological inhibitors such as wortmannin, expression of dominant-interfering mutants, or microinjection of blocking antibodies can completely prevent most actions of insulin (11,12). Consistent with the necessary requirement for the product of PI 3-kinase (PI-3,4,5P_3), overexpression of the PI 3′ phosphatase pTEN or the PI 5′ phosphatase SHIP also block the action of insulin.

Although these data demonstrate the necessity of PI 3-kinase, several lines of evidence indicate that activation of the enzyme is not sufficient. For example, dramatic overexpression of constitutively active mutants of PI 3-kinase can only partially stimulate the translocation of the insulin-responsive glucose transporter protein Glut4 (13). Other growth factors, such as PDGF and IL-4, or

certain adhesion molecules, stimulate PI 3-kinase to an extent similar to that seen with insulin, but have only a minor effect on glucose uptake and Glut4 translocation (14–16). In addition, cell-permeable derivatives of PIP_3 alone cannot mimic the stimulation of glucose uptake by insulin, but can stimulate when cells are pretreated with insulin and a pharmacological inhibitor of PI 3-kinase (17). Furthermore, several studies have demonstrated that exercise, contraction and hypoxia can all induce glucose uptake and Glut4 translocation in skeletal muscle independent of any detectable PI 3-kinase activation (18). Taken together, these data suggest that insulin must generate at least two independent signals to stimulate glucose transport: one dependent on and another independent of PI 3-kinase. Moreover, the activation of PI 3-kinase by non-insulinomimetic hormones suggests that the second pathway must be unique for insulin.

As indicated above, PI 3-kinase catalyzes the phosphorylation of phosphatidylinositol-4,5P_2 (PIP_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 serves as an allosteric regulator of the phosphoinositide-dependent kinase (PDK1) (19), that phosphorylates and activates both Akt (also known as PKB), as well as the atypical protein kinase C isoforms PKCζ and PKCλ (19,20). PIP_3 appears to mediate the translocation of Akt to the plasma membrane, via its pleckstrin homology (PH) domain (21). PKCζ?Λ and PDK1 translocate to the plasma membrane by this same mechanism. Although there are data to support an essential role for these kinases in glucose transport, the precise identity of the physiologically relevant kinase and the subsequent downstream targets remains uncertain (22,23). Also uncertain is whether these protein kinases migrate to new sites upon activation, or whether there is local activation that impacts on Glut4 translocation.

Glucose Transport Is Regulated by Changes in the Location of Glut4

As mentioned above, insulin-stimulated glucose uptake is primarily mediated by the facilitative transporter Glut4, one member of a large family of related transporters, that is highly expressed in adipose tissue, skeletal and cardiac muscle (24). In the basal state, Glut4 slowly recycles between the plasma membrane and vesicular compartments within the cell, where most of the Glut4 resides. Insulin stimulates the translocation of a pool of Glut4 to the plasma membrane, through a process of targeted exocytosis. At the same time, Glut4 endocytosis is attenuated, resulting in a dramatic increase in glucose uptake (25). Thus, the rate of glucose transport into fat and muscle cells is governed by the concentration of Glut4 at the cell surface, and the duration for which the protein is maintained at this site.

While there is substantial evidence that Glut4 exists in specialized vesicles sequestered within the cell, the precise intracellular location and trafficking pathways of these vesicles are unclear. Glut4 is localized in tubulovesicular and vesicular structures that are biochemically distinct from the recycling endosomal network. Furthermore, the Glut4 compartment is enriched in the v-SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) protein VAMP2 but not the related VAMP3/cellubrevin isoform that is present in recycling endosomes (25). Consistent with these data, ablation of transferrin receptor containing endosomes does not impair insulin-stimulated Glut4 translocation.

Substantial evidence suggests that the plasma membrane target for the GLUT4 vesicle is the t-SNARE, syntaxin 4 (Syn4) (26–29). Thus, the docking of the vesicle may be mediated by the interaction of Syn4 with the Glut4 vesicle v-SNARE protein, VAMP2. Several proteins have been demonstrated to bind to Syn4, including SNAP23 (30), Munc18c (31) and Synip (32), and thereby regulate the docking and fusion of VAMP2-containing GLUT4 vesicles. Among these, Synip has been shown to undergo an insulin-dependent dissociation from Syn4, allowing VAMP2 to bind to the protein (32). Moreover, recent evidence has suggested that Syn4 might reside in lipid raft subdomains of the plasma membrane (33,34), and further that Glut4 may transit through lipid rafts (35,36).

PI 3-Kinase-Independent Pathways in the Regulation of Glucose Transport: The Role of Caveolae

A potential clue towards identifying the PI 3-kinase-independent arm of insulin action emerged from the idea that signal initiation might be segregated into discrete compartments in the plasma membrane. One candidate for such a compartment is the caveolae, small invaginations of the plasma membrane that are a subset of lipid raft domains. These localized regions are enriched in lipid-modified signaling proteins, GPI-anchored proteins, glycolipids, sphingolipids and cholesterol (37). Insulin stimulates the tyrosine phosphorylation of caveolin, the major structural protein in caveolae (38–40). Further investigation into this pathway revealed that at least a fraction of the insulin receptor was segregated into these plasma membrane microdomains (41). Although the mechanism by which this sorting of the receptor occurs remains unknown, recent studies reveal that the insulin receptor associates with caveolin (40). Moreover, disruption of the Caveolin 1 gene results in the degradation of the insulin receptor in fat cells, and the subsequent disruption of insulin action in these cells, along with insulin resistance (42).

The identification of the insulin receptor in caveolae allows for the segregation of a signaling pathway separate from the activation of PI 3-kinase and downstream events. Insulin stimulates the phosphorylation of another insulin receptor substrate, the protooncogene c-Cbl (6). The

insulin-stimulated phosphorylation of Cbl occurs only in metabolically responsive cell lines, and not in other fibroblast lines, despite the presence of Cbl and an active receptor (6). This phosphorylation event requires the adapter protein APS, which recruits Cbl to the insulin receptor (4). APS has a PH domain that is required for membrane localization, and an SH2 domain that interacts with the phosphorylated receptor. After recruitment to the activated receptor, the protein subsequently undergoes tyrosine phosphorylation at a specific residue in the C-terminus of the protein. Upon phosphorylation, APS recruits Cbl by interacting with an atypical SH2 domain of the latter protein. Interestingly, both APS and Cbl are recruited to the receptor as dimers (Liu et al., submitted).

The Cbl-associated protein (CAP) was identified in a two-hybrid screen using Cbl as bait (43). CAP contains three carboxyl terminal SH3 domains, the last of which mediates its binding to Cbl. CAP is expressed predominantly in insulin-sensitive tissues and in differentiated 3T3-L1 adipocytes, but not in preadipocytes. Expression of the CAP gene is increased by the insulin-sensitizing thiazolidinediones (TZDs) (44). TZD activation of the nuclear receptor PPARγ directly activates the transcription of CAP through a PPARγ response element in its promoter (45). Moreover, TZD-stimulated increases in CAP expression lead to a more robust phosphorylation of Cbl in response to insulin (44), establishing a potential primary link between TZD-induced insulin sensitization and insulin signal transduction.

The localization of CAP in lipid rafts appears to result from its association with the hydrophobic protein flotillin. This interaction was localized to amino terminal sequences of CAP that contain homology to the gut peptide Sorbin, which we referred to as the Sorbin Homology (SoHo) domain (46). Dominant interfering SH3 deletion mutants of CAP that bind to flotillin, but not Cbl, or SoHo deletion mutants that bind to Cbl, but not flotillin, interfere with the localization of Cbl to lipid rafts. Moreover, these mutants specifically block insulin-stimulated Glut4 translocation and glucose uptake (46,47). The overall architecture found in CAP, with an amino terminal SoHo and carboxyl-terminal SH3 domains is found in a number of other proteins, including vinexin and ArgBP2 (46). It is likely that these proteins represent a new family that is responsible for the localization of signaling or cytoskeletal proteins to lipid rafts (46).

The G Protein TC10 Is Crucial to the Stimulation of Glucose Transport by Insulin

Once phosphorylated, Cbl can recruit the SH2-containing adapter protein CrkII to caveolae, along with the guanine nucleotide exchange factor C3G (48). C3G then appears to activate the two Rho family proteins TC10α and β (49). These small GTP binding proteins are expressed in fat and muscle, and can be acutely activated by insulin in a CAP-dependent but PI 3-kinase-independent manner. Activation of TC10 is specific for insulin, and disruption of its activation blocks insulin-stimulated glucose transport and Glut4 translocation (48). Although TC10 activation is required for insulin-stimulated glucose uptake, it is not sufficient. Constitutive activation of the protein by overexpression of C3G in 3T3L1 adipocytes does not mimic insulin action, but does potentiate the actions of the hormone, shifting the insulin dose-response curve (48). Moreover, coexpression of C3G with an active mutant of PI 3-kinase fully restores insulin action, suggesting that these two pathways provide synergistic signals in the regulation of glucose transport (Figure 1).

One clue to the specificity of TC10 activation lies in its unique carboxyl terminal-targeting motif. Unlike other members of the Rho family that undergo geranylgeranylation and interact with guanyl nucleotide dissociation inhibitors, TC10 is likely to undergo both farnesylation and palmitoylation in a manner analogous to that of H-Ras. These post-translational modifications are responsible for the targeting of TC10 to lipid raft domains (50). Moreover, mistargeting of TC10 into nonlipid raft regions of the plasma membrane prevents its activation by insulin and alters the ability of TC10 to modulate insulin-stimulated Glut4 translocation. In addition, disruption of lipid rafts with cholesterol-extracting drugs or by overexpression of inhibitory forms of caveolin completely blocks TC10 activation, as well as the stimulation of glucose transport by insulin (50).

While the precise effectors responsible for the actions of TC10 remain unknown, a potential clue towards understanding its function has emerged from prior studies on the established role of Rho proteins in the regulation of the actin cytoskeleton. In fibroblasts, Rho proteins control actin stress fibers, lamelipodia and filopodia (51). Multiple studies have also demonstrated a functional requirement for filamentous (F)-actin in insulin-stimulated Glut4 translocation and glucose uptake. For example, severing of F-actin with cytochalasin D or depolymerization by the monomeric actin-sequestering agent Latrunculin effectively inhibit insulin-stimulated Glut4 translocation (52–54). Similarly, inhibition of Rho function with *difficile* toxin B or expression of the amino terminal domain of TC10 disrupts the adipocyte actin cytoskeleton and inhibits Glut4 translocation (Hou and Pessin, in press). Although it remains to be determined whether TC10 mediates the regulation of cortical actin polymerization in adipocytes by insulin, this small GTP binding protein can directly interact with numerous effector molecules that are known to modulate cytoskeletal function in other cells (55).

Identification of TC10 Effectors in Insulin Action

While the precise role of TC10 in insulin's actions requires further investigation, a number of candidate molecules have emerged as effectors for the protein. One of these is a splice variant of the adapter protein, CIP4. This protein was first identified as an effector of Cdc42. The

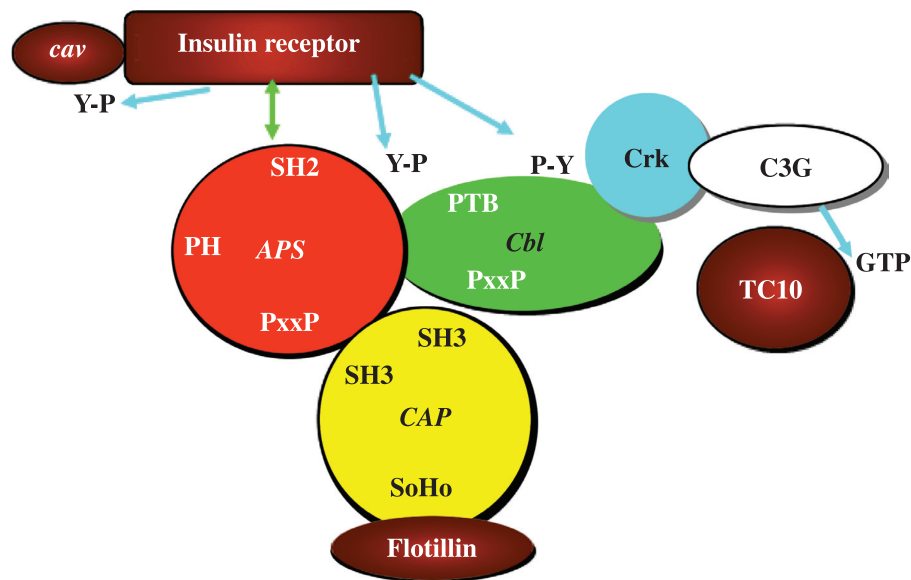

Figure 1: An insulin signaling pathway in caveolae. Two signaling pathways are required for the translocation of the glucose transporter GLUT4 by insulin in fat and muscle cells. A separate pool of the receptor interacts with caveolin, and catalyzes the tyrosine phosphorylation of this protein. Upon activation, the receptor recruits the adaptor protein APS, which undergoes phosphorylation on a single tyrosine. This results in the recruitment of the protooncogene Cbl, which subsequently undergoes phosphorylation by the receptor. Cbl constitutively interacts with CAP, which can bind to the lipid raft protein flotillin to stabilize the recruitment of this complex in caveolae. The recruitment of phosphorylated Cbl into the lipid raft results in the recruitment of the SH2/SH3 adapter protein CrkII, via an interaction of the SH2 domain of CrkII with phospho-Cbl. CrkII constitutively binds to the nucleotide exchange factor C3G, which can catalyze the exchange of GTP for GDP on the lipid raft-associated protein TC10. Upon activation, TC10 initiates a separate signaling pathway that, along with the PIP_3-dependent protein kinases, can stimulate the trafficking of the GLUT4 vesicle, along with its docking and fusion with plasma membrane.

multidomain structure of CIP4 isoforms suggests that this family of proteins may serve an adapter function. CIP4 contains an FCH domain, two coiled-coil domains, and an SH3 domain. The FCH domain interacts with microtubules, and the second coiled-coil domain interacts with TC10 in a GTP-dependent manner. CIP4 localizes to an intracellular compartment under basal conditions, and translocates to the plasma membrane upon insulin stimulation (56). Overexpression of constitutively active TC10 brings CIP4/2 to the plasma membrane, whereas overexpression of an inhibitory form of TC10 blocks the translocation of CIP4/2 produced by insulin. Overexpression of mutant forms of CIP4/2 containing an N-terminal deletion or with diminished TC10 binding inhibits insulin-stimulated Glut4 translocation, suggesting that CIP4/2 may play an important role in insulin-stimulated glucose transport as a downstream effector of TC10.

TC10 also interacts with one of the components of the exocyst complex, Exo70, in a GTP-dependent fashion (57). The exocyst complex has been implicated in the tethering or docking of secretory vesicles. Exo70 translocates to the plasma membrane in response to insulin via the activation of TC10, where it assembles a multiprotein complex that includes Sec6 and Sec8, as well as additional proteins that have not yet been identified. Overexpression of an Exo70 mutant blocks insulin-stimulated glucose uptake, but not the trafficking of Glut4 to the plasma membrane. However, this mutant did block the extracellular exposure of the Glut4 protein. These data suggest that the exocyst may play a critical role in the targeting of the Glut4 vesicle to the plasma membrane, perhaps directing the vesicle to the precise site of fusion.

Temporal and Spatial Determinants of Specificity in Insulin Action

Although the molecular targets of the PI 3-kinase and CAP/Cbl pathways are uncertain, one possibility is that they regulate different processes (Figure 1). For example, PIP_3-dependent kinases may phosphorylate proteins involved in the tethering of the Glut4 vesicle at intracellular sites, leading to the release and default trafficking of the vesicle to the plasma membrane. On the other hand, the CAP/TC10 pathway may regulate processes involved in the trafficking, docking and fusion of the vesicle at the plasma membrane.

Even in insulin-responsive cells and tissues, there are numerous other tyrosine kinases and G protein-coupled receptors that can lead to the activation of lipid and protein kinase pathways similar to those activated by insulin, without producing the unique metabolic effects of the

hormone. One potential clue towards solving this dilemma of specificity might involve temporal and spatial restrictions on signaling. For example, the rapid termination of insulin signaling by phosphatases or retrophosphorylation pathways could play a key role in delineating the downstream effects of the hormone, perhaps preventing the propagation of signals such as those produced by the persistent activation of the MAP kinase pathway that lead to oncogenesis or inflammation. The spatial compartmentalization of signaling might also play an important role in insuring specificity. For example, the spatial segregation of Cbl and downstream molecules allows for the activation of TC10 in a restricted domain of the plasma membrane, the lipid raft. In this way, TC10 activation is specific for insulin, and targets effectors in a well-defined region of the cell. While we may have a handle on many of the critical pathways that cells use to respond to hormones, it is likely that teasing out their relationship in space and time will allow us to understand how they produce their unique physiological effects.

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