Regulated Membrane Trafficking of the Insulin-Responsive Glucose Transporter 4 in Adipocytes

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Since the discovery of insulin roughly 80 yr ago, much has been learned about how target cells receive, interpret, and respond to this peptide hormone. For example, we now know that insulin activates the tyrosine kinase activity of its cell surface receptor, thereby triggering intracellular signaling cascades that regulate many cellular processes. With respect to glucose homeostasis, these include the function of insulin to suppress hepatic glucose production and to increase glucose uptake in muscle and adipose tissues, the latter resulting from the translocation of the glucose transporter 4 (GLUT4) to the cell surface membrane. Although simple in broad outline, elucidating the molecular intricacies of these receptor-signaling pathways and membrane-trafficking processes continues to challenge the creative ingenuity of scientists, and many questions remain unresolved, or even perhaps unasked. The identification and functional characterization of specific molecules required for both insulin signaling and GLUT4 vesicle trafficking remain key issues in our pursuit of developing specific therapeutic agents to treat and/or prevent this debilitating disease process. To this end, the combined efforts of numerous research groups employing a range of experimental approaches has led to a clearer molecular picture of how insulin regulates the membrane trafficking of GLUT4. (Endocrine Reviews 25: 177–204, 2004)

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I. Introduction

GENERATED BY THE carbon fixation or dark reactions of photosynthesis, glucose is extremely common in nature. Indeed, one of the polymeric forms of glucose, cellulose, is likely the most abundant organic molecule in the biosphere. Therefore, it is not surprising that glucose is the primary energy source used in the animal kingdom, and multiple mechanisms have evolved for its metabolism, synthesis, and storage. Because hydrophilic carbohydrate molecules are generally excluded from the hydrophobic core of cellular membranes, numerous transport mechanisms have evolved to move glucose and other sugars across lipid bilayers (1, 2). For example, bacteria employ several symport, antiport, and uniport systems for the uptake of glucose and other carbohydrates. In contrast, mammals use Na⁺-dependent cotransporters and facilitative uniporters. Moreover, many bacteria can survive with glucose as their only carbon source, and several mammalian tissues, notably the brain, are dependent on glucose as their primary energy source. In addition, to accommodate the glucose requirements of the
brain and other tissues, finely tuned regulatory systems have evolved in mammals to maintain remarkably stable plasma glucose concentrations despite intermittent food intake. Thus, during fasting periods the liver provides sufficient glucose output to maintain normal circulating glucose levels. In contrast, the increases in plasma glucose levels that occur immediately after a meal are minimized by enhanced glucose uptake in skeletal muscle and adipose tissue. This complex interplay between glucose absorption from the gut, output from the liver, and uptake by peripheral tissues is normally exquisitely balanced such that plasma glucose concentrations are maintained at 4–7 mM in humans. Deviations from this narrow range can have severe consequences. For example, low blood glucose levels can lead to seizures, coma, and death. In contrast, prolonged elevated glucose levels, as occurs in the diabetic state, can result in many complications, including renal failure, blindness, neuropathy, and cardiovascular disease.

There are several types of diabetes, with type 1 or insulin-dependent diabetes mellitus and type 2 or non-insulin-dependent diabetes mellitus accounting for the vast majority of pathological states of dysregulated glucose metabolism. Type 1 diabetes mellitus results from the autoimmune destruction of pancreatic β-cells of the islets of Langerhans, resulting in absolute insulin deficiency. In contrast, the hallmark of type 2 diabetes mellitus is peripheral insulin resistance, which refers to the inability of insulin to efficiently stimulate glucose uptake into muscle and adipose tissue and to suppress hepatic glucose production. The progression to overt diabetes in these individuals usually results when enhanced insulin secretion from pancreatic β-cells fails to compensate for peripheral insulin resistance. In addition to environmental risk factors, including diet, age, and exercise habits, type 2 diabetes is a complex polygenic disease. Type 2 diabetes accounts for more than 90% of all cases and is becoming increasingly common worldwide, both in developed and developing countries. Basic research into the molecular mechanisms of insulin signaling and glucose uptake thus represents an important research area for the global human community. Since the discovery of insulin in the early 1920s, much has been learned about how this peptide hormone triggers the tyrosine kinase activity of its cell-surface receptor and initiates intracellular signaling cascades. We now know that insulin induces the translocation of the glucose transporter 4 (GLUT4) from intracellular membrane compartments to the plasma membrane, where it catalyzes the uptake of glucose into adipose and muscle cells, the rate-limiting step for glucose metabolism. Research on the mechanisms of insulin-induced GLUT4 translocation has thus progressed toward a convergence of receptor tyrosine kinase-signaling processes and regulated membrane-trafficking events.

II. The Hexose Transporter Family

A. Family members, structure, and function

In mammals, glucose is cleared from the bloodstream by facilitative transporters (GLUTs), which comprise a family of highly related 12 transmembrane domain-containing proteins (Fig. 1) (3). As facilitative carriers, the GLUT proteins transport glucose down its concentration gradient in an energy-independent manner. Most mammalian cell types are net consumers of glucose and maintain low intracellular glucose concentrations, thus favoring glucose entry. Hepatocytes, however, are net producers of glucose during the periods of reduced food intake (low circulating insulin but high glucagon levels). During fasting periods, hepatic gluconeogenesis and glycogenolysis increase the concentration of intracellular glucose above that in the blood. This results in a net efflux of glucose from the liver and provides the brain and other tissues with a steady supply of glucose despite sporadic food consumption.

An extended family of hexose transporters has evolved to meet the specific metabolic needs of various tissues. The GLUT family contains 13 known members and can be divided into three classes based on conserved structural characteristics (4). Class I includes GLUTs 1–4, which are, by far, the best characterized transporters of the family. Class II is comprised of GLUT5 (a fructose-specific transporter), and GLUTs 7, 9, and 11, which remain poorly understood (3).
Class III includes GLUTs 8, 10, 12, and the proton-myoinositol symporter H⁺-myo-inositol cotransporter (HMIT1), all of which are largely uncharacterized (3). Because more is known about the class I GLUTs, we will briefly summarize the properties of GLUTs 1–4.

GLUT1 is expressed to some degree in most tissues; however, it is highly expressed in endothelial cells lining the blood vessels of the brain and in human erythrocytes (5, 6). Indeed, GLUT1 represents approximately 5% of the total erythrocyte membrane protein content, which facilitated its biochemical purification by Kasahara and Hinkle (7) in 1977. GLUT1 is also expressed in adipose and muscle tissue, which are the insulin-responsive sites for glucose disposal. Here, GLUT1 appears to provide a low, constitutive level of glucose transport required for basal cellular processes, probably in combination with several other GLUT isoforms (5). GLUT2 is a low-affinity [high Michaelis-Menten constant (Kₘ)] GLUT present in liver, intestine, kidney, and pancreatic β-cells (8). This transporter functions as part of the glucose sensor system in β-cells and in the absorption of glucose by intestinal epithelial cells. GLUT3 is expressed primarily in neurons, and, together, GLUT1 and GLUT3 allow glucose to cross the blood-brain barrier and enter neurons (6). GLUT4 is expressed primarily in striated muscle and adipose tissue and, unlike most other GLUT isoforms, is sequestered in specialized intracellular membrane compartments under basal conditions (9). As the major insulin-responsive GLUT isoform, the study of the structure, function, and regulation of GLUT4 has been a major focus of workers in the diabetes field.

**B. GLUT4**

1. **Translocation hypothesis.** In 1980, nearly a decade before the molecular identification and cloning of GLUT4, compelling evidence was presented from two independent groups that strongly supported the hypothesis that insulin causes the redistribution of what was then described as “glucose transport activity” from intracellular membrane compartments to the cell surface (10, 11). By measuring glucose binding and transport activity in membrane fractions isolated from rat adipocytes, both groups found that insulin decreased the number and function of GLUTs in the internal membrane fractions while at the same time causing a concomitant increase in transporter function in the plasma membrane fractions. Although these data were consistent with the translocation of a GLUT to the cell surface, without specific antibodies or a cloned transporter cDNA there was no unequivocal, independent means to verify these results. These early experiments, nevertheless, proved sufficient incentive, and in 1989 five independent groups reported the cloning of the cDNA encoding GLUT4 (12–16). This enabled the generation of isoform-specific antibodies and confirmed that the glucose transport activity identified in the 1980 experiments corresponded to the translocation of GLUT4 to the cell surface in response to insulin. Indeed, since its initial proposal, more than two decades of accumulated evidence has consistently supported the translocation hypothesis. However, in addition to stimulating translocation, recent work has suggested that insulin may also regulate the intrinsic transport activity of GLUT4 (reviewed in Ref. 17). Nevertheless, the redistribution of GLUT4 to the plasma membrane in response to insulin remains the dominant paradigm and can be readily visualized using a GLUT4-enhanced green fluorescent protein reporter construct transiently expressed in 3T3L1 adipocytes (Fig. 2). The molecular mechanism by which insulin causes this redistribution of GLUT4 remains unknown and is currently under intense investigation. Indeed, this research area holds promise for the identification of therapeutic agents that target specific molecules functioning proximal to the intracellular GLUT4 storage compartment, thereby bypassing the multifarious upstream insulin receptor (IR)-signaling cascades.

2. **GLUT4 knockout mouse models.** GLUT4 is the only known insulin-responsive GLUT highly and specifically expressed in muscle and adipose tissue, the major sites of postprandial glucose disposal. In addition, overexpression of the human GLUT4 gene in muscle and fat tissue of the diabetic db/db mouse, which lacks the leptin receptor, protected these animals from insulin resistance and diabetes (18). However, the genetic ablation of GLUT4 did not result in severe insulin resistance or diabetes (Table 1). Indeed, although GLUT4-null mice had decreased life spans, were growth retarded, and showed cardiac and adipose tissue abnormalities, they exhibited only mild defects in glucose homeostasis and were not diabetic (19). These results suggest that GLUT4 is not absolutely required for normal glycemic control in rodent model systems. However, it was subsequently observed that male mice heterozygous for GLUT4 (GLUT4+/−) showed progressive muscle insulin resistance accompanied by hyperinsulinemia and hyperglycemia, and by 8–12 months exhibited diabetic histopathologies of the heart and liver (20, 21). It remains unclear why the heterozygous GLUT4+/− mice display a more severe phenotype than the homozygous GLUT4+/+ mice. However, the GLUT4−/− animals are presumably protected from diabetes by the compensatory up-regulation of other GLUT isoforms (22), although this supposition has not yet been demonstrated experimentally.

Rather than globally eliminating or reducing GLUT4 in all tissues, another strategy for studying GLUT4 function in intact animals is the selective disruption of GLUT4 in specific tissues using the Cre-lox system. For example, genetic ablation of the GLUT4 gene specifically in muscle tissue resulted in insulin resistance and glucose intolerance in mice as young as 8 wk of age (23). Moreover, disruption of GLUT4 specifically in adipose tissue resulted in impaired glucose uptake and hyperinsulinemia (24). Not surprisingly, adipose tissue showed insulin resistance and impaired glucose uptake. However, liver and muscle also developed insulin resistance, despite the fact that GLUT4 was ablated only in fat tissue. In addition, muscle tissue showed normal levels of GLUT4 expression, although insulin-induced glucose uptake was markedly reduced in this tissue. These results suggest that the GLUT4-deficient adipose tissue secretes an unknown factor(s) that travels to liver and muscle via the bloodstream. Thus, it appears that the perturbation of GLUT4 expression levels in specific tissues could be a potential contributing factor for peripheral insulin resistance.
III. Intracellular GLUT4 Storage Compartments

A. The specialized GLUT4 storage compartment

Under basal conditions the majority of GLUT4 is excluded from the plasma membrane of adipose and muscle cells due to efficient endocytosis and intracellular sequestration within insulin-responsive storage compartments. Insulin increases the exocytosis of GLUT4 (9) and slightly inhibits endocytosis (25–28), resulting in an approximately 10- to 20-fold increase in transporter levels at the plasma membrane. Considerable attention has focused on identifying and characterizing the insulin-regulated GLUT4 storage compartment (29). In addition, several groups (30–34) have attempted to enhance our understanding of the compartmentalization and regulated trafficking of GLUT4 through comparisons with the insulin-stimulated secretion of other adipocyte proteins. For example, adipocytes secrete several small soluble proteins in response to insulin, including adipin, leptin, lipoprotein lipase (LPL), and adipocyte complement related protein of 30 kDa (ACRP30; also known as adiponectin or adipQ), among others. In all cases studied, GLUT4 localized to membrane compartments that were separate from those occupied by these secretory proteins. It therefore appears that adipocytes harbor several intracellular compartments that respond to insulin. Moreover, insulin stimulation results in about a 2-fold increase in the extent of exocytosis of these secreted proteins. In contrast, insulin causes a dramatic 10- to 20-fold increase in GLUT4 at the plasma membrane. Taken together, these data suggest that GLUT4 is targeted to specific membrane compartments that are exceptionally insulin responsive and that are separate from those occupied by secreted peptides.

Although the existence of a specialized insulin-responsive GLUT4 storage compartment is supported by abundant indirect evidence, it has been very difficult to biochemically or structurally characterize this compartment. In part this is because at steady state GLUT4 is distributed, at varying levels, throughout most of the endomembrane system (35). Indeed, after insulin-stimulated translocation, GLUT4 is retrieved from the plasma membrane by endocytosis and routed through a complex trafficking itinerary that appears to include endosome compartments and the trans-Golgi network (TGN), before returning to the insulin-responsive storage compartment. This complex itinerary has made it difficult to reliably distinguish between the insulin-responsive compartment and the general endosome recycling compartments occupied by GLUT4. In addition, there may be multiple interrelated insulin-responsive GLUT4 storage compartments with overlapping properties (36–40).

Nevertheless, several approaches including subcellular fractionation, vesicle immunoabsorption, membrane compartment ablation, and immunoelectron microscopy have begun to distinguish between the general versus specialized GLUT4 recycling compartments. For example, a horseradish peroxidase-transferrin receptor conjugate has been used in compartment ablation studies to show that the insulin-responsive pool of GLUT4 protein partitions into vesicular compartments that are largely separate from those occupied by cellubrevin and the transferrin receptor (41). Moreover, vesicle immunoabsorption experiments have demonstrated...
that although some vesicle populations contain both GLUT4 and general endosomal markers, other vesicles are enriched for GLUT4 and appear to exclude endosomal proteins (41–43). Similarly, studies using quantitative immunoelectron microscopy have demonstrated that the insulin-responsive pool of GLUT4 apparently consists of a unique population of vesicles that is largely devoid of constitutively recycling proteins such as the cation-dependent mannose-6-phosphate receptor (CD-MPR) (44). Moreover, cell fractionation studies employing iodixanol (Optiprep) gradients showed that, although GLUT4 was sorted into multiple intracellular compartments, those responsive to insulin were distinct from endosomes or the TGN (45).

**B. Insulin-regulated amino peptidase (IRAP)**

IRAP was identified as a major protein that colocalizes with GLUT4 in insulin-responsive storage vesicles (Refs. 46–49 and reviewed in Ref. 50). IRAP is a type II integral membrane protein with an amino-terminal cytoplasmic tail of 109 amino acids, a transmembrane domain, and an extracellular domain of 894 amino acids. Interestingly, the cytoplasmic domain of IRAP harbors dileucine and acidic cluster motifs that resemble those in the carboxyl terminus of GLUT4 (51). Membrane aminopeptidases are known to process regulatory peptides, and IRAP has been shown to cleave vasopressin, oxytocin, lys-bradykinin, met-enkaphalin, dynorphin, and angiotensins III and IV (50). In addition, IRAP has recently been identified as the angiotensin IV receptor (52). A possible connection between IRAP and GLUT4 was recently uncovered through the targeted ablation of IRAP (53). Interestingly, the IRAP−/− mice showed decreased levels of GLUT4 protein in skeletal muscle, heart, and adipose tissues, despite being normal for glucose homeostasis (53). Although the mechanism for the observed decrease in GLUT4 protein levels remains to be elucidated, these results nevertheless hint at a potential role for IRAP in regulating GLUT4 expression.

**C. GLUT4 targeting motifs**

The data described above support a model in which GLUT4 is partitioned into specialized insulin-responsive storage compartments that represent the primary site of insulin action. In addition, this model predicts that GLUT4 contains intrinsic targeting domains that direct its localization to the insulin-responsive compartment. Consistent with this prediction, the membrane compartment distribution of GLUT4 is dramatically different from the very similar GLUT1 transporter (65% amino acid identity), which localizes predominantly to the plasma membrane, even under basal conditions (54). This difference in intracellular localization suggests that the two transporters undergo differential targeting/sorting processes. In addition, when GLUT4 is heterologously expressed in fibroblasts, it is efficiently retained in intracellular compartments. This suggests that GLUT4 may harbor intrinsic targeting signals for efficient sequestration within the cell. Understanding the mechanism

**Table 1. Mouse models with global or tissue-specific targeted disruptions in some genes considered potentially relevant to the maintenance of glucose homeostasis**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tissue</th>
<th>Phenotype associated with targeted gene disruption</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT4−/−</td>
<td>Global</td>
<td>Mild insulin resistance, but not overtly diabetic</td>
<td>19</td>
</tr>
<tr>
<td>GLUT4−/−</td>
<td>Global</td>
<td>Muscle insulin resistance; hyperinsulinemia; hyperglycemia</td>
<td>20</td>
</tr>
<tr>
<td>GLUT4−/−</td>
<td>Muscle</td>
<td>Insulin resistance; glucose intolerance</td>
<td>23</td>
</tr>
<tr>
<td>GLUT4−/−</td>
<td>Adipose</td>
<td>Adipose, liver, and muscle insulin resistance</td>
<td>24</td>
</tr>
<tr>
<td>IR−/−</td>
<td>Global</td>
<td>Death at 3–7 d after parturition</td>
<td>80, 81</td>
</tr>
<tr>
<td>IR−/−</td>
<td>Global</td>
<td>No major metabolic abnormalities, normal glucose tolerance</td>
<td>80, 81</td>
</tr>
<tr>
<td>IR−/−/IRS1−/−</td>
<td>Global</td>
<td>Diabetes at 4–6 months</td>
<td>82</td>
</tr>
<tr>
<td>IR−/−/IRS1−/−</td>
<td>Muscle</td>
<td>Normal glucose tolerance, increased adipose tissue mass</td>
<td>83</td>
</tr>
<tr>
<td>IR−/−/IRS1−/−</td>
<td>Adipose</td>
<td>Protected against age- and diet-induced glucose intolerance; decreased fat tissue mass; increased longevity</td>
<td>86, 87</td>
</tr>
<tr>
<td>IR−/−/IRS1−/−</td>
<td>Liver</td>
<td>Reduced glucose tolerance and hyperinsulinemia</td>
<td>88</td>
</tr>
<tr>
<td>IR−/−/IRS1−/−</td>
<td>β-cell</td>
<td>Impaired first-phase insulin secretion; glucose intolerance</td>
<td>89</td>
</tr>
<tr>
<td>IRS1−/−</td>
<td>Global</td>
<td>Increased caloric consumption and adiposity; insulin resistance</td>
<td>90</td>
</tr>
<tr>
<td>IRS2−/−</td>
<td>Global</td>
<td>Mild insulin intolerance</td>
<td>94, 95</td>
</tr>
<tr>
<td>IRS2−/−</td>
<td>Normal</td>
<td>Diabetes; liver insulin resistance; decreased β-cell mass</td>
<td>92, 21</td>
</tr>
<tr>
<td>IRS3−/−</td>
<td>Global</td>
<td>No apparent abnormalities</td>
<td>96</td>
</tr>
<tr>
<td>IRS4−/−</td>
<td>Global</td>
<td>MILD growth defects; mild glucose intolerance</td>
<td>98</td>
</tr>
<tr>
<td>p85−/−</td>
<td>Global</td>
<td>Enhanced insulin sensitivity and glucose transport; hypoglycemia</td>
<td>113, 114</td>
</tr>
<tr>
<td>SHIP2−/−</td>
<td>Global</td>
<td>Death shortly after birth; severe hypoglycemia</td>
<td>117</td>
</tr>
<tr>
<td>SHIP2−/−</td>
<td>Global</td>
<td>Improved glucose tolerance; enhanced insulin sensitivity</td>
<td>117</td>
</tr>
<tr>
<td>PKBα−/−</td>
<td>Global</td>
<td>No apparent defects in glucose homeostasis</td>
<td>138</td>
</tr>
<tr>
<td>PKBβ−/−</td>
<td>Global</td>
<td>Mild glucose intolerance; impaired glucose uptake in skeletal muscle</td>
<td>135</td>
</tr>
<tr>
<td>aPKCγ−/−</td>
<td>Global</td>
<td>Impaired immune system function</td>
<td>148</td>
</tr>
<tr>
<td>IRK9−/− ob/ob</td>
<td>Global</td>
<td>Protecte against diet-induced insulin resistance</td>
<td>226</td>
</tr>
<tr>
<td>PTP1B−/−</td>
<td>Global</td>
<td>Resistance to diet-induced obesity; improved insulin sensitivity</td>
<td>268, 269</td>
</tr>
<tr>
<td>Syntaxin 4−/−</td>
<td>Global</td>
<td>Early embryonic death</td>
<td>289</td>
</tr>
<tr>
<td>Syntaxin 4−/−</td>
<td>Global</td>
<td>Impaired glucose uptake and GLUT4 translocation in skeletal muscle</td>
<td>289</td>
</tr>
<tr>
<td>Cellubrevin−/−</td>
<td>Global</td>
<td>No apparent disruption of normal glucose homeostasis</td>
<td>295</td>
</tr>
</tbody>
</table>

MIRKO, Muscle IR knockout; FIRKO, fat IR knockout; LIRKO, liver IR knockout; bIRKO, β-cell IR knockout; NIRKO, neuron IR knockout.
for targeting and retaining GLUT4 within intracellular compartments may provide important insights regarding the molecular mechanism by which insulin mobilizes GLUT4 vesicles. Several groups have therefore examined potential targeting motifs within the GLUT4 protein, both by mutating candidate motifs and by transplanting them onto heterologous reporter constructs (55). Attention has largely focused on the N and C termini of GLUT4, which contain sequence elements that are absent from the GLUT1 protein. However, results from several laboratories have been controversial and at times conflicting. For example, an early study found that neither the N nor the C termini contributed to the overall trafficking dynamics of GLUT4 in 3T3L1 adipocytes (56). Instead, two domains, one between residues 80 and 101 and the other between residues 316 and 343, were found to contribute to the intracellular sequestration of GLUT4. In contrast, a significant body of research has generally supported a role for either the N or the C termini in the intracellular trafficking of GLUT4 (Fig. 1). In general, the interpretation of these data is complicated by the fact that various researchers have used different cell lines and different heterologous GLUT4 chimeric constructs, making direct comparisons among investigators challenging.

1. The amino-terminal targeting motif. Studies carried out in the early 1990s suggested that a cytosolic N-terminal FQQI motif was important for the intracellular sequestration of GLUT4. Mutations in this sequence, particularly the aromatic phenylalanine residue, resulted in the accumulation of GLUT4 at the plasma membrane (57). Furthermore, when the FQQI motif was transplanted onto GLUT1 or the H1 subunit of the asialoglycoprotein receptor, the resulting heterologous reporter constructs were excluded from the plasma membrane and were instead retained in intracellular compartments (57). Initially, these results were interpreted as evidence that the FQQI motif played a necessary and sufficient role in targeting GLUT4 to intracellular compartments. However, by closely examining the endocytic behavior of GLUT4-chimeric constructs, it was subsequently discovered that the FQQI motif functions during the endocytosis of GLUT4 from the plasma membrane, rather than in its intracellular retention per se (58, 59). Because wild-type GLUT4 undergoes efficient endocytosis, in the absence of insulin the transporter is primarily localized to intracellular compartments. Because mutations in the FQQI motif inhibited internalization, over time GLUT4 accumulated at the plasma membrane under basal conditions. Conversely, transplanting the FQQI motif led to the efficient internalization of heterologous reporter constructs from the plasma membrane, resulting in an intracellular localization under steady-state conditions.

Although the FQQI motif is required for efficient endocytosis, some mutations in this motif do not completely block the uptake of reporter constructs. Thus, it has been possible to investigate whether the FQQI motif plays additional roles in intracellular GLUT4 vesicle trafficking. For example, it was recently shown that the FQQI motif functions in the sorting of internalized cell surface GLUT4 protein into specific intracellular compartments (60). Thus, mutation of FQQI to SQQI caused the mislocalization of the mutant transporters to late endosome and lysosome compartments. Moreover, it was independently shown that mutation of the FQQI to AQQI also caused GLUT4 to localize to endosomes (in this case recycling endosomes) (61). These results suggest that, in addition to playing an important role in endocytosis, the FQQI motif may also function during the intracellular trafficking of GLUT4 from endosome compartments into the insulin-responsive perinuclear storage compartment. However, these results must be interpreted with caution because the mutant transporters may be incorrectly folded and thus targeted for degradation in lysosomes. In addition, these data are based on the steady-state distribution of reporter constructs and may therefore reflect net changes in the trafficking rate constants between membrane compartments of the normal trafficking pathway, rather than mislocalization to compartments not normally occupied by the transporter.

2. Carboxyl-terminal targeting motifs. In addition to the N-terminal FQQI motif, the cytoplasmic C-terminal 30-amino-acid region of GLUT4 was also initially found to be essential for the intracellular localization of the transporter (61–63). However, similar to the FQQI sequence described above, subsequent work demonstrated that a dileucine (LL) motif located within this region functioned during the endocytosis of GLUT4 from the plasma membrane (64, 65). Thus, mutating the LL motif to AA significantly inhibited internalization, resulting in the cell-surface accumulation of the mutant transporter. Moreover, studies employing chimeric proteins consisting of the extracellular domain of the transferrin receptor fused to the intracellular dileucine motifs of either GLUT4 or IRAP also support a role for this sequence during endocytosis (66). In addition to its role in endocytosis at the plasma membrane, the LL motif has also been reported to function during exit of GLUT4 from the TGN (60, 67). However, a recent study using primary adipocytes isolated from rat epididymal fat pads found that mutation of the LL motif resulted in no detectable alteration in the trafficking of a GLUT4 reporter construct (58). The apparent conflicting results regarding the LL motif may reflect important differences in the trafficking properties of GLUT4 reporter constructs expressed in different cell types.

Other studies utilizing GLUT1/GLUT4 chimeras stably expressed in 3T3L1 adipocytes found that the carboxy-terminal 30 amino acids of GLUT4 were sufficient to correctly target chimeras to the insulin-responsive compartment (68). However, although the LL motif within this 30-amino-acid region was important for endocytosis from the plasma membrane, this motif was not critical for targeting chimeric constructs to the insulin-responsive compartment. These data are consistent with recent results implicating an acidic cluster motif (TELEYLGP) in GLUT4 trafficking. The TELTEYLGP sequence is located downstream of the LL motif and appears to function in the targeting of GLUT4 from endosomes to a subdomain of the TGN enriched in the t-SNARE proteins syntaxins 6 and 16 (51, 70). Once recycled from the cell surface back to the TGN, GLUT4 may undergo additional sorting processes, perhaps involving syntaxins 6 and 16, before becoming insulin responsive (70). However, further work is needed to clarify the functional roles of syntaxin 6 and 16 in GLUT4 sorting processes.
IV. Regulated GLUT4 Exocytosis

A. The IR: structure and function

The IR is a member of the family of transmembrane receptors with intrinsic tyrosine kinase activity (71). Encompassing more than 120 kb, the IR gene harbors 22 exons, 11 of which encode the α-subunit, with the remaining 11 exons encoding the β-subunit. Synthesized as a single polypeptide, the proreceptor is cleaved in the TGN by the serine protease furin. The mature cell surface IR is composed of two extracellular α- and two transmembrane β-subunits disulfide linked into an αβ2-heterotetrameric structure (Fig. 3) (72, 73). Moreover, owing to alternative splicing of exon 11 of the IR transcript, the human IR exists in two isoforms, IR-A and IR-B, that differ by the inclusion (IR-B) or exclusion (IR-A) of 12 amino acids at the carboxyl terminus of the α-subunit (74). IR-A is expressed predominantly in the developing fetus as well as a few adult tissues, including pancreatic β-cells (Refs. 75 and 76; reviewed in Refs. 77 and 78). In contrast, IR-B is expressed predominantly in adult tissues and mediates the metabolic effects of insulin in muscle, adipose, and liver. During development, IR-A is controlled mainly by IGF-II. In adult tissues, insulin is the major ligand for IR-B; however, in β-cells insulin appears to activate both IR-A and IR-B and may regulate different cellular processes through these receptor isoforms. Regardless of the receptor isoform, insulin binds to the extracellular α-subunits and generates a conformational change that allosterically regulates the intracellular β-subunit tyrosine kinase domain. Subsequently, the β-subunits undergo a series of intermolecular trans-autophosphorylation reactions that generate multiple phosphotyrosine sites, some of which serve distinct functional roles (73). For example, tyrosine phosphorylation at the juxtamembrane Y960 residue is necessary for appropriate substrate recognition (e.g., insulin receptor substrates, IRS1–4), whereas tyrosine phosphorylation at residues Y1146, Y1150, and Y1151 in the kinase activation domain relieves pseudosubstrate inhibition, thereby further enhancing the receptor’s tyrosine kinase activity (73).

B. The IR: knockout mouse models

Mutations in the human IR are extremely rare and account for less than 5% of all cases of type 2 diabetes. Nevertheless, through genetic manipulation of the IR in mice, much has been learned about the roles specific tissues play in the pathophysiology of insulin resistance (79) (Table 1). Whereas homozygous knockout of the IR caused death at 3–7 d post parturition, heterozygous mice showed no major metabolic abnormalities and were normal for glucose tolerance (80, 81). However, mice doubly heterozygous for null alleles of the IR and Irs1 (Irs1 null animals are mildly insulin intolerant) developed frank diabetes at 4–6 months of age (82). In addition, using the Cre-lox system, a range of tissue-specific IR knockouts has been generated, sometimes with surprising results. For example, although skeletal muscle accounts for more than 80% of postprandial glucose disposal, the ablation of the IR specifically in muscle resulted in normal glucose tolerance (83). How is glucose cleared from the bloodstream in this situation? These animals have increased adipose tissue mass and appear to compensate, at least in part, with increased glucose uptake in their excess fat reserves. In addition, an increase in both IGF receptor signaling and exercise-mediated signaling has also been reported in muscle lacking the IR (84, 85). In contrast, ablation of the IR specifically in adipose tissue resulted in a protective effect against age- and diet-induced obesity and a marked resistance to the development of glucose intolerance (86). These animals also had decreased overall fat tissue mass, with a polarized distribution of large and small adipose cells. Intriguingly, the fat-specific IR knockout mice also showed increased life span in the absence of caloric restriction (87). Knockout of the IR specifically in liver resulted in reduced glucose tolerance and hyperinsulinemia (88). The β-cell IR is part of the glucose-sensing mechanism, and knockout of the receptor in this cell type resulted in a dramatic decrease in glucose-stimulated first-phase insulin secretion accompanied by progressive,
age-dependent glucose intolerance (89). Finally, ablation of the IR specifically in neurons resulted in increased caloric consumption, increased adiposity, and insulin resistance (90). Together, these studies shed light on how insulin-responsive tissues functionally interact to maintain glucose homeostasis in intact model organisms, and illustrate how insulin resistance can potentially result from the combinatorial effects of impaired insulin action in specific tissues.

C. The IR: complex signaling networks

Many receptor tyrosine kinases directly recruit downstream effector molecules to their phosphorylated cytoplasmic domains. In contrast, the insulin receptor phosphorylates several scaffolding proteins that in turn recruit various downstream effector proteins (91). A number of scaffolding proteins downstream of the activated IR have been identified, including the four members of the insulin receptor substrate family (IRS1, -2, -3, and -4), Gab1, Shc, SIRPS, Cbl, and adapter protein containing PH and SH2 domains (APS). The best studied of these scaffolding proteins is the IRS family (92), and the genetic ablation of IRS2 resulted in a diabetic phenotype (93). This resulted from a combination of peripheral insulin resistance and impaired β-cell function, similar to what occurs in typical type 2 diabetes. IRS1 knockouts showed mild insulin intolerance (94, 95), whereas ablation of IRS3 resulted in no apparent abnormalities (96). However, IRS3 does not appear to be functional in humans (97). IRS4 knockout mice showed mild growth defects and were slightly glucose intolerant (98). The insulin-dependent tyrosine phosphorylation of IRS proteins generates docking sites for several downstream effectors, including the p85 regulatory subunit of the type 1A phosphatidylinositol 3-kinase (PI3K), the SH2-containing protein tyrosine phosphatase (SHP2), the Src family member kinase Fyn, and the small adaptor proteins Grb2 and Nck (91). Although the precise signaling function of these various adapters/effectors remains poorly understood, they appear to serve distinct yet overlapping biological roles in insulin signaling. For example, Grb2 primarily functions in Ras activation by insulin through engagement of the Ras guanyl nucleotide exchange factor Son-of-Sevenless (SOS). This pathway leads to ERK activation (via Raf and MAPK kinase) and is an important cascade regulating several transcription events and eventually mitogenesis. In addition, Ras can also regulate the activity of the PI3K through interactions with its p110 catalytic subunit (99). The PI3K functions in many cellular responses, including the regulation of transcription, mitogenesis, apoptosis, protein synthesis, glycogen synthesis, and glucose transport (100–102). Moreover, PI3K functions in the regulation of the actin cytoskeleton, which is under the control of Rac and Rho family members (103). Defining the interrelationship between all these possible signaling events and the in vivo specificity required for a defined biological response represents a challenging problem for future investigators. Here our discussion will be limited to two independent signaling pathways that function downstream of the IR to regulate GLUT4 translocation (Fig. 4). A key player of the first, and more established, pathway is PI3K. The second pathway is characterized by the APS/CAP (Cbl-associated protein)/Cbl complex, which is compartmentalized at specialized microdomains at the plasma membrane.

D. The PI3K and GLUT4 translocation

Among the many PI3K isoforms, the class 1A enzymes have been directly implicated in insulin signaling leading to the translocation of GLUT4 in adipocytes. Class 1A PI3K function as heterodimers and are composed of a regulatory p85 subunit and a catalytic p110 subunit (101, 102). The p85 subunit binds phosphotyrosine residues through SH2 (Src homology 2) domain interactions and allosterically regulates the catalytic activity of the p110 subunit. PI3Ks catalyze phosphorylation of the 3′-OH moiety of membrane myoinositol lipids (100). Although PI3K can phosphorylate several substrates, the type 1A isoforms prefer the substrate phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to generate phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] (100).

Insulin stimulation results in the tyrosine phosphorylation

Fig. 4. Schematic model for the interrelationship between the two IR-mediated signaling pathways. Insulin stimulation results in the tyrosine phosphorylation of IRS proteins, which induces the association, activation, and/or targeting of the PI3K. Production of PI[3,4,5]P3 results in the recruitment and activation of PDK1, which phosphorylates and activates both αPKCs and PKB. In parallel, insulin appears to phosphorylate Cbl through the adaptor protein APS. CAP also associates with Cbl and targets it to lipid raft microdomains through its interaction with the caveolar protein flotillin. Cbl then recruits the CrkII-C3G complex, resulting in the regulation of the lipid raft-associated Rho GTP-binding protein, TC10.
of the IRS proteins generating docking sites for the SH2 domains of the p85-regulatory subunit (78). This interaction apparently provides a dual function by both activating the PI3K and targeting it to the plasma membrane localized substrate PI(4,5)P2. In turn, the local increase in PI(3,4,5)P3 in the membrane provides a lipid-based platform for the recruitment and anchorage of downstream signaling molecules that contain pleckstrin homology (PH) domains (100). PH domains are 100- to 120-amino acid modules with a characteristic α-sandwich fold that binds, with varying degrees of specificity and affinity, to membrane phosphoinositides (104). Although most PH domains studied to date preferentially bind various phosphoinositides, the PH domain of IRS1 has been found to mediate interaction with a recently identified protein termed PHIP for PH domain-interacting protein (105). Interestingly, this interaction appears to be required for several insulin-dependent processes, including GLUT4 translocation.

In any case, it has been well established that the insulin-stimulated production of PI(3,4,5)P3 is essential for GLUT4 translocation. For example, inhibition of PI3K activity with relatively selective pharmacological inhibitors (wortmannin and LY29004) prevents insulin-dependent GLUT4 translocation (106–108). Similarly, introduction of PI3K-blocking antibodies or expression of dominant-interfering PI3K mutants also inhibits insulin-stimulated GLUT4 translocation (109, 110). On the other hand, expression of a constitutively active p110 subunit or addition of a PI(3,4,5)P3 analog to cells induces GLUT4 translocation, albeit not to the same extent as insulin (111, 112). Surprisingly however, p85-regulatory subunit knockout mice paradoxically display enhanced insulin sensitivity and increased glucose transport in adipose and skeletal muscle, leading to a hypoglycemic phenotype (113, 114). This appears to result, at least in part, from the preferential use of the p50α-regulatory subunit splice variant in mice lacking full-length p85. It has also been suggested that the p85 subunit may function as a competitive inhibitor of PI3K signaling in the monomeric state (115). Reduced levels of p85 may thus improve the p85/p110/IRS stoichiometry and enhance insulin signaling through this pathway.

Additional evidence favoring PI3K function in insulin action comes from investigations on the mechanisms for inactivating the PI(3,4,5)P3 second messenger. Attenuation of PI3K signaling occurs through the activity of type II SH2-domain-containing inositol 5-phosphatase (SHIP2), a phosphatase that removes the 5′-phosphate from PI(3,4,5)P3, generating phosphatidylinositol-3,4-bisphosphate (PIP3) (116). SHIP2 negatively regulates insulin signaling, and its genetic ablation resulted in enhanced insulin sensitivity (117). Although SHIP2-null animals died shortly after birth and were severely hypoglycemic, heterozygotes showed improved glucose tolerance and enhanced insulin sensitivity. This appeared to be due, at least in part, to enhanced insulin-dependent recruitment of GLUT4 to the plasma membrane (117). In contrast to SHIP, phosphatase and tensin homolog (PTEN) is a 3′-specific PI(3,4,5)P3 phosphatase that generates PI(4,5)P2, and overexpression of PTEN prevents the accumulation of PI(3,4,5)P3 and also inhibited insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes (118). Together, these data provide compelling evidence that the PI3K lipid product, PI(3,4,5)P3 is an essential intermediate in mediating insulin-stimulated GLUT4 translocation.

E. Protein kinase B (PKB) and atypical protein kinase C (aPKC)

Insulin rapidly activates the serine/threonine kinase PKB (also called Akt). This occurs through PI3K activity and the generation of PI(3,4,5)P3, which recruits PKB to the plasma membrane through specific interactions with its amino-terminal PH domain (119). At the plasma membrane, PKB is activated when two key residues are phosphorylated, threonine 308 (T308) and serine 473 (S473) (119, 120). T308 lies within the kinase activation loop and is phosphorylated by another PH domain-containing kinase, the 3′-phosphoinositide-dependent kinase 1 (PKD1) (121). The carboxyl-terminal PH domain of PKD1 binds PI(3,4,5)P3 with high affinity, which also recruits PKD1 to the plasma membrane (121). Although PKD1 appears to be maintained in an active, phosphorylated state even under basal conditions, binding of PI(3,4,5)P3 may also potentiate the catalytic activity of PKD1 (122, 123). S473 is located near the carboxyl terminus of PKB and may undergo autophosphorylation (124). Alternatively, there has been an extensive search for a putative PDK2, and several kinases including the integrin-linked kinase, MAPK-activated protein kinase 2, protein kinase C-related kinase 2, and others have been implicated in the phosphorylation of S473 (reviewed in Ref. 119).

Although the precise activation mechanism of PKB remains unclear, results from cell culture systems have generally supported a role for PKB function in insulin-stimulated GLUT4 translocation (125–130), although others have reported evidence against such a role (131, 132). Nevertheless, stable expression of a constitutively active membrane-bound form of PKB in 3T3L1 adipocytes resulted in increased glucose transport and persistent localization of GLUT4 to the plasma membrane (72, 128, 133). In addition, coexpression of an epitope-tagged GLUT4 with dominant-interfering PKB mutants was also reported to inhibit insulin-stimulated GLUT4 translocation (134). Moreover, there are three PKB isoforms, α, β, and γ, and knockout of PKBβ resulted in mild glucose intolerance, impaired glucose uptake in skeletal muscle, and the failure of insulin to suppress hepatic glucose production (135–137). In contrast, the ablation of PKBα resulted in no observable defects in glucose homeostasis (138). These results are consistent with an important role for PKBβ in insulin signaling and glucose uptake. However, although numerous substrates are known for PKB (119, 120, 139), how PKB activity influences GLUT4 translocation remains undefined (139).

In addition to PKB, the aPKC isoforms, ζ and λ/τ, have also been implicated in insulin-dependent GLUT4 translocation (see Ref. 140 for discussion of isoforms). Unlike the conventional and novel PKCs, aPKCs are not activated by diacylglycerol. However, although aPKCs lack PH domains, PI(3,4,5)P3 and other acidic phospholipids bind to the regulatory domains of these kinases and apparently induce conformational changes that lead to aPKC activation (141, 142). Activation of aPKC activity has also been reported to occur through autophosphorylation, phosphorylation by PDK1,
and relief from pseudosubstrate inhibition (143, 144). As with PKB, results from cell culture experiments also support a role for aPKC-γ and α/β in insulin-regulated GLUT4 translocation (140). These include inhibition of aPKC activity with a pseudosubstrate peptide (145) and with general PKC inhibitors (145, 146). In addition, expression of kinase-dead or activation-resistant aPKCs inhibited GLUT4 translocation and glucose transport, whereas expression of constitutively active aPKCs promoted glucose transport (131, 147). However, knockout of the aPKCζ isoform resulted in mice without gross abnormalities but with some impairment of immune system function (148).

F. A second insulin-signaling pathway regulating GLUT4 translocation?

Although PI3K appears to be necessary for insulin-stimulated GLUT4 translocation, substantial evidence indicates that the generation of PI(3,4,5)P3 is not sufficient to recruit GLUT4 to the plasma membrane (Refs. 112, 149–152; reviewed in Ref. 153). Furthermore, expression of an active PDK1 mutant was found to phosphorylate and activate both PKB and aPKCζ/λ without any significant effect on glucose uptake (154). Together, these data imply that additional signaling pathways may function in parallel with the PI3K pathway. Recently, a candidate second signaling cascade that is functionally organized within caveolin-enriched lipid raft microdomains has been partially characterized (Fig. 4) (155–159). Caveolae are 50- to 100-nm invaginations of the plasma membrane and represent a subset of lipid raft domains (160). In addition to the caveolin and flotillin structural proteins, these liquid-ordered domains are enriched in palmitoylated signaling proteins, glycosylphosphatidylinositol (GPI)-anchored proteins, glycolipids, sphingolipids, and cholesterol (161). Lipid rafts are thought to spatially organize signaling cascades, and the tyrosine kinase activity of the IR is reportedly enhanced by virtue of its association with caveolae (162). It is generally recognized that the cell cytoskeleton plays important roles in various membrane trafficking events and in the retention of organelles at specific locations within cells (172–175). In the case of GLUT4, the integrity of the GLUT4 storage compartment is disrupted by cytoskeleton-perturbing reagents such as nocodazole (for microtubules) and latrunculin (for actin filaments) (176, 177), and subsequently insulin-induced glucose uptake and GLUT4 translocation are severely impaired by these treatments (158, 177–184). Nevertheless, recent studies have revealed the importance of the actin cytoskeleton on the acute metabolic actions of insulin (185, 186). Furthermore, the PI3K and TC10 insulin signaling pathways have profound influence on the actin cytoskeletal organization in various cell types (111, 187). Although a substantial amount of evidence strongly supports a critical role of the cytoskeleton in insulin-induced GLUT4 translocation, the precise functional roles and cytoskeleton-regulatory mechanisms remain enigmatic.

A. Actin

Monomeric globular actin (G actin) is highly conserved and is the most abundant cytoskeletal protein in mammalian...
cells. G actin polymerizes in a head to tail fashion to form filamentous actin (F actin) (188). Currently, more than 150 actin-binding proteins have been identified, which not only modulate the behavior of actin but also directly impinge upon the function of intracellular signaling cascades (189, 190). Stimuli such as attachment to the extracellular matrix and growth factors including insulin integrate these various actin-binding proteins to regulate the actin-based cytoskeletal system. Spatial and temporal regulation of dynamic actin rearrangements play important roles in a wide array of cell functions such as motility, chemotaxis, cytokinesis, phagocytosis, and intracellular vesicle trafficking, as well as for cell polarity and differentiation processes (191–194).

In fibroblasts, F actin is well known to form stress fibers, lamellipodia, and filopodia. However, the actin cytoskeleton is dramatically changed during the differentiation of preadipocytes (fibroblast-like) into adipocytes (Fig. 5). Although preadipocytes contain well-defined stress fibers, after differentiation into adipocytes this F actin converts to a cortical actin lining the inner face of the cell surface membrane (158, 195). Concurrent with the changes in F actin during this differentiation process, the levels of caveolin mRNA and protein expression increase 20-fold, and the number of caveolae increases 10-fold (196, 197). This marked induction of caveolin, and hence caveolae, results in the clustering of the individual caveolae (50–80 nm) into large ring-like arrays (caveolae rosettes) that can be visualized by fluorescent microscopy (157, 195, 197, 198). A recent report demonstrates that these caveolae-rosette structures are a complex of both caveolae and other membrane components that form large caves (199). Intriguingly, fully differentiated 3T3L1 adipocytes display patches of punctate F actin that emanate from the organized caveolae-rosette/cave structure composing the relatively thick cortical actin (Fig. 5) (158, 195). In addition, the F actin localized along the inner circumference of the caveolae-rosettes/caves disappears after the disruption of caveolae by depleting cholesterol from the cells. This unique F actin structure has therefore been designated as caveolin-associated F actin (Cav-actin) (195).

Currently, the molecular basis underlying the conversion from stress fiber type F actin to the Cav-actin structure in adipocytes has not been identified. However, this dramatic structural change suggests that actin modulators and their regulatory mechanisms are essential for normal adipocyte function. Indeed, the insulin-induced rapid stress fiber break down and appearance of lamellipodia/membrane ruffling are observed only in predifferentiated and relatively immature 3T3L1 cells, whereas fully differentiated 3T3L1 adipocytes...
cytes do not display pronounced lamellipodia/membrane ruffling (158). Similarly, isolated primary rat adipocytes do not have significant amounts of stress fibers, lamellapodia, or filopodia but instead they have a layer of cortical F actin and diffuse reticular F actin near the cell pole (181).

The role of F actin in relation to insulin action has been examined using actin depolymerizing agents such as cytochalasin D, latrunculin A and B, and all these compounds inhibited insulin-induced glucose uptake and GLUT4 translocation (178–182). More recently, using enhanced yellow fluorescent protein-tagged G actin and real-time imaging techniques, insulin was shown to induce dynamic actin rearrangements at both the cortical and perinuclear regions in differentiated 3T3L1 adipocytes (158). Consistent with these findings, latrunculin, which directly binds to the monomeric G actin to prevent its polymerization (200), has a more potent inhibitory effect on GLUT4 translocation than cytochalasin D, which inhibits further polymerization of the existing F actin by capping the elongating ends (181). These results suggest that de novo actin polymerization may play an important role in insulin-dependent GLUT4 translocation. Furthermore, jasplakinolide, a cell-permeable F actin stabilizer, results in massive actin polymerization at both cortical and the perinuclear regions and inhibits insulin-induced dynamic actin rearrangements and GLUT4 translocation (158).

The necessity of actin dynamics has been further supported by the recent observation that myosin 1C (Myo1c) function is required for insulin-stimulated GLUT4 translocation (201). In a series of experiments, it was shown that expression of wild-type Myo1c enhanced insulin-dependent GLUT4 translocation, whereas a dominant-interfering mutant form of Myo1c inhibited GLUT4 translocation. Moreover, small interfering RNAs were used to reduce the expression of endogenous Myo1c, and this maneuver inhibited insulin-stimulated glucose uptake.

In addition to the importance of nonconventional myosin families, multiple mechanisms have been proposed for the function of actin in the control of vesicle-trafficking events (174). For example, several studies have shown that a proper actin regulation is required for the TGN exit (202, 203), spatial targeting of secretory proteins, and the maintenance of the Golgi complex structure (204–206) including the GLUT4 storage compartment (177). In different cell systems, regulated secretion events apparently require distinct properties of actin with some fusion events promoted by F actin stabilization (207) and others by F actin disassembly (208) or dynamic actin rearrangements such as depolymerization/repolymerization (209, 210). Recently, one exciting mechanism has emerged from the study of the N-WASP isoform of the Wiskott-Aldrich syndrome protein (WASP) (211). N-WASP, a ubiquitously expressed member of the WASP family, contains several multifunctional domains including the WASP homology/Ena vasodilator-stimulated phosphoprotein homology domain, IQ motif, basic region, Cdc42/Rac interactive binding/GTPase binding domain, proline-rich region, and VCA (verpolin, cofilin-like, and acidic) region (212, 213). In concert with PI(4,5)P2, N-WASP is regulated by direct binding of the Rho family small GTP binding proteins such as Cdc42 (211, 212, 214), TC10 (187, 215), and recently identified RhoT (216). The interaction of activated Rho proteins with N-WASP induces a conformational change in N-WASP that exposes its VCA region. In turn, this activates the Arp2/3 complex, resulting in a burst of de novo actin polymerization in response to extracellular stimuli (188, 213). This N-WASP-dependent actin polymerization, referred to as actin-comet tails, has been shown to provide a driving motor force to propel vesicles (159, 217–219), viruses, and infectious bacteria (220–222).

In vitro reconstitution with endogenous vesicles and exogenous artificial vesicles containing PI(4,5)P2 or PI(3,4,5)P3 resulted in the nucleation of actin comet-tails and actin-based motility in a manner dependent upon Cdc42, N-WASP, Arp2/3, and tyrosine phosphorylation (214, 217, 219). Similarly, TC10 appeared to be capable of inducing the nucleation of actin-comet tails on the vesicles in an N-WASP-dependent manner (187). Intriguingly, using the same in vitro reconstituted system, GLUT4-containing vesicles were also found to induce actin-comet tails and actin-based motility stimulated by insulin stimulation (159). Furthermore, GTPyS plus sodium vanadate treatment is known to induce insulin-like actions including glucose uptake and GLUT4 translocation in adipocytes and muscle cells (223–225). These agonists also induced actin-comet tails on the GLUT4 vesicle and actin-based motility in the in vitro reconstituted system. The GLUT4 vesicle mobility was inhibited by latrunculin B, toxin B, and a dominant-interfering N-WASP/YCA-lacking VCA region that is necessary for Arp2/3-dependent actin polymerization (159). Furthermore, expression of N-WASP/YCA in 3T3L1 adipocytes partially, but significantly, inhibited insulin-stimulated GLUT4 translocation (159, 226). Taken together, these data suggest that the N-WASP-dependent actin polymerization on the GLUT4-containing compartments somehow plays a role in the process of the translocation of GLUT4 protein from the intracellular storage compartments to the plasma membrane.

Although these findings support a required role for actin function in the regulation of insulin-stimulated GLUT4 vesicle trafficking, they also raise several questions, especially with regard to the insulin-signaling pathways involved. Actin is an exceedingly complex molecule in terms of its dynamics and interactions with other proteins; however, two key regulators are well established: as mentioned above, one is phosphoinositides and another is the Rho family small GTP-binding proteins (191, 227). The insulin-induced dynamic actin rearrangements necessary for GLUT4 translocation apparently result from a combination of the PI3K signal and spatially compartmentalized TC10 signal that both are activated by insulin stimulation.

It is well established that phosphoinositides play critical roles in modulating actin cytoskeleton and membrane trafficking (227, 228). The concentration and composition of cellular phosphoinositides are tightly regulated by both inositol lipid kinases including the type IA p58/P110 PI3K and phosphatases such as PTEN and SHIPs. In particular, the PI(4,5)P2 and PI(3,4,5)P3 appeared to be important regulators of the actin cytoskeleton in response to multiple extracellular stimuli (229). In many cell types, activation of PI3K and subsequent production of PI(3,4,5)P3 from PI(4,5)P2 stimulated by growth factors/hormones including insulin induce dynamic actin rearrangements (111, 230). The most recent link of PI3K...
to the actin cytoskeleton is a role for these enzymes in the regulation of chemotaxis, the migration of cells along a chemical gradient (231–233). There are several potential pathways for these lipid products to regulate the actin cytoskeleton. For example, Rac-GEFs such as Vav-1 and Tiam-1 interact with PI(3,4,5)P3 through their PH domains and are thereby activated (234, 235). Consistent with these results, treatment of cells with PI3K inhibitors blocks Rac activation by Vav-1 and Tiam-1 and PI3K-dependent actin rearrangements in fibroblasts (235, 236).

Another important molecular link between PI3K and actin includes members of the ARF (ADP ribosylation factor) family of small GTP-binding proteins. ARFs are well established as important regulators of intracellular membrane trafficking through controlling the assembly and disassembly of vesicle coat proteins (237, 238). Recent evidence has emerged that ARF1 and ARF6 are involved in reorganizing the actin cytoskeleton (239–242). ARFs are regulated by ARF-GEFs and ARF-GAPs (GTPase-activating proteins) (237, 238). Two classes of ARF-GEFs can be distinguished on the basis of their sensitivity to brefeldin A. Although members of the brefeldin-sensitive type do not contain a PH domain and control Golgi apparatus traffic and integrity, the cytohesin family of ARF-GEFs that is insensitive to brefeldin contains a PH domain, a coiled-coil domain, and a Sec7 catalytic exchange factor domain (243). The PH domains of ARF nucleotide-binding site opener, cytohesin-1, and general receptor for cytoplasmic domain-specific inhibitor, cytohesin-3 preferentially bind PI(3,4,5)P3 over PI(3,4)P2 and other phosphoinositides (244, 245). On the other hand, the PH domain-containing ARF-GAPs have recently been grouped in the centaurin family (246). These proteins display a PI3K-dependent requirement to the plasma membrane through their PH domains upon activation of PI3K (247). Although the precise roles played by ARF1 and ARF6 during actin reorganization remain unclear, some evidence indicates that ARF1 may potently activate F-actin-stimulated stress fiber formation (242). In contrast, ARF6 may function in Rac-stimulated membrane ruffling (248).

In addition to these possible roles for PI(3,4,5)P3 function, PI(4,5)P2 also plays an important role in regulating actin organization through modulating the activities of multiple actin-binding proteins. For example, PI(4,5)P2 inhibits the activities of actin-severing proteins (e.g., ADF/cofilin), capping proteins (e.g., gelsolin), and profilin. In contrast, PI(4,5)P2 activates actin cross-linking proteins (e.g., α-actinin) and links actin to the plasma membrane through vinculin, talin, and ezrin/radixin/moesin proteins (248). Recent studies have revealed that WASP family proteins are activated by PI(4,5)P2 in conjunction with Cdc42 and TC10 leading to Arp2/3 complex-induced actin nucleation (187, 249, 250). As mentioned above, phosphatidylinositol 4-phosphate 5-kinase α expression induces actin-comet tails that are predominately nucleated from sphingolipid-cholesterol-rich rafts (217). Moreover, local increases in PI(4,5)P2 production might facilitate vesicle budding by enhancing membrane curvature (251).

As indicated above, phosphoinositide-based actin polymerization appears to function in cooperation with small GTP-binding proteins of the Rho family, particularly, RhoA, Rac, and Cdc42. As previously discussed, TC10 is an unusual member of this family and has been implicated in the insulin regulation of GLUT4 translocation (156, 157). In vitro binding assays have indicated that active GTP-bound TC10 can bind several potential effectors that were originally identified as binding partners for Cdc42 and/or Rac. These include mixed-lineage kinase 2 (MLK2), myotonic dystrophy-related Cdc42 kinase (MRCK), p21-activated protein kinases (PAK), the Borg family of interacting proteins, the mammalian partition-defective homolog Par6, and N-WASP (215, 252–255). To date, more than 20 Cdc42 target proteins have been identified in mammalian cells, and most of these are also potentially able to interact with TC10. However, despite their high degree of sequence similarity, TC10 and Cdc42 may play distinct functional roles in adipocytes and other cell types (156, 157, 187, 256).

For example, overexpression of Cdc42 wild type (Cdc42/WT) in fibroblasts caused a decrease in actin stress fibers along with the formation of plasma membrane microspikes, whereas overexpression of wild-type TC10 (TC10/WT) was without effect (255). However, expression of constitutively active TC10 (TC10/Q75L) is fully capable of inducing plasma membrane microspikes in fibroblasts. However, expression of constitutively active TC10 (TC10/Q75L) is fully capable of inducing plasma membrane microspikes. These data suggest that the signaling pathways required for TC10 activation are not present and/or functional in fibroblasts. This is in marked contrast to adipocytes where expression of Cdc42 has no significant effect on the actin cytoskeleton or insulin-induced GLUT4 translocation (156, 187). In contrast, a more recent report has indicated that expression of a constitutively active mutant version of Cdc42 (V12) can induce GLUT4 translocation (257). In any case, overexpression of TC10/WT, TC10/Q75L, or even the GDP-bound inactive mutant (TC10T31N) results in a total disruption of cortical and Caveolin-1 actin, leading to a marked inhibition of insulin-induced GLUT4 translocation. One interpretation of these data is that the ability of TC10 to regulate actin function is dependent upon specific cell types that express unique subsets of actin regulators. In addition, the existence and amount of lipid raft/caveolae are also crucial for actin regulation by TC10, because targeting to these specialized microdomains through C-terminal lipid modification is necessary for TC10 activation and its subsequent biological effects (157). Consistent with a role of TC10 in the control of Caveolin-1, the Rho family-specific inhibitor, Clostridium difficile toxin B, blocks TC10 activation, disrupts Caveolin-1, and prevents insulin-stimulated GLUT4 translocation without affecting PI3K signaling (158).

B. Microtubules

Microtubules are composed of repeating subunits of α and β-tubulin polymerized into long, hollow cylinders. Typically, microtubules have one end attached to a single microtubule-organizing center (MTOC) called a centrosome. Microtubules play critical roles in the long-range transport of intracellular organelles and vesicles in neurons (258). The balance of molecular motor activity directed toward the plus ends (cell periphery) vs. the minus ends (perinuclear region) of micro-
tubules is an important mechanism implicated in the regulation of membrane trafficking (172).

Several studies have implicated the microtubule cytoskeletal network as important structural and regulatory elements in insulin-induced GLUT4 translocation (176, 177, 183, 184, 259). For example, α-tubulin and the intermediate filament protein vimentin have been copurified with intracellular GLUT4-containing vesicles (176), and expression of a dominant-interfering vimentin peptide dispersed the perinuclear localized GLUT4 protein. Furthermore, inhibition of the microtubule motor proteins dynein and kinesin reduced insulin-stimulated GLUT4 translocation (176, 184). Most recent work has demonstrated that conventional kinesin KIF5B is highly expressed in adipocytes, and dominant negative mutants of conventional kinesin light chain blocked GLUT4 translocation in response to insulin (260). In addition, microtubule depolymerizing agents (nocodazole, colchicine, and vinblastine) dispersed the perinuclear localized GLUT4 protein and partially inhibited insulin-stimulated glucose uptake and GLUT4 translocation (177, 183, 184, 259). Furthermore, the microtubule minus end motor dynein has been found to function with Rab5 in regulating the endocytosis of GLUT4 (28). These data support a model wherein the microtubule cytoskeleton participates in insulin-induced GLUT4 translocation.

In contrast, other studies have demonstrated that disruption or stabilization of microtubule structure has no significant effect on insulin-stimulated GLUT4 translocation (185, 186). In addition, although the initial rate of GLUT4 endocytosis was not affected by microtubule disruption, the internalized transporter was not correctly recycled back to the perinuclear region. Furthermore, nocodazole appears to inhibit glucose uptake through a direct interaction with the transporter itself, without affecting the ability of GLUT4 to translocate to the plasma membrane in response to insulin (185, 186). Together, these data demonstrate that microtubular organization may play a role in the recycling of endocytosed GLUT4 from early endosome compartments back to the perinuclear region and in the retention of GLUT4 storage compartments under basal conditions. However, the potential role of microtubules in insulin-stimulated translocation of GLUT4 to the cell surface remains unclear. Because microtubule depolymerization generates short, disorganized microtubule tracks, additional studies are still needed to clarify the potential role of microtubule motors in the process of GLUT4 translocation.

### VI. The IR: Attenuation of Receptor Signaling

Two basic mechanisms are known for attenuating IR signaling: 1) serine phosphorylation of the IR and IRS proteins, and 2) tyrosine dephosphorylation of the receptor and its substrates (261). Although a great deal of attention has recently focused on the ubiquitination of receptor tyrosine kinases, particularly the epidermal growth factor receptor (262), so far this mechanism has not been demonstrably associated with the IR (see, however, Ref. 165). IR serine phosphorylation appears to decrease insulin-stimulated tyrosine kinase substrate activity, leading to a reduction in IRS tyrosine phosphorylation (78). Similarly, serine/threonine phosphorylation of IRS has been reported to decrease its effectiveness as an IRS and may also promote interactions between 14-3-3 proteins (263). 14-3-3 Proteins bind phosphoserine/threonine residues and have been proposed to desensitize insulin signaling, although the mechanism remains unclear (264).

Several serine/threonine kinases have been implicated in the desensitization process, including PKB, PI3K, glycogen synthase kinase-3, mammalian target of rapamycin, MAPKs (ERK and c-Jun N-terminal kinase), and the inflammatory kinase pathway mediated through IkB kinase β (IKKβ). Evidence that the IKKβ pathway plays a critical role in the development of insulin resistance has been provided by historical clinical studies coupled with the use of reverse mouse genetics. Several historical clinical studies had observed that treatment of patients with high-dose salicylates improved glucose tolerance (reviewed in Ref. 265). More recently, it was observed that salicylates at these doses are inhibitors of IKKβ and that salicylate treatment of rodents prevents lipid infusion-induced insulin resistance (266). Moreover, overexpression of IKKβ in cultured cells inhibited insulin signaling, and inhibition of IKKβ activity reversed insulin resistance (266). The role for IKKβ in insulin resistance was further substantiated in IKKβ(+/-) heterozygotic mice that were protected against high-fat diet-induced insulin resistance when crossed into the obese (ob/ob) background (266). Although substantial work is still needed to establish the IKKβ signaling cascade in mediating insulin resistance, this pathway is quite appealing, as many other manipulations known to cause insulin resistance, such as increased fatty acid levels, activation of PKC-θ, and proinflammatory cytokines, all result in activation of IKKβ.

In addition to serine/threonine phosphorylation as a negative regulator, several protein tyrosine phosphatases have also been associated with the attenuation of IR signaling. Recently, considerable attention has focused on PTP1B. The activated IR tyrosine phosphorylates PTP1B, thereby increasing its enzymatic activity (267). Furthermore, PTP1B associates only with activated receptors and can dephosphorylate both the IR and IRS proteins. Genetic ablation of PTP1B resulted in resistance to diet-induced obesity, enhanced tyrosine phosphorylation of the IR and IRS proteins, and improved insulin sensitivity (268, 269). More recently, it has been suggested that increased insulin sensitivity in PTP1B deficiency in vivo is an indirect effect due to enhanced leptin signaling (270). In either case, the pharmacological inhibition of PTP1B holds promise for enhancing insulin sensitivity. Indeed, this phosphatase has become an attractive target for the development of small-molecule inhibitors that could potentially be used for the treatment of type 2 diabetes.

Several receptor tyrosine kinases (e.g., platelet-derived growth factor, c-Met, and epidermal growth factor) are thought to undergo rapid homologous down-regulation through ubiquitination and lysosomal degradation (167–169). However, the IR undergoes a very slow desensitization and down-regulation process. Although one study has reported an insulin-induced ubiquitination of the IR, this is unlikely to account for impaired insulin signaling (165). On the other hand, IRS protein levels are reduced in several
rodent models of insulin resistance and are down-regulated in cultured cell systems with impaired insulin signaling (92, 271). More recently, the IRS proteins were observed to undergo ubiquitination followed by 26S proteasome degradation during insulin stimulation or during cellular stress (272). Interestingly, proinflammatory cytokines not only activate the IKKβ pathway, they are also potent inducers of the SOCS (suppressor of cytokine signaling) family of proteins that were originally identified as negative regulators of cytokine receptor signaling (273). More recently, insulin has been observed to induce the up-regulation of SOCS3, and several SOCS proteins have been found to promote ubiquitination of IRS1 and IRS2 (272) and to negatively regulate insulin signaling (274). This may provide an additional mechanism to inhibit insulin action and promote glucose intolerance during chronic states of metabolic stress, infection, or inflammation.

VII. GLUT4 Vesicle Docking and Fusion with the Plasma Membrane

A. SNARE proteins

Results from classic membrane trafficking studies suggested that specific interactions between cognate receptor proteins expressed on the cytoplasmic surfaces of vesicle and target membranes control lipid bilayer fusion events. Several key components of the fusion machinery were initially identified in experiments that reconstituted intercisternal Golgi transport in vitro, including two cytosolic proteins, the N-ethylmaleimide (NEM)-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) (275). With the soluble components identified, their membrane-bound partners, termed SNAREs for SNAP receptors, were soon discovered. These studies, together with results from purified synaptic vesicles, led to the discovery of the target membrane SNAP receptors (t-SNAREs), which are members of the syntaxin family, and the vesicle membrane SNAP receptors (v-SNAREs), which are members of the VAMP family. Subsequently, another target membrane SNARE, called SNAP25 for synaptosome-associated protein of 25 kDa, was found to stably interact with syntaxin. The key functional significance of v- and t-SNAREs for synaptic vesicle exocytosis was shown in neurons by utilizing the tetanus and botulinum clostridial neurotoxins, which block neurotransmitter release in an irreversible manner by selectively cleaving VAMP2, syntaxin 1, or SNAP25 (276). Moreover, in vitro studies using v- and t-SNARE partners reconstituted into separate liposome populations demonstrated that interactions between cognate SNARE pairs form a molecular bridge that brings the respective vesicles into sufficiently close proximity to induce lipid bilayer mixing (277). In addition, structural studies have shown that cognate v- and t-SNAREs interact through coiled-coil domains to form a very stable four-helix bundle that may be sufficient to overcome the energetic barrier to membrane fusion (278, 279). Thus, v- and t-SNAREs may represent the minimal physiologic machinery required for membrane fusion. In this model, the v- / t-SNARE complex forms first during the fusion event, allowing the subsequent binding of SNAPs followed by NSF. The SNARE complex is subsequently disassembled through ATP hydrolysis by NSF, thereby freeing the SNAREs for another round of membrane fusion.

Although v- and t-SNAREs may have the potential to interact promiscuously in vitro, under in vivo conditions specific pairing of SNARE partners appears to contribute to the fidelity of the membrane fusion process (280, 281). Both the v- and t-SNAREs represent large families of proteins that localize to specific intracellular membrane compartments. SNARE proteins may therefore help to demarcate the membrane compartments that have the potential to fuse with each other. Because the distribution of SNAREs may be critical for maintaining membrane compartment identity, several investigators have examined the targeting mechanism by which SNAREs localize to specific membrane compartments. For VAMP2, a specific signal within an α-helical domain was shown to specify localization to synaptic vesicles (282). More recently, two independent signals that cooperate to maintain syntaxin 6 in the TGN were identified, including an α-helical motif that traps syntaxin 6 in the TGN and a retrieval signal that returns wayward syntaxin 6 molecules from the plasma membrane back to the TGN (283). These results suggest that cells actively maintain the specific membrane compartment localization of various SNARE proteins. Thus, the specificity of membrane fusion events may be maintained at least in part by localizing the fusogenic SNARE proteins in spatially segregated compartments. Maintaining membrane compartment identity and fusion specificity is particularly important for GLUT4 and other proteins that traffic through multiple compartments during their biogenesis, intracellular storage, exocytosis, and retrieval from the cell surface. Although SNAREs play central roles in membrane fusion, several additional proteins also regulate the fusion process and may help ensure membrane fusion specificity (284, 285).

Syntaxin 4 is the only syntaxin isoform that has been implicated in insulin-stimulated GLUT4 vesicle trafficking in adipocytes (Fig. 6) (reviewed in Refs. 286–288). Endogenous syntaxin 4 function has been disrupted by several experimental approaches in cell culture systems, including overexpression of the cytosolic domain of syntaxin 4 or introduction of inhibitory syntaxin 4 antibodies. These experimental maneuvers inhibited insulin-stimulated GLUT4 translocation, apparently by blocking the docking or fusion of GLUT4 vesicles with the plasma membrane. In addition, although the homozygous knockout of syntaxin 4 caused early embryonic lethality, heterozygous mice showed impaired insulin-stimulated glucose uptake and GLUT4 translocation in skeletal muscle (289). Moreover, the syntaxin 4 binding partner SNAP23 (a SNAP25 homolog in adipocytes) is a functional component of the t-SNARE complex and also has been implicated in GLUT4 vesicle fusion with the plasma membrane (290).

Synaptobrevin 2 (also known as VAMP2) and cellubrevin (also known as VAMP3) are two potential GLUT4 vesicle v-SNAREs expressed in adipocytes. Both of these isoforms partially colocalize in intracellular membrane compartments with GLUT4 and both translocate to the plasma membrane after insulin stimulation. In addition, these two v-SNAREs can form stable complexes with syntaxin 4, and expression of the cytosolic domains of either synaptobrevin 2 or cellubrevin inhibited insulin-stimulated GLUT4 translocation.
Although these two proteins are divergent at their N termini, they differ by just one amino acid in their central t-SNARE binding coiled-coil domains. Based upon these close structural properties, a series of elegant experimental approaches has been employed to distinguish the functional differences between synaptobrevin 2 and cellubrevin. Initial studies using relatively selective proteases found that cleavage of synaptobrevin 2, but not cellubrevin, inhibited insulin-stimulated GLUT4 translocation (291). Consistent with a specific role for synaptobrevin 2, introduction of short peptides corresponding to the unique amino-terminal extension of this v-SNARE had a small inhibitory effect on the translocation of GLUT4 to the plasma membrane (290). In addition, membrane compartment ablation studies showed that cellubrevin localized predominantly to endosomal compartments defined by the transferrin receptor (292–294). These compartments contained about 50% of the GLUT4 protein but only a minor fraction of the total population of synaptobrevin 2 protein. Furthermore, ablation of the endosomal population enriched for cellubrevin and the transferrin receptor resulted in a minor effect on insulin-stimulated GLUT4 translocation but significantly inhibited GTPγS-stimulated GLUT4 translocation (294). Together, these results strongly indicate that synaptobrevin 2 is the primary v-SNARE for insulin-stimulated GLUT4 translocation. In contrast, other stimuli such as GTPγS appear to function through the recruitment of alternative GLUT4 transport vesicles and/or other SNARE isoforms. Consistent with this hypothesis, the genetic ablation of cellubrevin caused no observable disruption of glucose homeostasis (295).

B. SNARE-associated proteins in GLUT4 translocation

Several proteins in addition to NSF, SNAPs, and SNAREs participate in the membrane fusion process. One important syntaxin interacting partner was initially identified in yeast as sec1 and subsequently as unc18 in Caenorhabditis elegans, ROP in Drosophila melanogaster, and nSec1 or Munc18a in mammals (296). In in vitro assays, nSec1 interacts with high affinity with syntaxin 1 and competes with the ability of syntaxin 1 to bind synaptobrevin 2 and SNAP25. Thus, nSec1 effectively prevents the formation of the syntaxin 1-synaptobrevin 2-SNAP25 ternary complex (297, 298). Moreover, in D. melanogaster increased ROP expression inhibited neurotransmitter release in vivo, consistent with a negative regulatory role for nSec1 (299). However, in contrast to the above results, null or temperature-sensitive mutants of Sec1 homologs in Saccharomyces cerevisiae, C. elegans, and D. melanogaster blocked secretion at specific steps. These results suggest that Sec1 proteins play a positive role during membrane fusion (300–302). Recent structural analyses of the nSec1-syntaxin 1 complex have suggested that nSec1 maintains syntaxin 1 in a closed, inactive conformational state that prevents promiscuous membrane fusion (303). In this model, nSec1 is postulated to undergo a conformational change that leads to the conversion of syntaxin 1 to the open conformational state, thereby allowing membrane fusion. nSec1 may therefore regulate the transition state between the open and closed conformations of syntaxin 1.

Although nSec1/Munc18a was originally characterized as a neuronal-specific isoform, two additional ubiquitously expressed isoforms, termed Munc18b and Munc18c, were subsequently identified. Because only Munc18c binds to syntaxin 4 with high affinity, substantial attention has therefore focused on this isoform for its potential role in GLUT4 vesicle trafficking. Several groups have shown that overexpression of Munc18c inhibits GLUT4 vesicle translocation, apparently by binding to syntaxin 4 and blocking its ability to interact with synaptobrevin 2 (304–306). Consistent with this hypothesis, Munc18c blocked the interaction of synaptobrevin 2 with syntaxin 4 in both the yeast two-hybrid assay and in

Fig. 6. SNARE and associated proteins implicated in GLUT4 trafficking. The v-SNARE synaptobrevin 2 is present in GLUT4-containing compartments. After insulin stimulation, synaptobrevin 2 interacts with the t-SNAREs syntaxin 4 and SNAP23 at the plasma membrane. This interaction brings the GLUT4 vesicle into close contact with the plasma membrane and may be sufficient to drive bilayer fusion. Although the exact sequence of events remains to be elucidated, synap and Munc18c may dissociate from syntaxin 4 after insulin stimulation, thus allowing the productive formation of the synaptobrevin 2/SNAP23/syntaxin 4 heterotrimeric fusion-competent complex.
**C. Exocyst in GLUT4 translocation**

As discussed above, SNARE proteins are thought to play an essential role during membrane fusion. However, before SNARE-mediated fusion, vesicles appear to undergo a tethering (pre-SNARE binding) step with target membranes. A recent study has suggested that the exocyst protein complex may function in such a manner for GLUT4 vesicles (308). The exocyst is an evolutionarily conserved octameric protein complex composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (309). The exocyst component Exo70 has been identified as a direct target for activated TC10, and insulin activation of TC10 through CAP-Cbl signaling cascade recruits Exo70 together with Sec6 and Sec8 (presumably with other exocyst proteins) to the plasma membrane. Although Exo70 does not possess an identifiable CRIB domain, the amino-terminal region of Exo70 contains a coiled-coil domain that is required for the interaction with TC10. In addition, the carboxy-terminal region of Exo70 appears necessary for its recruitment to the plasma membrane, probably due to an interaction with other proteins. Intriguingly, the amino-terminal fragment of Exo70 specifically prevented the tethering and/or docking of GLUT4-containing vesicles, a necessary prerequisite for plasma membrane fusion. However, the amino-terminal fragment did not disrupt the intracellular membrane trafficking of GLUT4 en route to the cell surface, because GLUT4 vesicles accumulated underneath the plasma membrane. These data suggest that TC10 and Exo70 recruit the exocyst complex during the final steps of GLUT4 exocytosis.

In addition to TC10, recent studies have demonstrated that the exocyst complex is regulated by several small GTP-binding proteins, including Rab, and Rho (309). Sec4, a member of yeast Rab family, was originally reported to interact with Sec15 (310). In the case of the yeast Rho family, Rho1 and Cdc42 interact with Sec3, whereas Rho3 has been shown to associate with Exo70 (311, 312). Mammalian exocyst Sec5 has been reported to interact with Ral A small GTP-binding protein (313, 314), and this Ral A-Sec5 interaction appeared to be involved in basolateral, but not apical, membrane traffic in polarized epithelial cells (314). Thus, the exocyst appears to play an important role in multiple aspects of exocytosis, apparently being regulated by interactions between specific exocyst components. Further work is clearly needed to identify a functional role of the exocyst complex in the insulin-induced GLUT4 translocation process.

**D. Rab and ARF proteins in GLUT4 translocation**

With more than 60 known mammalian isoforms, Rab proteins comprise the largest branch of the Ras superfamily of small GTP-binding proteins. Rabs localize to the cytoplasmic
surfaces of intracellular compartments and regulate membrane transport between organelles. In addition, distinct Rab isoforms localize to specific intracellular compartments and may contribute to the specificity of bilayer fusion events (reviewed in Refs. 315 and 316). Like other small GTPases, Rabs are considered molecular switches that oscillate between a GDP-bound (off) and a GTP-bound (on) state. In the GTP-bound state, Rabs interact with effector proteins that regulate several steps of membrane transport, including vesicle budding, motility, tethering, and fusion (311, 312). After a round of vesicle fusion, the Rab protein returns to its GDP-bound state. In addition, GDP-Rabs are extracted from their target membrane by soluble guanylnucleotide dissociation inhibitor proteins and recycled back to the donor membrane for another round of vesicle transport (311, 312).

Although insulin-responsive cells express many Rab isoforms, to date Rab4, Rab5, and Rab11 have been implicated in GLUT4 trafficking processes (reviewed in Refs. 286, 288, and 317). In addition, a Rab GAP has also recently been implicated in insulin-stimulated GLUT4 trafficking (139). In fibroblasts, Rab4 localizes to early endosomes and has been implicated in protein sorting and recycling processes (315). In addition, in cell fractionation studies Rab4 colocalized with GLUT4 in the low-density microsome fraction of adipocytes (318) and muscle (319). Moreover, insulin stimulated nucleotide exchange on Rab4 and caused the redistribution of Rab4 from the microsome fraction to the cytosolic fraction, and this occurred in parallel with the translocation of GLUT4 to the cell surface (318, 320). Insulin-simulated GLUT4 translocation was also inhibited both by the introduction of a peptide corresponding to the carboxyl terminus of Rab4 and by expression of a carboxyl-terminal Rab4 truncation mutant (321–323). In addition, overexpression of wild-type Rab4 or a hydrolysis-deficient (GTP-bound) mutant also inhibited GLUT4 translocation, presumably because the cytosolic forms of these proteins function as dominant interfering inhibitors when present at high levels (323–325). Interestingly, Rab4 has been reported to interact directly with syntaxin 4 and to regulate SNARE protein interactions in a GTP/GDP-dependent manner (326).

Rab5 was originally identified as a regulator of the fusion between internalized vesicles and early endosomes. Recent work has shown that insulin may inhibit Rab5 activity, thereby decreasing the rate of GLUT4 endocytosis and favoring plasma membrane localization of the transporter (28). Rab11 localizes to perinuclear recycling endosomes of fibroblasts. In rat cardiomyocytes, Rab11 has been shown to colocalize with GLUT4 vesicles and to redistribute to the plasma membrane in response to insulin (327). However, recent work has indicated that Rab11 may function in the endosomal sorting steps that return GLUT4 to the insulin-responsive compartment (328).

The ADP-ribosylation factors (ARFs) are another important Ras subfamily of small GTP binding proteins involved in vesicle trafficking (329). ARFs are thought to function in the early stages of vesicle budding, during the recruitment of coat proteins to the donor membrane compartment. In the GDP-bound state, ARFs remain in the cytosol; however, upon GTP loading they associate with donor membranes and recruit cytosolic coat components to the site of vesicle bud formation. The coat proteins then deform the donor membrane into a nascent vesicle and interact with cargo molecules. Given their widespread roles in membrane trafficking processes, it seems likely that one or more ARF isoforms participate in the complex intracellular trafficking itinerary of GLUT4. Indeed, ARF6 has been implicated in GLUT4 vesicle trafficking; however, results have not always been consistent. For example, the introduction of myristoylated peptides corresponding to the amino terminus of ARF6 inhibited GLUT4 vesicle translocation by approximately 50% in permeabilized adipocytes (330). In contrast, other studies using a dominant-interfering mutant of ARF6 observed no significant effect on basal or insulin-stimulated glucose transport (331). Thus, although members of the ARF family are likely to participate in GLUT4 vesicle trafficking, additional work is needed to clarify our understanding of the potential roles played by ARF6 and perhaps other ARF isoforms.

VIII. GLUT4 Endocytosis

Although insulin is well known to enhance the rate of GLUT4 exocytosis, insulin also decreases the rate of GLUT4 endocytosis approximately 2- to 3-fold (332–334), and this may occur at least in part through inhibition of Rab5 activity (28). A complete understanding of the molecular mechanism underlying GLUT4 endocytosis could create opportunities for developing novel drug therapies aimed at slowing the rate of GLUT4 internalization and thus enhancing glucose uptake under diabetic conditions. Currently, we have only a rudimentary understanding of the molecular mechanisms responsible for GLUT4 endocytosis. Nevertheless, several lines of evidence suggest that GLUT4 is internalized through clathrin-coated pits. For example, GLUT4 localizes to clathrin-coated pits as determined by immunofluorescence and electron microscopy analysis (42, 335, 336). In addition, inhibition of clathrin-mediated endocytosis by a variety of methods also prevents GLUT4 internalization (65, 337).

The mechanism by which clathrin-coated pits invaginate and form free vesicles remains unclear. However, the GTPase dynamin plays a key role in this process. Dynamin is a 100-kDa cytosolic protein with an amino-terminal GTPase domain, a central pleckstrin homology domain, and a carboxyl-terminal proline-rich region (338). Dynamin encircles the necks of invaginating vesicles in a spiral conformation, a process that was initially observed in studies examining the D. melanogaster dynamin homolog shibire. Temperature-sensitive mutations in shibire result in a paralytic phenotype at the nonpermissive temperature (339). Analysis of the presynaptic termini of these flies revealed an absence of synaptic vesicles and an abundance of clathrin-coated pits with electron-dense collars around their necks, suggesting that a late stage of endocytosis was inhibited (340). The shibire temperature-sensitive mutation occurs near the GTPase domain, and together with experiments employing GTPγS it was proposed that dynamin’s GTPase activity was necessary for the vesicle scission process. In this model, GTP hydrolysis causes a conformational change in dynamin such that the necks of invaginating vesicles are constricted to the point
where membrane scission occurs and free vesicles released (341). However, in another model for dynamin function, the energy derived from GTP hydrolysis has been proposed to cause the elongation of dynamin spirals, resulting in the stretching and eventual scission of the vesicle neck (342). Alternatively, dynamin may instead function as a classical GTPase molecular switch that recruits downstream effector molecules, such as endophilin, which then participate in the formation of released vesicles (343).

Although the details of its molecular mechanism remain controversial, dynamin clearly plays an important role in GLUT4 endocytosis. Several studies have used dominant-interfering dynamin mutants or specific peptides to disrupt dynamin function, resulting in a significant inhibition of GLUT4 endocytosis (344–346). However, it remains unclear how insulin might regulate dynamin function. Nevertheless, the proline-rich domain of dynamin is known to associate with various SH3 domain-containing proteins, which in turn enhance dynamin’s GTPase activity (347). For example, Grb2 associates with dynamin and induces the binding of the dynamin-Grb2 complex to tyrosine-phosphorylated Shc and IRS1 proteins (348). These results suggest that this higher-order complex either directly inhibits dynamin activity and/or results in the sequestration of dynamin away from the GLUT4-containing clathrin-coated pits. Alternatively, insulin reportedly induces the tyrosine phosphorylation of dynamin (349). In either case, the functional significance of these events remains to be determined, as there is currently no in vivo evidence that insulin regulates the localization, GTPase activity, or pinchase function of dynamin.

IX. GLUT4 Activation

Although it is clear that insulin induces the translocation of GLUT4 from intracellular compartments to the plasma membrane, it remains possible that the intrinsic glucose transport activity of GLUT4 may also be regulated (reviewed in Ref. 17). For example, early studies showed that isoproterenol and other β-adrenergic agonists inhibit insulin-stimulated glucose transport in muscle and adipose tissue, without changing the plasma membrane content of the transporter. In addition, agents that stimulate cAMP production (e.g., forskolin), inhibit cAMP phosphodiesterase (e.g., 3-isobutyl-1-methylxanthine), or mimic cAMP ( dibutyryl-cAMP) all inhibit glucose transport (350–354). One interpretation of these data is that isoproterenol and other cAMP stimulators modulate the intrinsic transport activity of GLUT4. Indeed, it was subsequently demonstrated that isoproterenol can stimulate phosphorylation of the carboxyl terminus of GLUT4 at serine 488 in vivo, and that the cAMP-dependent protein kinase A can phosphorylate the same site in vitro (355). These data suggest that the observed attenuation of glucose transport activity upon β-adrenergic stimulation may result from phosphorylation of GLUT4. However, other models have been proposed to explain the effects of isoproterenol on glucose transport, including the occlusion or incomplete fusion of GLUT4-containing vesicles at the plasma membrane (356, 357).

In addition to the possible modulator effects of β-adrenergic agonists on intrinsic GLUT4 transport activity, a number of investigators have reported a discrepancy between the fold increase in glucose uptake with the increase in GLUT4 transporter translocation (reviewed in Ref. 17). In general, the reported fold increase in glucose uptake exceeded the fold increase in translocation, although results varied considerably. In addition, time course experiments employing a GLUT4 reporter construct with an engineered exofacial myc epitope have shown that GLUT4 translocation occurs before glucose transport (358). One interpretation of these data is that insulin enhances the glucose transport activity of GLUT4 transporters at the plasma membrane above basal activity levels. Moreover, recent evidence has implicated p38 MAPKs in GLUT4 activation. For example, it was shown that the SB202190 and SB203580 inhibitors of p38 MAPK attenuate glucose transport without affecting the ability of GLUT4 to translocate to the cell surface in response to insulin (359). However, although insulin increases p38 MAPK activity (360), insulin is also known to reduce the amount of phosphorylated GLUT4 at the plasma membrane (353). Thus, future work is necessary to resolve the potential mechanism by which p38 MAPK may regulate the intrinsic transport activity of GLUT4.

X. Summary and Future Directions

Since the discovery of insulin in the early 1920s it took an additional 60 yr to make the seminal observation that insulin stimulates glucose uptake by inducing the translocation of GLUT proteins from intracellular storage sites to the plasma membrane. This discovery provided the conceptual framework that continues to guide the research efforts of workers in the insulin-signaling and GLUT4-trafficking fields. Indeed, in the intervening decades since the translocation hypothesis was proposed, we have identified multiple members of the facilitative GLUT family and demonstrated that the GLUT4 isoform is the predominant insulin-responsive transporter in striated muscle and adipose tissue. Moreover, inroads have been made with regard to the signaling processes downstream of the IR, and it now appears that two distinct cascades are required to stimulate GLUT4 translocation. One pathway, characterized by the PI3K, has been intensely studied, and several downstream effectors including PKB, PDK, and aPKC isoforms, have been implicated in GLUT4 translocation. The second pathway, characterized by the Rho-family GTPase TC10, has only recently been implicated in GLUT4 translocation, and much remains to be learned regarding downstream signaling molecules.

In addition, over the past several years gene knockout and transgenic technologies have provided important new insights into how specific molecules function with respect to insulin signaling in the context of intact model organisms. Furthermore, the genetic disruption of individual genes has yielded valuable and, at times, surprising new information on how specific tissue types interact to regulate glucose disposal. In particular, it has become increasingly clear that cross-talk exists between adipose and other tissues, and that adipocytes may secrete factors that regulate glucose uptake in muscle tissue. Newer technologies, including microchip arrays and RNA interference, are also likely to yield impor-
tant and surprising insights into insulin signaling and GLUT4 translocation.

Despite these advances, it remains completely unknown how insulin mobilizes GLUT4-containing vesicles. This is an important area for future investigations as it is the elucidation of insulin signaling events occurring proximal to the GLUT4 storage compartment. Indeed, a key goal is to characterize the GLUT4 storage compartment at the molecular level, with the aim of identifying the molecular machinery that allows the insulin-dependent mobilization of GLUT4 vesicles. Unlike synaptic vesicles, the small, uniform size of which facilitated their isolation and characterization in almost unprecedented molecular detail, the apparently heterogeneous insulin-responsive GLUT4 storage compartment has so far remained recalcitrant to biochemical isolation and characterization. This represents a major technical hurdle for future work, because the ability to isolate GLUT4 vesicles and reconstitute insulin-dependent GLUT4 trafficking steps in vitro holds tremendous promise for identifying the specific molecules that link insulin signaling processes to GLUT4 trafficking events. The identification of molecules that act directly on the GLUT4 storage compartment and regulate transporter mobilization in response to insulin could potentially serve as targets for the rational design of therapeutic interventions for treating or preventing the development and progression of type 2 diabetes.

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