Perspectives in Diabetes

Moving GLUT4

The Biogenesis and Trafficking of GLUT4 Storage Vesicles

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The GLUT4 system in muscle and fat cells plays an important role in whole-body glucose homeostasis. Insulin stimulates the translocation of GLUT4 from an intracellular storage compartment to the cell surface. The nature of this compartment remains largely unknown. We review recent studies describing the biogenesis and molecular constituents of the GLUT4 storage compartment and conclude that it is segregated from the endosomal and biosynthetic pathways. Further, we present evidence to suggest that the GLUT4 storage compartment moves directly to the plasma membrane in response to insulin and, hence, is analogous to small synaptic vesicles in neurons. We propose that the GLUT4 storage compartment be referred to as GLUT4 storage vesicles or GSVs. *Diabetes* 46:1667–1677, 1997

Mammals maintain glucose homeostasis despite major shifts in energy consumption and storage. A disruption in this balance leads to diseases such as morbid obesity and diabetes mellitus and to cardiovascular disorders. One of the pivotal components used in maintaining this balance is a unique glucose transport system in muscle and adipose tissue that in a matter of minutes can orchestrate a 10- to 40-fold increase in glucose uptake.

Paradoxically, this transport system is used in muscle to both sustain energy requirements during exercise and mediate glycogen storage in the postprandial state. Three mechanisms exist whereby muscle and fat could induce such a rapid change in their rate of glucose uptake: *1*) a conformational alteration in a cell surface glucose transporter leading to increased transport activity; *2*) the rapid synthesis of new transporters; and *3*) the recruitment of transporters to the cell surface from an intracellular storage site. The latter model has received widespread experimental support, and it is now accepted that this type of mechanism is utilized by a variety of cells to regulate transiently the flux of various metabolites and ions across their cell surfaces.

The discovery of this mechanism, which was first documented in 1980 (1,2), has inspired many students of metabolism to turn their attention to the cell biology of protein trafficking. Progress in this area has been spectacular over the past 5 years with the realization that the rules and types of molecules that govern membrane protein trafficking both between different organelles and within completely different organisms, such as yeast and mammals, are essentially the same (reviewed in 3–5). By applying this knowledge to regulated glucose transport in muscle and fat cells, it has been possible to unveil some of the key molecules that are intimately involved in translocating glucose transporters to the plasma membrane upon demand.

GLUT4

The integral membrane protein GLUT4 represents the acutely regulated glucose transporter of fat and muscle cells (reviewed by 6–8). GLUT4 belongs to a family of six or more facilitative glucose transporters that transport glucose down a concentration gradient. GLUT4 expression during myogenesis and adipogenesis parallels the induction of insulin and/or exercise-regulated glucose responsiveness in these tissues. Figure 1 shows the immunolabeling of GLUT4 in 3T3-L1 adipocytes and two of the features that distinguish it from other GLUTs. In the absence of insulin, GLUT4 is almost completely sequestered in an intracellular compartment. With insulin, this compartment is quickly mobilized to the cell surface, resulting in a substantial increase in both surface GLUT4 levels and glucose transport.

While it is generally accepted that GLUT4 translocation is the major mechanism for increasing glucose uptake in muscle and adipocytes, there are several issues that require clarification. GLUT1, which is ubiquitously expressed, also undergoes insulin-dependent translocation in adipocytes and myocytes. Further, a number of fibroblast cell lines when cultured under certain conditions also exhibit insulin-dependent GLUT1 translocation (9). In all cases, however, the magnitude of this response is small because a large fraction of GLUT1 is constitutively targeted to the cell surface, even in the absence of insulin (Fig. 2). Also, it appears that a variety

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Received for publication 8 July 1997 and accepted in revised form 4 August 1997.

ANF, atrial naturetic factor; αSNAP, alpha soluble NSF attachment protein; CHO, Chinese hamster ovary; EM, electron microscopy; GEF, GTP exchange factor; GSV, GLUT4 storage vesicle; MDCK, Madin Darby Canine Kidney; Munc, mouse Unc homolog; NSF, *N*-ethylmaleimide sensitive factor; SNAP25, synaptosome-associated 25-kDa protein; SNARE, SNAP receptor; SSV, small synaptic vesicle; TFR, transferrin receptor; TGN, *trans*-Golgi network; VAMP, vesicle-associated membrane protein.

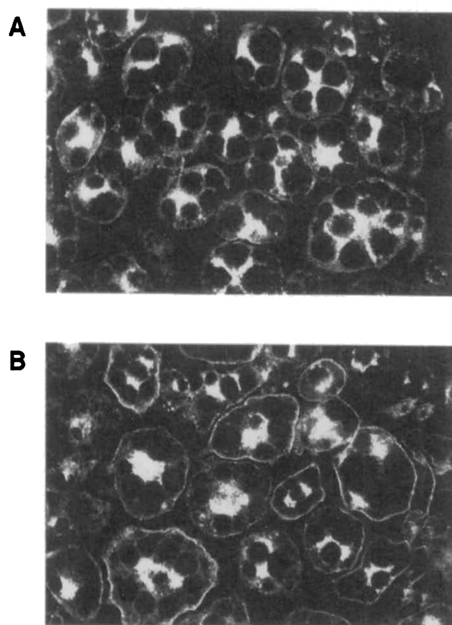

FIG. 1. The effect of insulin on the subcellular distribution of GLUT4 in 3T3-L1 adipocytes. Adipocytes were incubated in either the absence (*A*) or presence (*B*) of a maximal concentration of insulin for 15 min at 37°C. The cells were then fixed and immunolabeled using an antibody specific for the cytoplasmic C-terminus of GLUT4 as described previously (22). In the absence of insulin, most of the GLUT4 labeling is found within the cell with very little on the cell surface. Insulin causes a marked increase in surface labeling. Although not obvious in this figure, EM studies conclusively reveal that insulin leads to a commensurate decrease in intracellular labeling (20).

of stimuli, including viral infection, hypoglycemia, stress, and numerous growth factors, can trigger GLUT1 but not GLUT4 translocation (10,11). The limited repertoire of agonists for GLUT4 translocation in muscle and fat may play an important role in protecting the whole body against hypoglycemia during viral infection and rapid growth bursts and also against muscle glucose toxicity that arises when cells encounter chronically high glucose levels.

A puzzling observation has been the discovery of GLUT4 in cell types that are not generally considered insulin responsive. This includes neurons of the cerebellum, hippocampus, medulla oblongata, ventricle, and thalamus (12); endothelial cells of the blood-brain barrier (13); and specific parts of the kidney, namely in the thick ascending limb of the distal tubule and in granular cells of the juxtaglomerular apparatus and afferent arterioles (14). However, as illustrated in Fig. 2, GLUT4 is targeted to intracellular tubulo-vesicular structures, irrespective of its cellular environment. This is a property not shared with the other members of the GLUT family. Hence, it is feasible that GLUT4 translocation is triggered by different stimuli in different cell types. In the kidney, the physiological trigger may be hyperosmolarity (15), whereas in the brain appetite-regulating factors may be the relevant secretagogues.

The lack of marked insulin resistance in GLUT4 null mice has cast some doubt on the central role of GLUT4 in whole-body glucose metabolism (16). Establishing the basis for the adaptive response that enables these animals to maintain normal blood glucose levels may shed new light on the processes leading to the development of insulin resistance. Skeletal muscle has been implicated as the predominant site for acute peripheral glucose utilization (17,18). Although adaptive changes in other tissues may compensate to some extent for the absence of GLUT4 in muscle of null mice, it is clear that an alternate transport system must exist in this tissue. The only other glucose transporter expressed in normal adult muscle is GLUT1. However, in GLUT4 null mice, there is no change in the expression of either GLUT1 or other known isoforms (GLUT2, GLUT3, and GLUT5) (16). Hence, either the efficiency of GLUT1 to transport glucose must be markedly elevated or a novel transporter isoform must compensate for the absence of GLUT4. Future studies should distinguish between these possibilities. It is important to point out that the adaptive response observed in GLUT4 knockout mice may only occur on a null background. The expression of GLUT4, although detectable in embryos, begins to peak around postnatal days 3–7 in insulin-sensitive tissues (19). It is possible that the adaptive response can only be invoked in the complete absence of GLUT4 and not when its expression is moderately suppressed, as might be observed in animals carrying a targeted disruption in only one allele at the GLUT4 locus.

SORTING AND STORING GLUT4: AN ELABORATE STORY

The intracellular GLUT4 compartment. Numerous integral membrane proteins continually recycle between the plasma membrane and specific intracellular loci by way of a series of discontinuous tubular and vesicular structures, collectively referred to as the endosomal compartment. Using immunoelectron microscopy, GLUT4 has been localized to several elements of the recycling pathway including the *trans*-Golgi network (TGN), clathrin-coated vesicles, and endosomes. However, the vast majority of GLUT4 (~60%) is found in tubulo-vesicular elements clustered in the cytoplasm, often just beneath the cell surface (20,21).

Although it has been difficult to distinguish the GLUT4 compartment from other elements of the constitutive recycling pathway, several observations suggest that it represents a separate compartment, which we will subsequently refer to as the GLUT4 storage compartment. Such observations include the following. First, double-label immunofluorescence microscopy in 3T3-L1 adipocytes has revealed differential intracellular targeting of GLUT1 and GLUT4 (22). Second, internalization of transferrin conjugated to horseradish peroxidase in adipocytes and its subsequent ablation using diamino benzidine chemistry has shown that endosomal recycling proteins, such as the transferrin receptor and cellubrevin, can be specifically ablated, whereas a large fraction of GLUT4 cannot (23). Third, vesicle immunoadsorption studies have revealed subpopulations of vesicles in adipocytes, some of which are enriched in GLUT4 but not endosomal markers, while others are enriched in both GLUT4 and endosomal markers (22,24–26). Finally, glycerol gradient centrifugation has been used to identify a population of small GLUT4-positive vesicles in adipocytes that are segregated from endosomes (27).

Three additional observations also support the notion that the GLUT4 storage compartment is a separate entity from the endosomal recycling system. First, at least one other protein, the aminopeptidase vp165, has been found to co-localize identically with GLUT4 in muscle and fat cells, hence implying that the GLUT4 storage compartment contains other pro-

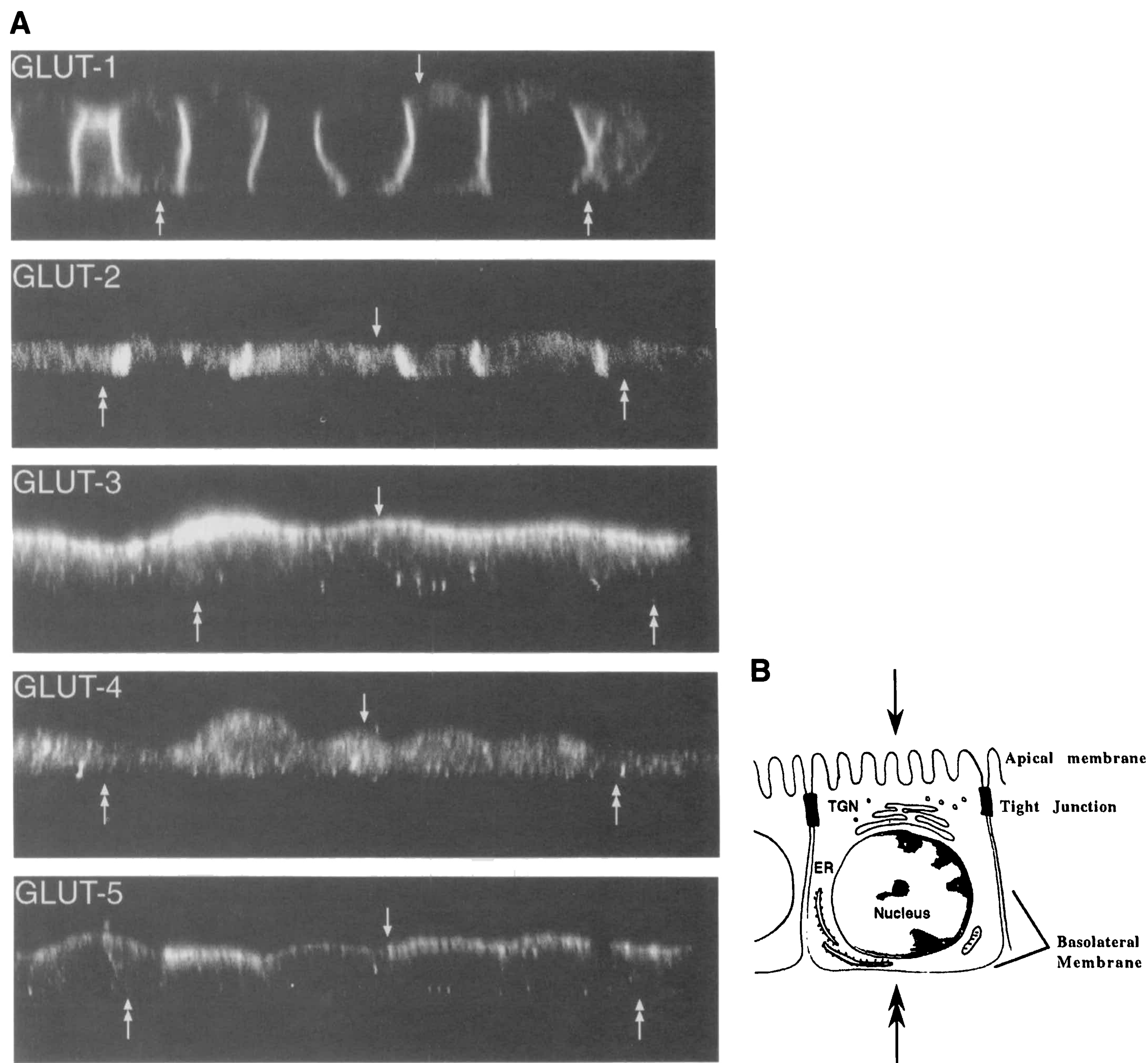

FIG. 2. *A*: GLUT 1–5 show a heterologous subcellular distribution when expressed in polarized epithelial cells. Madin Darby Canine Kidney (MDCK) cells express high levels of the facilitative glucose transporter GLUT1. To compare the distribution of endogenous GLUT1 with other GLUT isoforms, cells were stably transfected with GLUTs 2–5 as previously described (89). Cells were then fixed and immunolabeled with antibodies specific for each isoform. Shown is an X-Z image generated by confocal laser immunofluorescence microscopy, illustrating the heterologous distribution of these molecules. Although all images are of the same magnification, it is evident from the figure that MDCK monolayers exhibit height variation both within and between clonal cell populations. Single-headed arrows mark the apical surface, while double-headed arrows mark the basal surface. GLUT1 and -2 are enriched in the basolateral membrane, GLUT3 and -5 in the apical membrane, while GLUT4 is highly enriched in intracellular structures. At higher magnification, these structures are tubulo-vesicular in nature (89). The intracellular staining seen for GLUT2 was present in control sections (89). *B*: sketch of an X-Z sectioned MDCK cell. Tight junctions, which normally function to maintain distinct compartmental boundaries and restrict mixing of apical and basolateral membrane proteins, are shown. ER, endoplasmic reticulum.

teins aside from GLUT4 (28–30). Second, by incubating permeabilized adipocytes with functional SNARE domains, it has been feasible to uncouple the insulin-dependent translocation of GLUT1 and GLUT4, indicating that they arise from different populations of vesicles (S. Rea, D. James, unpublished observations). Finally, GLUT4 is enriched in regulated secretory granules when expressed in endocrine cells, indicating that it must contain sorting signals that enable it to be distinguished from other recycling proteins (31,32).

The above observations reinforce the finding that while insulin causes a two- to fourfold increase in cell surface levels of many recycling proteins, including the transferrin

receptor, the mannose 6-phosphate receptor, and α2-macroglobulin, the translocation of vp165 and GLUT4 is considerably greater (10- to 40-fold) (reviewed in 33). This dichotomous effect presumably depicts the major function of the intracellular GLUT4 storage compartment, which is to sequester GLUT4 and the other co-inhabitants of this compartment in the basal state and to facilitate their rapid access to the cell surface in response to stimuli such as insulin or exercise. Elucidation of the signal transduction pathways that insulin utilizes to regulate these trafficking pathways is of major interest and several proteins including IRS1, phosphatidylinositide 3′-OH kinase, and protein kinase B have been implicated. These molecules and their regulation by growth factors have been thoroughly covered in recent reviews (34,35). The remainder of this review will focus on GLUT4 trafficking and the types of molecules that regulate the intracellular localization of GLUT4 in adipocytes.

Formation of the GLUT4 storage compartment. There has been relatively little progress in mapping the biogenesis of the GLUT4 storage compartment largely because of the lack of specific cell surface labeling techniques. However, with the availability of exofacial tags in GLUT4 (36) or the use of other proteins in the GLUT4 compartment as reporters, progress on this front is imminent. It is known that like other recycling molecules, GLUT4 is internalized via clathrin-coated pits and at some stage transits through a sorting endosome (20). However, the intervening steps are not clear at present. One can envisage at least three models for the entry of GLUT4 into the intracellular storage compartment after leaving the plasma membrane (see Fig. 3).

Model A. The GLUT4 storage compartment may bud directly from endosomes. This is supported by the differential overlap between endosomal proteins and GLUT4 (23) and also by the potential role of Rab4 (see below) in GLUT4 trafficking (37,38). Electron microscopy (EM) studies have clearly revealed GLUT4 clustered in tubulo-vesicular elements adjacent to sorting endosomes, consistent with a specific sorting step at this location (20). Furthermore, the PI3-kinase inhibitor wortmannin disrupts both GLUT4 trafficking and endosomal sorting, consistent with a common modality of action (39). Finally, unique coats, the material that orchestrates vesicle budding, have been identified on endosomal tubules (40).

Model B. GLUT4 may be internalized via a separate endosome, compared with other recycling proteins. This model has recently been proposed for the reformation of synaptic vesicle–like membranes in PC12 cells (41). A characteristic feature of the specialized endosome in these cells is that traffic out of this compartment is blocked at low temperature. Intriguingly, the prolonged incubation of adipocytes at 18°C inhibits insulin-stimulated GLUT4 translocation, and this may be consistent with this model (42). However, it is also possible that in model A the specific sorting of GLUT4 out of the recycling endosome is blocked at low temperature. In addition, model B does not account for the colocalization between GLUT4 and recycling proteins in intracellular membranes (23). Hence, unless there is mixing between these endosomes subsequent to their formation, it is difficult to rationalize this model at present.

Model C. GLUT4 might traffic back to the TGN, and the specific GLUT4 storage compartment may bud from this structure. By EM, GLUT4 is enriched in the TGN in all insulin-sensitive cells (20,21,43). It has recently been shown that a large proportion of GLUT4 enters ANF-containing secretory granules in atrial cardiomyocytes, apparently at the level of the TGN (32). This did not appear to represent newly synthesized GLUT4, suggesting that a large proportion of GLUT4 recycles via the TGN in these cells. Furthermore, there is considerable overlap between GLUT4 and the Golgi-specific coat subunit AP1 (23). However, since AP1-containing coated vesicles are thought to fuse with endosomes and since the Golgi may be located at some distance from the majority of the cell surface in skeletal myocytes and adipocytes in vivo, model C is debatable. In addition, although vp165 and GLUT4 co-localize in tubulo-vesicular elements of atrial cardiomyocytes, vp165 unlike GLUT4, does not appear to be enriched in either the ANF granules or the TGN of these cells (28).

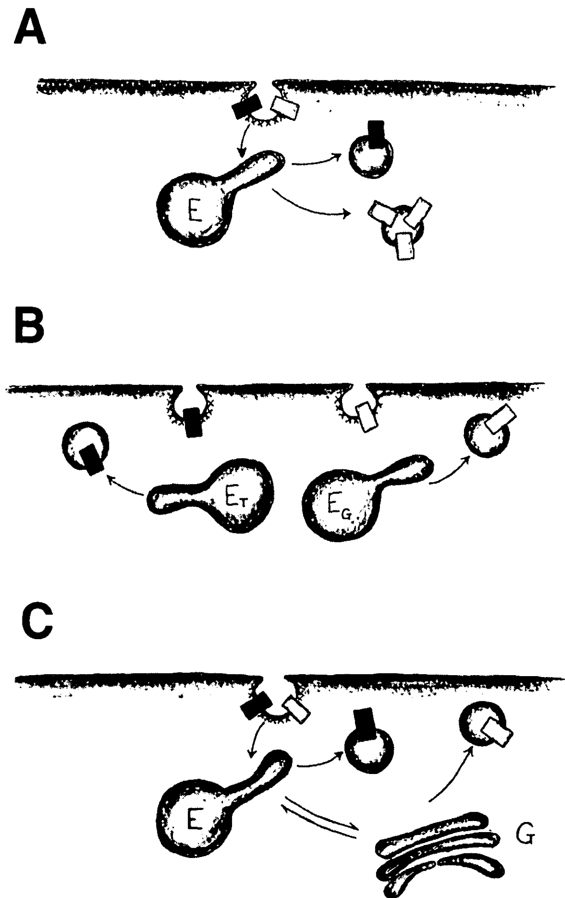

FIG. 3. Putative models for the biogenesis of the GLUT4 storage compartment in insulin-sensitive cells. Each model (A–C) compares the trafficking of GLUT4 (□) to a constitutively recycling protein such as the TFR (■) throughout the endosomal (E) and/or Golgi (G) systems. While models A and C suggest that GLUT4 and TFR traffic through the same endosome, model B suggests that each molecule may traffic via distinct endosomes (E_T and E_G).

An additional possibility is that the GLUT4 storage compartment may emanate from both endosomes and the TGN. Two arguments lend support to this notion. First, recent studies have shown that sorting signals regulating internalization of membrane proteins from the cell surface may also facilitate sorting in the Golgi (44). Similarly, the signal in GLUT4 that mediates its entry into the GLUT4 storage compartment might also be operative at two sites, possibly functioning at both the

TGN and endosomes. Second, a consideration of the gross architecture of fat and muscle cells reveals that both cell types are extremely compartmentalized. White adipocytes exhibit a characteristic signet shape, with the nucleus at one pole and a thin rim of cytoplasm extending around a large lipid droplet. Likewise, skeletal muscle is a syncytium composed predominantly of densely packed myofibres interspersed with an elaborate sarcoplasmic reticulum. Nuclei are scattered on the periphery of the myocyte and deep invaginations connect the interior of the cell with the extracellular milieu. Hence, in some places of the cell, the regeneration of the GLUT4 storage compartment may occur independently of the Golgi in a manner analogous to the regeneration of small synaptic vesicles (SSVs) in neurons. In the latter cells, the specialized architecture of the neurite dictates the need for a Golgi-independent vesicle recycling system.

GLUT4 trafficking signals. What types of motifs do GLUT4 molecules possess to maneuver themselves throughout the intracellular labyrinth of tubules and vesicles, and how is it they are distinguished from the many other membrane proteins that are also shuttling through these compartments simultaneously? Based on our current knowledge of GLUT4 trafficking in adipocytes (see Fig. 3), there should be at least three signals: one to facilitate GLUT4 entry into coated pits at the cell surface, another involved in TGN sorting, and a third that mediates biogenesis of the GLUT4 storage compartment. By studying the distribution of chimeric, truncated, or point-mutated transporters, two discrete motifs have been identified in GLUT4, both of which regulate internalization from the cell surface. Intriguingly, both motifs resemble well-described targeting motifs that have been identified in numerous other recycling proteins.

The first is an aromatic-based motif found in the GLUT4 N-terminus (FQQI). Mutation of the phenylalanine in this motif to alanine results in an accumulation of GLUT4 at the cell surface, and this is at least in part due to impaired entry of this mutant into cell-surface clathrin-coated pits (45). Furthermore, the FQQI motif functions as an internalization signal in the context of heterologous reporters (46). The second signal comprises a di-leucine motif in the cytoplasmic C-terminus of GLUT4 (47). Mutation of these residues to alanines also leads to accumulation of GLUT4 at the cell surface under steady-state conditions but, distinct from the FQQI mutant, only when the mutant transporter is expressed at high levels (48,49). Regardless of expression levels, however, it is clear that mutation of this di-leucine motif impairs GLUT4 internalization (49). Interestingly, the di-leucine signal is also apposed to the major phosphorylation site in GLUT4 (serine 488), and it has been shown that mutation of this residue impairs GLUT4 internalization in Chinese hampster ovary (CHO) cells (50). This site is phosphorylated by cAMP-dependent protein kinase (51), so it remains to be determined whether phosphorylation of this residue by counter-regulatory hormones that act through cAMP, such as catecholamines and glucagon, play a role in modulating glucose transport in insulin-sensitive cells by altering the rate of GLUT4 endocytosis.

Based on the kinetic and morphological analysis of each of the mutants that have been characterized so far, it is unlikely that either the FQQI or di-leucine motifs regulate entry of GLUT4 into the GLUT4 storage compartment. Hence, it seems likely that GLUT4 must contain a separate motif that facilitates this function. It has been suggested that this signal might exist in the cytoplasmic C-terminus of GLUT4. Because vp165 is also targeted to the GLUT4 storage compartment, a similar targeting motif might be expected to exist in this protein. Interestingly, there is a short stretch of residues in the cytoplasmic tail of vp165 that shows some homology with the extreme C-terminus of GLUT4 (30).

The cytoplasmic tail of vp165 does not contain an aromatic-based targeting signal similar to that found in the GLUT4 N-terminus. How this protein is internalized and whether it traffics identically to GLUT4 is currently under investigation. Two di-leucine motifs, including one apposed to the putative GLUT4 storage compartment localization signal, are present in the cytoplasmic tail of vp165. It is possible that one or both of these fulfill an internalization role. The possibility also exists that the trafficking pathways of vp165 and GLUT4 may not be identical. The localization experiments in atrial cardiomyocytes, outlined above, support this notion. An additional and enticing possibility is that when in an appropriate environment, vp165 and GLUT4 may hetero-oligomerize, and such an interaction might therefore facilitate the formation of the GLUT4 storage compartment. This idea is in some ways reminiscent of studies in neurons where it has been shown that many of the membrane proteins of SSVs can be co-immunoprecipitated when solubilized in nonionic detergents (52). Hence, self-assembly may be a general mechanism for compartmental biogenesis in eukaryotic cells.

MOBILIZING THE GLUT4 STORAGE COMPARTMENT: THE ROLE OF SNARES

So far we have described evidence to suggest that in insulin-responsive tissues, GLUT4 is stored in a unique compartment that is segregated from both the endosomal and biosynthetic pathways. Furthermore, it appears that entry into this compartment requires a specialized sorting event that probably occurs in the endosome and/or at the TGN. Despite the uncertainties in its biogenesis, several attractive features are associated with storing GLUT4 in a segregated compartment. Notably, the compartment itself could be made mobile, its mobility could be regulated by factors such as insulin, and such a mobile compartment could be made to fuse directly with the cell surface. The controlled release of small synaptic vesicles in neurons provides a paradigm for this type of regulated storage compartment. These tiny vesicles freight neurotransmitters and specific membrane proteins to the surface of presynaptic neurons in response to various depolarizing stimuli (reviewed in 3–5). Several molecules have been identified that specifically mediate the targeting, docking, and fusion of SSVs with the neuronal plasma membrane. Recently, homologues of many of these molecules were identified in both adipocytes and myocytes, and it is now becoming apparent that the trafficking of the GLUT4 storage compartment in response to insulin and/or exercise is highly analogous to SSV exocytosis. For the remainder of this review, we will discuss evidence suggesting that the GLUT4 storage compartment is in fact a regulated vesicular compartment. Concurrently, we will describe some of the molecular circuitry that appears to facilitate docking and unloading of GLUT4 vesicles with their primary port of call, the plasma membrane.

The SNARE hypothesis. The identification of SNARE proteins in 1993 heralded a turning point in understanding how vesicles traffic to specific compartments (53). Before this time, only the cytosolic proteins NSF and αSNAP were

known to play a joint and generic role in numerous membrane trafficking events (54–56). However, by constructing an affinity column with both proteins, Rothman and colleagues (53) identified the first of a related group of membrane receptors for these factors from detergent solubilized brain extracts. Two of the proteins they identified had previously been localized to the plasma membrane of presynaptic neurons and another to SSV membranes. This provided the enticing possibility that different sets of proteins found in different membrane compartments were capable of interacting in a highly specific way, much like receptors and ligands. The SNARE hypothesis was subsequently formulated, and it proposed that for all membrane trafficking events, a high-affinity match between a ligand in a transport vesicle (v-SNARE) and a receptor in the target membrane (t-SNARE) would be required to consummate a docking and fusion reaction. In the case of regulated exocytosis in neurons, the v- and t-SNAREs were VAMP2 and Syntaxin 1A/SNAP-25, respectively.

A corollary of the SNARE hypothesis is that there will be large gene families of v- and t-SNAREs, essentially one set for each eukaryotic vesicle transport reaction. Consistent with this notion, several techniques have led to the discovery of numerous Syntaxin, SNAP-25, and VAMP homologues, and the mammalian Syntaxin family alone appears to contain at least 15–20 members (57). Initial attempts at localizing these different proteins also support the prediction that SNAREs will be found at discrete intracellular locations.

The fundamental requirement for SNARE proteins in vesicular transport reactions of eukaryotic cells is illustrated by studies from yeast and the mammalian synapse. In yeast, mutation of discrete SNARE proteins results in highly specific transport defects. Mutating yeast plasma membrane Syntaxins, for example, leads to an accumulation of secretory vesicles between the Golgi and the cell surface, yet traffic between other organelles is not disrupted (58). In the neuron, the clostridial neurotoxins tetanus and botulinum irreversibly block SSV exocytosis by specifically proteolyzing VAMP2, Syntaxin1A, or SNAP-25 (59). Despite the overwhelming evidence for the critical function of SNARE proteins in vesicle transport events, it is apparent that these molecules do not act alone. As discussed below, additional molecules act to either positively or negatively regulate the assembly of SNARE proteins.

v-SNAREs of the GLUT4 storage compartment. In light of their resemblance, it has come as no surprise to see that the types of molecules involved in the insulin- and exercise-stimulated release of GLUT4 are very similar to factors that are involved in SSV exocytosis. In some cases, these proteins are identical. Several SNARE and SNARE-binding proteins have been identified that play critical roles in the stimulated translocation of GLUT4 to the plasma membrane.

The expression of neuronal VAMP homologues in adipocytes was first reported by Lienhard and colleagues (60). It was subsequently shown that adipocytes express both VAMP2 and an additional v-SNARE cellubrevin (23,61). Paradoxically, both proteins appeared to co-localize with GLUT4 in adipocytes (61). However, a specific role for VAMP2 in GLUT4 trafficking was recently proposed based on the use of the transferrin-conjugated horseradish peroxidase ablation technique in 3T3-L1 adipocytes (23). Whereas the majority of cellubrevin was in endosomes, consistent with previous localization studies (62), a large fraction of VAMP2 was targeted to the GLUT4 storage compartment (23). Moreover, synthetic peptides that comprise unique VAMP2 domains block GLUT4 exocytosis but not the constitutive trafficking of GLUT1 in permeabilized 3T3-L1 adipocytes (L. Martin, D. James, unpublished observations). Hence, a plausible model based on these data is that cellubrevin mediates the constitutive endosomal trafficking of GLUT4, while VAMP2 specifically regulates the docking of GLUT4 vesicles in response to insulin and exercise stimulation. This model is appealing since it provides scope for the differential effects of insulin on endosomal trafficking and mobilization of the GLUT4 storage compartment, respectively.

What and where is the t-SNARE? Among the Syntaxins thought to regulate recycling through the endosomal system, only Syntaxins 1A and 4 have been shown to bind to VAMP2. Syntaxin 1A is predominantly expressed in neurons (53,63), but is absent from insulin-sensitive cells. Syntaxin 4, however, is expressed at high levels in fat and muscle cells and is predominantly targeted to the plasma membrane (64,65). A functional role for Syntaxin 4 in GLUT4 trafficking was recently established when it was shown that the introduction of either a recombinant fusion protein encoding the cytoplasmic tail of Syntaxin 4 or antibodies directed against Syntaxin 4 specifically blocked insulin-stimulated GLUT4 translocation in permeabilized 3T3-L1 adipocytes (64,65).

The reason for insulin-sensitive cells employing the same v-SNARE as neurons, but a different t-SNARE, is not completely understood, but it may reflect the differences in the types of signals used to activate fusion in each system. In neurons, SSV exocytosis is triggered by an elevation in cytosolic calcium. During this process, the calcium binding protein synaptotagmin, as well as N and P/Q type calcium channels, directly interact with Syntaxin 1A (reviewed by 66). No direct link between insulin-regulated GLUT4 translocation and intracellular calcium has been shown. Hence, the types of molecules that interact with Syntaxin 4 in adipocytes are likely to be quite different to those that regulate the function of Syntaxin 1A in neurons. Interestingly, calcium has been implicated in exercise-induced glucose transport in skeletal muscle. Furthermore, exercise and insulin appear to regulate glucose transport via distinct mechanisms in this tissue (67,68). It is conceivable that these differences arise from the use of different sets of SNAREs. One prediction then is that muscle will display a dual expression pattern of SNAREs, one set more analogous to those found in neurons and the other set more like those found in adipocytes.

The NSF debate: what is the bottom line? Originally, Rothman and colleagues proposed that NSF and αSNAP could play a role in the docking and fusion step of vesicle transport. They suggested that the ATPase activity of NSF might in fact catalyze the fusion reaction by either disrupting the SNARE complex or conformationally destabilizing it (53). Recent studies, however, have placed the function of NSF much earlier in the transport reaction and indeed suggest that the major function of this protein occurs before vesicle docking. The most convincing support for this altered role of NSF has come from the work of Mayer et al. (69) studying homotypic fusion in the yeast vacuole. In this system, it appears that both NSF and αSNAP cooperate to activate v- and t-SNAREs independently into a docking competent state. Additional molecules then function to direct the interaction of the v- and t-SNAREs into a fusion-competent SNARE complex.

Regardless of the precise action of NSF, what is apparent from both models is that regulating the interaction of SNAREs is critical for controlling vesicle fusion. Several molecules have been discovered that directly and specifically modulate the interaction of SNARE partners. Studies from independent systems converge on distinct families of proteins with conserved SNARE binding functions. Importantly, homologues of these proteins have been discovered in fat and muscle cells, some of which specifically dictate the interaction of VAMP2 with Syntaxin 4.

SNARE-binding proteins implicated in GLUT4 exocytosis. In the neuron, several Syntaxin-binding proteins have been identified. In addition to synaptotagmin and the specific calcium channels mentioned earlier, these include Munc-13, Munc-18, and SNAP-25 (reviewed in 3–5). Homologues of the latter two proteins have been identified in adipocytes. Although the precise role of many of these proteins has yet to be elucidated, in the case of Munc-18 and SNAP-25, it appears that these two proteins modulate vesicle docking efficiency by directly regulating the availability of Syntaxin (70).

In neurons, SNAP-25 is anchored to the plasma membrane via palmitoylation, and alone it exhibits a high affinity for Syntaxin 1A (71). When both proteins are mixed with VAMP2 in vitro, these three proteins form a tight complex that is SDS resistant (72). Hence, it appears that SNAP-25 is a positive modulator for the interaction between VAMP2 and Syntaxin. Munc-18a is a peripheral membrane protein that binds to Syntaxin 1A in neurons. This interaction has been reconstituted in vitro and shown to reduce the affinity of Syntaxin 1A for VAMP2 (70). Interestingly, protein kinase C, which stimulates calcium-dependent exocytosis in various types of secretory cells, phosphorylates Munc-18a in vitro, thus preventing its interaction with Syntaxin 1A (73). Hence, it appears that in contrast to SNAP-25, Munc-18 may be a negative regulator of vesicle docking.

The inability to detect significant expression of SNAP-25 in adipocytes drew attention to another recently described SNAP-25 homolog, known as Syndet (74). Syndet is ubiquitously expressed, and it interacts with Syntaxin 4 in vitro (S. Rea, D. James, unpublished observations). It is also expressed at high levels in mouse adipocytes, and in these cells it has a distribution that is indistinguishable from Syntaxin 4 (74). Peptides based on the C-terminus of Syndet inhibit GLUT4 translocation (S. Rea, D. James, unpublished observations). The role of Syndet in the trafficking of GLUT4 within muscle cells is less certain. Evidence suggests that there may in fact be an additional Syndet-like isoform in this tissue, which is also distinct from SNAP-25 (75).

Recently, it was shown that there are at least three different Munc-18 isoforms participating in the endosomal recycling system of adipocytes (Munc18a-c) (64). Of these, Munc-18c appears to be linked to GLUT4 exocytosis. Munc-18c is expressed at high levels in adipocytes, and despite having no membrane attachment domains, it is primarily targeted to the cell surface. In fact, its subcellular distribution is indistinguishable from that of Syntaxin 4 (64). In addition, Munc-18c reduces the interaction between Syntaxin 4 and VAMP2 in vitro (64) similar to the effect observed for Munc-18a on the interaction between Syntaxin 1A and VAMP2 (70). These data suggest that Munc-18c may play a pivotal role in the insulin-regulated movement of GLUT4 and provides further support for the similarity between the regulation of GLUT4 exocytosis in fat cells and synaptic vesicles in neurons.

Modulating the availability and affinity of Syntaxin 4 for VAMP2 by the modification of key regulatory proteins like Munc-18c and Syndet provides an attractive model for how insulin and exercise might stimulate the fusion of GLUT4 vesicles with the cell surface. In Fig. 4, we have presented a model illustrating how SNARE proteins and the regulation of their association might be involved in GLUT4 trafficking after insulin stimulation. In line with the terminology used to describe the SSVs of neurons, it is evident from this figure that the GLUT4 storage compartment may in fact be better referred to as GLUT4 storage vesicles (GSVs).

SNAREing the facts: a convergence of multiple themes. From the above discussion, it is evident that the insulin-dependent movement of GLUT4 in muscle and adipocytes shares many morphological and biochemical features with synaptic vesicle exocytosis in neurons. Indeed, it was the recognition of this fact several years ago that catalyzed the search for neuronal SNAREs and other synaptic vesicle proteins in adipocytes. This search has of course been very fruitful, and it led to the realization that there is a remarkable conservation between both systems. Several regulated recycling systems of physiological significance have also been recognized in other cell types, most notably the vasopressin-regulated water channel system in kidney (76) and the H,K-ATPase in gastric parietal cells (77). It is highly likely that these systems will also utilize similar types of proteins to those that have been described above. With regard to GLUT4 trafficking, only a handful of molecules involved have thus far been elucidated. However, by extending work from the mammalian synapse, it is clear that many more are involved and some of these will likely intersect with the insulin signal transduction pathway. The v-SNARE binding protein synaptophysin is an attractive candidate. Although existing isoforms have not been detected in fat or muscle, in neurons this protein has been shown to bind the N-terminus of VAMP2. In addition, synaptophysin binding to VAMP2 is mutually exclusive to Syntaxin 1A binding (78). One function of Synaptophysin, then, might be to sequester VAMP2 from the actions of NSF and αSNAP until an appropriate stimulus is provided. A similar function may also apply to another relatively poorly characterized VAMP2 binding protein VAP-33 (79).

The Rab family of small GTP-binding proteins are known to play an important role in vesicle trafficking. More than 30 Rabs have been identified, and like SNARE proteins, these molecules function at specific transport steps throughout the cell (80). While their removal results in transport specific defects, the precise role of Rab proteins in vesicular trafficking is only just becoming understood. Recent studies suggest that these proteins function after NSF and αSNAP to catalyse the union of v- and t-SNAREs in the vesicle docking step (81). In the yeast vacuole, this function is dependent upon additional factors (82), and presumably the same will apply to all transport steps involving Rabs. Lupashin and Waters (83) recently showed that Ypt1, a Rab-like molecule involved in yeast ER to Golgi trafficking, directly bound the Syntaxin homolog Sed5 in vitro and in vivo. Interestingly, Sly1p, a Munc-18 homolog, modulated this interaction. A weak but reproducible interaction was also detected between Ypt1 and the endoplasmic reticulum of v-SNARE Sec22p in vitro. Hence, in line with the GDP-GTP switching function of G-pro-

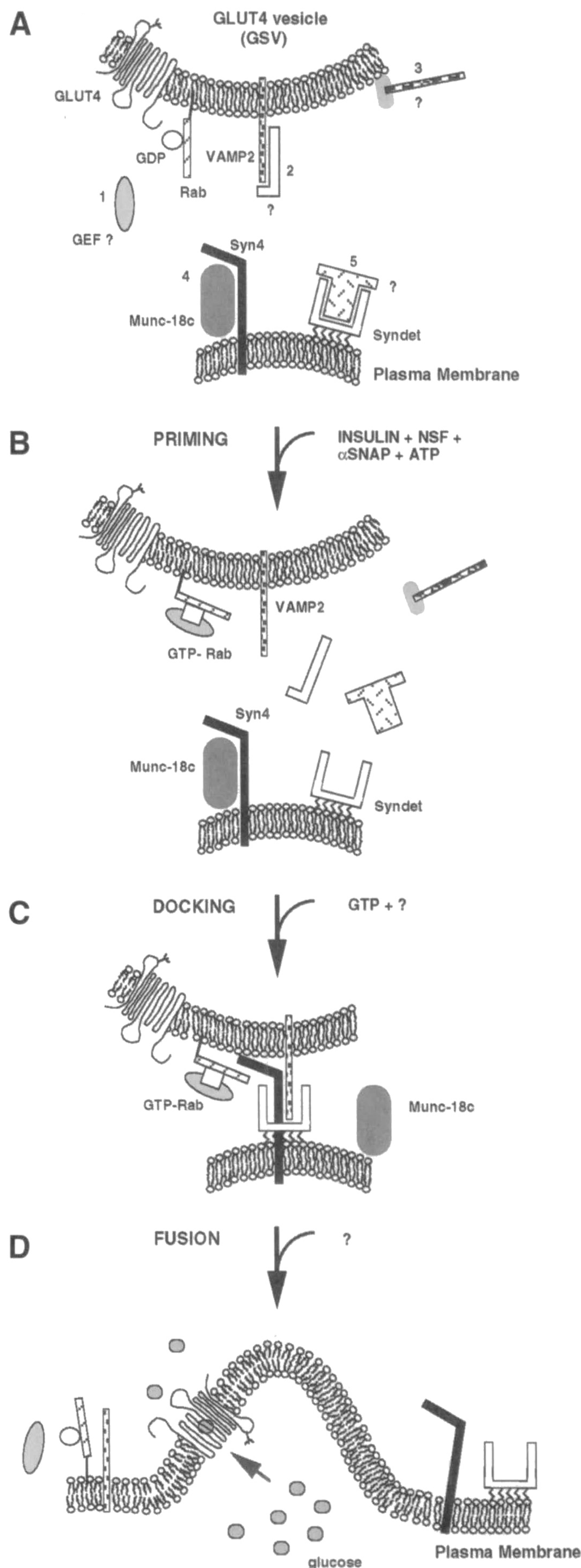

FIG. 4. The potential role of SNARE proteins in GLUT4 trafficking: insulin modulates SNARE availability. Evidence suggests that the GLUT4 storage compartment traffics directly to the cell surface in response to insulin (see text for details). Vesicle targeting, docking, and fusion are mediated by SNARE proteins. Various molecules appear to modulate the fidelity of these interactions and are therefore likely candidates for insulin regulation. Based on the neuronal system, the following scheme of events is proposed for the insulin-dependent translocation of GLUT4 to the cell surface. *A*: in the noninsulin stimulated state, the core SNARE proteins VAMP2, Syntaxin 4, and Syndet are prevented from interacting. Additional molecules predicted to be important for directing the interaction of SNAREs are also in an inactive state. Numbers indicate potential sites of insulin action and include: *1*) activation of a GLUT4 vesicle specific Rab GTP exchange factor (GEF); *2*) release of a putative VAMP2 blocking protein; *3*) detachment of GLUT4 vesicles from the actin cytoskeleton (Spectrin has been implicated in tethering the GLUT4 storage compartment to actin filaments in the basal state [90]); *4*) modulation of the affinity of Munc-18c for Syntaxin 4; and *5*) release of a putative Syndet blocking protein. *B*: following the action of insulin, NSF and αSNAP prime v- and t-SNAREs into a docking competent state in an ATP-dependent manner. GEF loads the GLUT4 vesicle rab with GTP and the latter protein adopts an activated conformation and/or recruits additional molecules to the vesicle surface (not shown). *C*: free of the cytoskeleton, GLUT4 vesicles may be actively transported to the cell surface where a SNARE complex is assembled. GTP-Rab displaces Munc-18c from Syntaxin 4. This may be the rate limiting step in formation of the SNARE complex. *D*: following SNARE docking, several additional steps may be necessary for lipid mixing and bilayer fusion. Lipid kinases and other GTPases are probably involved. Ultimately, insulin leads to an increase in the number of GLUT4 molecules at the cell surface and to the net rate of glucose transport into the cell. Question marks (?) refer to unidentified and/or hypothetical steps and proteins.

teins, the emerging picture is that Rabs act to accelerate productive SNARE complex formation and in this way increase vesicular traffic.

Several low molecular weight GTP-binding proteins have been observed in GLUT-4 enriched vesicles by radioactive overlay (84). Many of these activities are likely to be of endosomal origin and, hence, involved in the general recycling of GLUT4. Three independent studies support a specific role for Rab4 in the insulin-dependent release of GLUT4. Le Marchand-Brustel and colleagues (37) showed that transient co-expression of Rab4 with an epitope-tagged GLUT4 in rat adipocytes led to the enhanced retention of GLUT4 in the basal state, but had no effect on the insulin dependent release of GLUT4. Paradoxically, when overexpressed at ~200-fold endogenous levels, in addition to decreasing the amount of GLUT4 at the cell surface, Rab4 also blocked the insulin-induced recruitment of GLUT4 to the cell surface. These results suggest that Rab4 functions in both the genesis of the GLUT4 storage compartment and in directing GLUT4 to the cell surface in response to insulin. Overexpression of Rab4 resulted in an increase of the cytosolic form of this protein, and the inhibitory effect observed was probably due to sequestration of an insulin-activated signaling molecule. In support of this idea, it was also shown that a Rab4 mutant lacking the membrane anchoring geranylgeranylation site had an identical effect to the wild-type Rab4 when overexpressed at high levels. In addition, Kojima and colleagues (38) have recently shown that electroporation of a peptide corresponding to the hypervariable carboxy terminal domain of Rab4 into rat adipocytes inhibits insulin-stimulated GLUT4 translocation by ~50%. Inhibition ranged from 30–90%, and the differences were attributed to electroporation efficiencies.

Independent studies also support a role for Rab4 in the biogenesis of the GLUT4 storage compartment. As in the case of rat adipocytes, heterologous co-expression of Rab4 and GLUT4 in Xenopus oocytes (85) leads to an increase in the intracellular retention of GLUT4 in these cells. In this system, as well as in the two mentioned above, the expression of related Rabs or use of peptides based upon them were without effect on GLUT4 trafficking, seemingly implying that the effect of Rab4 overexpression on GLUT4 movement is specific.

The above findings are, however, in striking contrast to the original studies characterizing Rab4 overexpression in CHO cells. There, it was reported that the recycling transferrin receptor (TFR) was predominantly retargeted from the endosome to the cell surface (86). One explanation that is consistent with both sets of observations is that when overexpressed, Rab4 rapidly leads to the retrieval of both GLUT4 and the TFR into the recycling endosome. However, whereas TFR subsequently traffics to the cell surface, the GLUT4 storage compartment might bud directly from the recycling endosome (Fig. 3*A*). Under normal circumstances, Rab4 might be passively carried into GLUT4 vesicles and there its hyper-variable region inactivated, perhaps through interaction with VAMP2. Among other effects, the addition of insulin would lead to the re-activation of Rab4, and GLUT4 vesicles would then be free to fuse with the cell surface via a Rab4 mediated trafficking event. Consistent with this model, it was recently shown that the activation of PI3 kinase by insulin directly leads to an increase in the GTP loading of Rab4 (87). Furthermore GRP1, a molecule that specifically binds the product of PI3 kinase, phoshoinositide-3,4,5-trisphosphate, was recently discovered in adipocytes and shown to contain a domain that is possibly involved in GTP exchange on small GTP-binding proteins (88). The role of insulin in activating this protein, or molecules like it, remains to be explored.

FUTURE PERSPECTIVES

Trafficking GLUT4 in an insulin- and exercise-responsive manner is evidently a complicated process. The molecules involved encompass general factors used by all endosomal recycling proteins to the highly specific GLUT4 sorting machinery and the specialized targeting, docking, and fusion machinery of the GLUT4 storage compartment. The goal of the next 10 years will be to define these molecules precisely. Ultimately, the hope is to use this information to rationally design insulinomimetic drugs for the treatment of diabetes. Such drugs might conceivably be cocktails of molecules catalyzing different steps of the GLUT4 life cycle so that GLUT4 can be ferried in and out of the cell at desired times.

ACKNOWLEDGMENTS

D.E.J. is a Wellcome Trust Professorial Research Fellow. The authors would like to thank Sharon Clark for critical reading of the manuscript and Timo Meerloo, Wendy Pascoe, and Kerri James for help in preparing Figs. 1, 2, and 3, respectively.

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