Insulin-regulated Glut4 Traffic In Muscle Cells:
A Concerted Action of the Cytoskeleton, Selective Fusion Proteins and Endosomal Sorting Mechanisms

Preface
It is the greatest honour to receive an award in the name of a foremost Canadian woman scientist and mentor. Dr. Jeanne Manery Fisher, Harvard and Rochester educated, biochemist, taught at the University of Toronto from 1932 to 1948. An expert in the physiology of ion transport and the generation of energy, she has inspired my work on insulin regulation of glucose and Na/K-transport in muscle. Jeanne Manery Fisher’s work was supported initially by grants from the Insulin Research Fund, later from the Medical Research Council and the Muscular Dystrophy Association of Canada. Her scientific accomplishments were numerous, as were her efforts on behalf of the recognition of women in the academic world. She worked ardently with the Royal Canadian Institute to promote the cause of science and women’s equality in academia. She was the President of the Toronto Biochemical and Biophysical Society and Chairman of the Equal Opportunities Committee of the Canadian Biochemical Society. She trained numerous students who continued to train the current generation of young researchers, and themselves excelled in their scientific endeavours. We humbly follow in her footsteps.

Introduction
Glucose entering the circulation during a meal is rapidly taken up by muscle and fat cells, for both energy generation and storage as glycogen. Glucose enters all mammalian cells via a family of integral membrane proteins, the glucose transporters of the GLUT family. One of these proteins, GLUT4, is specific to muscle and fat cells, and is distinct from all other transporters in that it undergoes both constitutive recycling (Satoh et al. 1989) and insulin-regulated exocytosis (James et al. 1989). The importance of this phenomenon is highlighted by evidence that insulin-dependent GLUT4 externalization, and hence the stimulation of glucose uptake, is defective in the pathophysiological state of insulin resistance underlying type 2 diabetes (King et al. 1992, Zierath et al. 1996, Zierath et al. 2000). In spite of extensive work, the intracellular GLUT4 donor compartments remain poorly defined, as do the effects of insulin on the route or the velocity of inter-compartmental GLUT4 traffic. This paucity in knowledge is largely due to shortcomings of biochemical approaches to isolate and characterize the diverse intracellular compartments populated by GLUT4, and by the limited intracellular space available in primary fat and muscle tissue and in cultured adipocytes for detailed immunolocalization.


An important protein in the insulin signalling pathway regulating GLUT4 traffic is type 1 phosphatidylinositol 3-kinase (PI3-K) (Kotani et al. 1995), an enzyme that possesses lipid kinase activity and has been demonstrated to interact with all the known insulin receptor substrate (IRS) proteins (Shepherd et al. 1998, White 1998). This enzyme consists of an 85-kDa regulatory subunit, p85, and
a 110-kDa catalytic subunit, p110. The type I, PI3-K enzyme is required for the insulin-dependent mobilization of vesicles containing GLUT4 glucose transporters to the plasma membrane (Cheatham et al. 1994, Kotani et al. 1995, Shepherd et al. 1998).

In addition to inducing translocation of glucose transporters to the cell surface, one of the early events in the insulin response is the reorganization of actin filaments. Two types of insulin-dependent morphological changes in actin filaments have been described, depending on the cell type studied: an increase in actin stress fiber formation (Goshima et al. 1984, Ridley & Hall 1992) and a bundling of actin below the plasma membrane, more commonly referred to as ‘membrane ruffling’ (Ridley & Hall 1992). The actin cytoskeleton participates in a variety of cellular functions including the compartmentalization of intracellular macromolecules and organelles. It is therefore conceivable that insulin-derived signalling intermediaries might redistribute in response to actin reorganization. However, until recently, the link between the actin microfilament network, the location of insulin signalling molecules and the location of GLUT4 organelles have remained poorly understood.

Once at the cell surface, GLUT4 is readily removed to enter the endosomal system. GLUT4 removal from the cell surface occurs via clathrin-coated vesicles (Nishimura et al. 1993) assisted by the GTPase dynamin (Al-Hasani et al. 1998, Kao et al. 1998, Volchuk et al. 1998). Whether or not insulin inhibits GLUT4 endocytosis in fat cells is still debatable (Czech & Buxton 1993, Jhun et al. 1992, Satoh et al. 1993, Yang & Holman 1993). The contribution of exocytic and endocytic pathways to insulin action in muscle cells has not been explored. Transmembrane proteins removed from the cell surface are directed first to the early endosome. From here, proteins destined for recycling will enter the recycling endosome (Mukherjee et al. 1997). This pathway is thought to work in a constitutive fashion but little was known until now about possible regulation of the individual steps, i.e. direction and speed of sorting out of the early endosome, fusion with the recycling endosome and exit from this compartment.

This chapter summarizes results from our laboratory that address the steady state distribution of GLUT4 in basal and insulin-stimulated muscle cells; the rates of externalization and internalization of this transporter; and the mechanisms regulating such traffic. Particular attention is given to the role of the insulin-induced actin remodelling in recruiting signalling molecules and GLUT4 vesicles beneath plasma membrane ruffles, and to the regulation of the transit of GLUT4 through the recycling endosome to reconstitute the specialized vesicle that fuses with the plasma membrane. For these studies we have made use of L6 muscle cells, which differentiate in vitro from myoblasts into multinucleated myotubes by multiple cell fusions (Yaffe 1968). In the myotube stage, these cells contain a large cytoplasmic space rich in actin filaments and are highly amenable to morphological analysis at the light microscopic level. The clonal line used for these studies has been stably transfected with GLUT4 encoding a 14 amino acid myc sequence at its first extracellular loop. This epitope is used to detect the presence of GLUT4 at the cell surface and to follow its journey within the cell.

**GLUT4 compartmentalization and insulin-dependent externalization**

In the L6 skeletal muscle cell line, GLUT4 expression occurs after differentiation from myoblasts into myotubes (Mitsumoto & Klip 1992). We have previously reported that expression of GLUT4myc in L6 myoblasts leads to the segregation of the protein to a GLUT4-specific pool, conferring insulin sensitivity to glucose uptake (Ueyama et al. 1999). This conclusion is based on the finding that in L6-GLUT4myc myoblasts, the intracellular GLUT4myc compartment contains the majority of the insulin-regulatable amino peptidase (IRAP) but less than half of the GLUT1 housekeeping glucose transporter. By expression of GLUT4myc alone the sensitivity of glucose uptake to insulin is markedly improved, compared to parental myoblasts, which do not yet express GLUT4 at this undifferentiated stage.

The exofacial myc epitope of GLUT4myc allowed us to estimate the proportion of this protein exposed at the cell surface and its total cellular content by analyzing intact and permeabilized cells, respectively. The amount of myc epitope exposed at the surface of non-permeabilized cells was determined by a quantitative assay based on the detection of anti-myc antibody bound to a monolayer of L6 cells. The total myc epitope present in L6 myoblasts was determined by permeabilization with 0.1% Triton X-100 before immunolabelling with anti-myc antibody. The primary antibodies bound are then reacted with HRP-conjugated sec-
ondary antibody coupled to a densitometric assay. The intracellular GLUT4myc content was then estimated by subtracting the amount present on the cell surface from the total cellular content. We found that, under basal conditions, 90.0 ± 0.6% of the GLUT4myc resides intracellularly. Stimulation for 30 min with 100 nM insulin elevates the cell surface content of GLUT4myc to 31.0 ± 1.7%, with a commensurate reduction in intracellular GLUT4myc.

We next examined the time course of GLUT4myc appearance at the cell surface in the presence of insulin. To measure GLUT4myc externalization, L6 myoblasts were treated with insulin in culture medium at 37°C for increasing times, and thereafter the cell surface-exposed myc epitope was reacted with anti-myc antibody at 4°C followed by the densitometric detection assay. Insulin triggered a rapid redistribution of GLUT4myc to the cell surface with a t½ of approximately 4 min. This rapid insulin response peaked by 30 min (Fig. 1).

**Insulin-induced actin remodelling and p85 relocalization**

Recent evidence points to the potential participation of the actin cytoskeletal network in the insulin-dependent compartmentalization of GLUT4-containing organelles. Indeed, in response to insulin, actin filaments undergo remodelling in L6 myotubes, leading to the formation membrane ruffles (Tsakiridis et al. 1994). Pretreatment of L6 myotubes or 3T3-L1 adipocytes with cytochalasin D (CD) or latrunculin B (LB), structurally unrelated drugs which cause actin filaments to depolymerize, prevents insulin-stimulated glucose transport (Tsakiridis et al. 1994, Wang et al. 1998b). CD and LB also reduce insulin-dependent translocation of GLUT4 vesicles to the plasma membrane of L6 myotubes, 3T3-L1 adipocytes and primary adipocytes (Omata et al. 2000, Tsakiridis et al. 1994, Wang et al. 1998a, Wang et al. 1998b). These findings suggest that actin reorganization is involved in the insulin-dependent relocalization of GLUT4 from intracellular storage sites to the cell surface and the subsequent stimulation of glucose uptake. In the experiments that follow we employ fluorescence and scanning electron microscopy to compare the spatial and temporal changes in actin, relocalization of PI3-K (p85) and the changes in the localization of the GLUT4 compartment.

L6 myotubes were treated with insulin and subsequently examined for morphological changes of the cell surface. Scanning electron micrographs of the L6 myotube monolayer were obtained after 0, 3 and 10 minutes of insulin stimulation (Fig. 2). In unstimulated myotubes, very little distortion of the dorsal cell surface was observed. At 3 minutes of insulin stimulation, the plasma membrane showed structures resembling membrane ruffles, particularly above the nuclei. By 10 minutes of insulin treatment, the protrusions from the plasma membrane were more pronounced.

We used indirect immunofluorescence to compare the time-dependent distribution of intracellular actin filaments in response to insulin with the membrane ruffling observed in Fig. 2. Under basal conditions (t = 0), rhodamine-phalloidin stained long filamentous structures that were aligned along the longitudinal axis of the cell (Fig. 3, a). Insulin treatment (100 nM) resulted in a rapid reorganization of cortical actin into structures reminiscent of those observed by scanning electron microscopy. The time course and pattern of insulin-dependent cortical actin remodelling (Fig. 3, a-c) was consistent with the dynamics of membrane ruffling observed by scanning electron microscopy.
Comparative analysis of the cellular localization of the p85 subunit of PI3-K with actin filaments over the same time period is illustrated in the center panels of Fig. 3, d-f. In the basal state (t = 0), p85 staining was diffusely punctate. Staining for p85 occurred throughout the myoplasm but was more concentrated in the region of the myonuclei, where it appeared to overlap with actin filaments. Within 3 minutes of insulin stimulation however, a fraction of p85 was found to relocate into the newly formed structures of actin filaments. The detection of p85 in these structures continued to parallel temporally actin staining, becoming most prominent at 10 minutes of insulin treatment. Therefore for all time points of insulin examined, a fraction of p85 staining colocalized with remodelled cortical actin. A similar pattern was observed for the p110α subunit of PI3-K, but not for p110β (results not shown). Neither antibodies against PKC-β or the insulin receptor, nor control antibodies became concentrated with actin at any time after insulin treatment (results not shown).

Given the multiple actions of PI3-K, it is conceivable that changes in its subcellular localization in response to insulin are critical to its ability to elicit the stimulation of GLUT4 translocation and glucose transport. The p85 subunit of PI3-K rapidly associates with the GLUT4-enriched compartment in insulin-stimulated 3T3-L1 adipocytes (Heller-Harrison et al. 1996, Wang et al. 1998b). It was suggested that this interaction involves cytoskeletal elements because pretreatment of cells with CD or LB prevents the subsequent detection of PI3-K on GLUT4-containing membranes (Wang et al. 1998a, Wang et al. 1998b). Conversely, PDGF activates PI3-K at the plasma membrane and does not cause GLUT4 translocation (Nave et al. 1996, Ricort et al. 1996). These results suggest that robust activation of PI3-K alone is not sufficient for glucose transport activation. We propose that the realocalization of PI3-K to actin-rich structures may facilitate the propagation of some PI3-K-dependent signals that are necessary for the translocation of glucose transporters to the cell surface. This hypothesis led us to the following experiments.

**Recruitment by insulin of GLUT4 vesicles into the actin-rich structures**

Given that the p85 and p110α subunits of PI3-K colocalized with the subcortical actin structures formed in response to insulin, we searched for a possible link between actin remodelling and the...
intracellular localization of the GLUT4 protein (Fig. 4). In the basal state, GLUT4myc was concentrated around the myonuclei. GLUT4myc staining remained perinuclear after the first 3 minutes of insulin treatment while actin filaments formed the cortical structures described above. After 10 minutes of stimulation with insulin, a portion of the GLUT4myc immunofluorescence began to colocalize with actin structures. Hence, in contrast to the observations with p85, there was a significant delay in the recruitment of GLUT4 into the cortical actin structures. These findings suggest that as the dorsal actin-rich structures begin to form, GLUT4-containing vesicles are slowly recruited into them, where they come to the vicinity of PI3-K.

**Role of Rac in insulin-dependent actin reorganization and GLUT4myc translocation**

It was previously shown that insulin-stimulated membrane ruffling in Swiss 3T3 cells is mediated by the Rho GTP-binding protein family member, Rac (Ridley & Hall 1992). To test a requirement for Rac in the actin remodelling events mediated by insulin in L6 muscle cells, we transfected a dominant inhibitory Rac1 mutant, Rac1-N17 (Zhang et al. 1995) into L6 myotubes. Cotransfection of Rac1-N17 cDNA with enhanced green fluorescent protein (EGFP) cDNA was performed to facilitate recognition of transfected cells. As expected, expression of Rac1-N17 did not affect the abundance of long actin stress fibres in either the basal or insulin-stimulated states (results not shown). Rac1-N17 expression prevented cortical actin remodelling which was otherwise clearly seen in the adjacent non-transfected cells (Fig. 5). We therefore examined the consequence of Rac1 inhibition on the translocation of GLUT4myc to the cell surface upon insulin stimulation. In the basal state, cells transfected with Rac1-N17 displayed the same density of GLUT4myc staining on the cell surface as adjacent, untransfected cells in the same optical field (not shown). However, the insulin-dependent appearance of GLUT4myc was largely prevented in cells transfected with dominant negative Rac1 (Fig 5). These results suggest that Rac1-dependent cortical actin remodelling may be causally linked to the incorporation of GLUT4-containing vesicles into the plasma membrane in L6 myotubes. However, they do not rule out that the dominant negative Rac1 interferes with GLUT4 traffic by means other than inhibiting the endog-

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**Figure 4.** Insulin causes redistribution of GLUT4myc-containing vesicles to cortical actin structures. L6 GLUT4myc myotubes, without or with insulin stimulation (3 and 10 min) were analyzed by indirect immunofluorescence to detect the distribution of GLUT4myc (using a monoclonal anti-myc antibody coupled to a FITC-labelled secondary antibody, green) and filamentous (F) actin (using rhodamine-labelled phalloidin, red).

**Figure 5.** Dominant inhibitory Rac-1 blocks insulin-dependent cortical actin remodelling and GLUT4myc externalization. L6 GLUT4myc myotubes were transiently transfected with 0.3 µg of enhanced green fluorescent protein cDNA together with 1.2 µg of Rac1-N17 cDNA. After 48 hours, cells were exposed to insulin for 10 (a) or 20 (b). (a) F-actin was detected in permeabilized cells, using rhodamine-phalloidin. (b) Surface GLUT4myc was detected in intact cells using anti-myc antibody coupled to Cy3-labelled secondary antibody.
In particular, it is conceivable that Rac1-N17 may scavenge activators of several small molecular weight G proteins, which in turn may be required for GLUT4 traffic. In this regard, it is possible that G3C, a guanine exchange factor of Rac1 and TC10, among other small molecular G proteins, could be tied up by Rac1-N17 and prevent the GTP loading of TC10. The latter protein has recently been suggested to be required for GLUT4 translocation (Chiang et al. 2000).

**Fusion of GLUT4 vesicles with the plasma membrane requires VAMP2**

Like neuronal synaptic vesicles, intracellular GLUT4-containing vesicles must dock and fuse with the plasma membrane, thereby facilitating insulin-regulated glucose uptake into muscle and fat cells. The backbone of the fusion machinery consists of a v-SNARE protein in the incoming vesicle and two t-SNARE proteins in the target membrane (See Foster & Klip 2000 for review). Specific SNARE isoforms are expressed in muscle and fat cells: the v-SNAREs VAMP2 and VAMP3/cellubrevin (hereafter called VAMP3) (Cain et al. 1992, Volchuk et al. 1994, Volchuk et al. 1995), and the t-SNAREs syntaxin4 (Sumitani et al. 1995, Volchuk et al. 1996) and SNAP-23 (Rea et al. 1998, Wang et al. 1997, Wong et al. 1997). Biochemically, the intracellular GLUT4 colocalizes in part with the vesicle-SNAREs VAMP2 and VAMP3. The participation of syntaxin4 and SNAP-23 in GLUT4 translocation has been implicated using various molecular and biochemical approaches such as introduction of botulinum toxins and neutralizing antibodies into 3T3-L1 adipocytes (see Foster & Klip 2000). Likewise, there is strong evidence for the participation of a vesicle (v)-SNARE in this process. The *Clostridial* tetanus and botulinum B or D neurotoxins specifically cleave and inactivate VAMP2 and VAMP3 (Jahn et al. 1995, Niemann et al. 1994, Schiavo et al. 1992). Introduction of botulinum neurotoxin D into streptolysin O (SLO)-permeabilized 3T3-L1 adipocytes (Cheatham et al. 1996) and microinjection of cytoplasmic VAMP2 soluble peptides and fusion proteins diminished the appearance of GLUT4 at the surface of 3T3-L1 adipocytes (Cheatham et al. 1996, Macaulay et al. 1997, Martin et al. 1998, Olson et al. 1997). While these experiments support the notion of a need for VAMPs in GLUT4 translocation, they do not distinguish which one, VAMP2 or VAMP3, is the protein responsible for GLUT4 arrival at the plasma membrane.

In the following experiments, we used GLUT4myc-expressing L6 myoblasts to compare the functional involvement of VAMP2 and VAMP3 in GLUT4 translocation. Myoblasts were chosen because of the ease to transfect these mononucleated cells. Transient transfection of proteolytically active tetanus toxin light chain (TeTx) cleaved both VAMP2 and VAMP3 proteins. Importantly, the transient transfection of TeTx markedly reduced the insulin-stimulated incorporation of GLUT4myc to the cell surface by about 70% (Fig. 6). Upon co-transfection of tetanus toxin with individual vesicle-SNARE constructs, only toxin-resistant VAMP2 rescued the inhibition of insulin-dependent GLUT4 translocation by tetanus toxin. Moreover, GLUT4 and VAMP2, but not VAMP3, were clustered in the insulin-induced remodelled cortical actin mesh (Fig. 7). We therefore propose that VAMP2 is a resident protein of the insulin-sensitive GLUT4 compartment, and that the integrity of this compartment is required for GLUT4 translocation.

![Figure 6. Tetanus toxin inhibits insulin-stimulated GLUT4myc translocation in L6-GLUT4myc myoblasts. L6-GLUT4myc myoblasts were transfected with 0.6 mg of wild type or toxin-resistant (VW) V2-GFP or V3-GFP cDNA in conjunction with 0.9 µg of pcDNA3 or TeTx cDNA. After 48h, cells were exposed to 100 nM insulin for 30 min. Surface GLUT4myc was detected by immunofluorescence in non-permeabilized cells. Results from several experiments were quantitated using NIH Image software. A value of 100% was assigned to the insulin response above basal in untransfected cells treated with insulin in each field of view. p < 0.01 relative to (*) untransfected cells or VAMP-transfected cells.](image-url)
protein is required for GLUT4-vesicle incorporation into the cell surface in response to insulin.

**Insulin does not delay the removal of GLUT4 from the cell surface**

Once at the cell surface, GLUT4myc undergoes rapid endocytosis. Half of the surface-labelled GLUT4myc is internalized within 3 min (Fig. 8). The rate of GLUT4myc disappearance from the cell surface was not appreciably slowed down in the continued presence of insulin, showing approximately the same $t_{1/2}$ of 3 min. These results suggest that insulin does not regulate GLUT4 internalization in L6-GLUT4myc myoblasts. This contrasts with observations made in fat cells where a small proportion of insulin-induced gain in surface GLUT4 appears to be due to inhibition of GLUT4 endocytosis (Jhun et al. 1992, Yang & Holman 1993).

**GLUT4myc travels through the early endosome to the recycling endosome**

Morphologically, different endosomes are defined by the presence of marker proteins. The Rab5 effector early endosome antigen 1 (EEA1) is a putative tethering protein that helps to bring vesicles in close proximity with the early endosome and is found solely on early endosomes (Mu et al. 1995). Numerous proteins undergo constitutive recycling between the plasma membrane and the recycling endosome. The transferrin receptor (TfR) is responsible for iron entry into the cell via binding to transferrin and is constitutively recycled back to the cell surface (Witt & Woodworth 1978). Myoblasts were again selected for these experiments because they can be manipulated to round up, thereby offering a better opportunity to label and differentiate intracellular organelles by confocal fluorescence microscopy, which otherwise would come too close together in flattened cells.

To follow the transit of GLUT4myc through the early endosome and recycling endosome, surface GLUT4myc labelled with anti-myc antibodies was allowed to internalize at $37^\circC$ for different times up to 20 min. Fig. 9a shows the localization of labelled GLUT4myc and EEA1 at the onset of rewarming. Two min after initiation of internalization, some GLUT4myc could be detected in a compartment positive for EEA1 staining (Fig. 9b) and remained in this compartment for at least 5 min (Fig. 9c). At 10 min, there was no detectable labelled GLUT4myc in the perinuclear, TfR-positive

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**Figure 7. VAMP2, but not VAMP3, colocalizes with the cortical actin structure following insulin stimulation.** L6-GLUT4myc myoblasts were left untreated (A) or stimulated with insulin (B), and processed for indirect immunofluorescence for endogenous VAMP2 or VAMP3 with anti-VAMP2 (top) or anti-VAMP3 (bottom) antibodies, along with F-actin staining using Oregon green (OG)-conjugated phalloidin. Open and closed arrows show recruitment of VAMP2 or absence of VAMP3, respectively. Scale bar: 25 µm.

**Figure 8. Insulin has little effect on the internalization of GLUT4 myc.** L6-GLUT4myc myoblasts were stimulated with 100 nM insulin at $37^\circC$ for 30 min, reacted with anti-myc antibody at $4^\circC$ for 1 h to label cell surface GLUT4myc and then re-warmed to allow endocytosis in the absence or presence of 100 nM insulin for 2 to 60 min. At the indicated times, the myc antibody-labeled GLUT4myc remaining on the surface was measured by the optical densitometric detection assay.
GLUT4myc began to collect there and by 20 min (Fig. 9f) it reached a steady-state where a large portion of the labelled GLUT4myc overlapped with TfR. This distribution remained for up to 30 min.

**Insulin accelerates the transit of GLUT4 through the endosomal system**

GLUT4myc was allowed to internalize in the continued presence of insulin. The hormone did not appear to affect the rate of appearance of surface-labelled GLUT4myc in the EEA1-positive compartment (Fig. 10a-d). As before, in unstimulated cells surface-labelled GLUT4myc was not detected in the recycling endosome by 10 min (Fig. 10a); in the continued presence of insulin, labelled GLUT4myc was detected in the TfR-positive compartment 10 min after initiation of internalization (Fig. 10c). In contrast, labelled GLUT4myc internalized in the absence of insulin required 20 min to be detected in the same compartment (Fig. 10b). Moreover, by 20 min, the labelled GLUT4myc internalized in the continued presence of insulin was no longer detectable in the TfR-containing endosomes (Fig. 10d).

**Figure 9. Internalized GLUT4myc travels through the early and recycling endosomes.** Rounded-up L6 GLUT4myc myoblasts were stimulated with insulin, GLUT4myc was labelled with anti-myc antibody at 4°C, then cells were re-warmed to allow GLUT4myc endocytosis. Confocal fluorescence images are shown of surface-labeled GLUT4myc after 0, 2, 5, 10, 15 and 20 min of GLUT4myc internalization. At the end of each internalization period, GLUT4myc and other antigens were detected by indirect immunofluorescence. Red staining is GLUT4myc, blue staining is DNA, green staining is EEA1 (a-c) or TfR (d-f). Areas outlined by white boxes (a-c) are expanded 4x in insets. Filled arrowheads indicate areas of colocalization (yellow) while open arrowheads highlight areas of non-colocalization. Scale bar: 10 µm.

**Figure 10. Effect of insulin on inter-endosomal GLUT4 traffic.** Confocal micrographs of surface-labeled GLUT4myc after 2 (a, c), 5 (b, d), 10 (e, g) and 20 (f, h) min of endocytosis in the absence (a, b, e, f) or presence (c, d, g, h) of 100 nM insulin (con, ins respectively). Red staining is GLUT4myc, blue staining is DNA, green staining is EEA1 (a, b, c, d) or TfR (e, f, g, h). Filled arrowheads indicate areas of colocalization (yellow) between GLUT4myc and EEA1 or TfR, open arrowheads highlight TfR staining not colocalized with GLUT4myc. Scale bar: 10 µm.
reaction, PI 3,4,5-P$_3$ and/or PI 3,4-P$_2$ may then bind and activate downstream effectors such as Akt/protein kinase B or PKC-λ/ζ to trigger the insertion of GLUT4 vesicles into the plasma membrane. This last step is mediated by the v-SNARE VAMP2 and t-SNAREs on the membrane ruffles.

The left hand side of Fig. 12 illustrates that internalized GLUT4 travels through the early endosome, defined by the presence of EEA1 and progresses to the recycling endosome defined by TfR. Insulin accelerates GLUT4 arrival at the recycling endosome. From the recycling endosome, surface-labelled GLUT4myc would generate specialized exocytic vesicles destined for the cell surface. Our model implies that sorting of GLUT4 likely occurs in the recycling endosome, but does not rule out that a portion of the specialized vesicle pool may form directly from the early endosome.

The results presented raise the hypothesis that insulin input is required at distinct loci in the cycle of GLUT4 traffic. PI3-K (and PKB) appear to be

Figure 12. Model of GLUT4 traffic and sites of insulin input. GLUT4 is internalized in an unregulated fashion from the plasma membrane via clathrin-coated pits. After removal from the plasma membrane, GLUT4 moves to the early endosome characterized by early endosome antigen (EEA1). From the early endosome GLUT4 can travel to the juxtanuclear, recycling endosome marked by transferrin receptor (TfR) or to the specialized vesicles. Transit to the recycling endosome is regulated by a PI 3-K- and PKB-dependent signal from insulin. Once in the recycling endosome GLUT4 is presumably packaged into specialized vesicles and this step may also be accelerated by insulin in a PI3K- and PKB-dependent manner. Insulin can cause cortical actin remodelling through the action of PI3K and a small molecular weight G-protein (SMG, possibly Rac or TC10). Actin remodelling is blocked by cytochalasin D (CD) and latrunculin D (LB). The remodelled actin brings the exocytic GLUT4 vesicles to the vicinity of the plasma membrane containing t-SNAREs, for vesicle incorporation into the membrane.
required for the acceleration of GLUT4 transit through the recycling endosome, as well as to facilitate arrival of GLUT4 vesicles to areas beneath the cell surface, aided by the remodelled actin mesh. VAMP2 on these recruited vesicles would then participate in forming a SNARE complex with the plasma membrane t-SNAREs for the ultimate fusion of the vesicles with the membrane. The overall effect of insulin on inter-endosomal GLUT4 traffic would be to expedite movement of GLUT4 through the endosomal system, culminating in the genesis of the GLUT4 vesicles. These diverse inputs of insulin would provide the cell with a means to maintain levels of plasma membrane GLUT4 in the presence of a continued insulin challenge by regulating the production of plasma membrane–destined GLUT4 vesicles.

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