



Ready, set, internalize: mechanisms and regulation of GLUT4 endocytosis

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Synopsis

The facilitative glucose transporter GLUT4, a recycling membrane protein, is required for dietary glucose uptake into muscle and fat cells. GLUT4 is also responsible for the increased glucose uptake by myofibres during muscle contraction. Defects in GLUT4 membrane traffic contribute to loss of insulin-stimulated glucose uptake in insulin resistance and Type 2 diabetes. Numerous studies have analysed the intracellular membrane compartments occupied by GLUT4 and the mechanisms by which insulin regulates GLUT4 exocytosis. However, until recently, GLUT4 internalization was less well understood. In the present paper, we review: (i) evidence supporting the co-existence of clathrin-dependent and independent GLUT4 internalization in adipocytes and muscle cells; (ii) the contrasting regulation of GLUT4 internalization by insulin in these cells; and (iii) evidence suggesting regulation of GLUT4 endocytosis in muscle cells by signals associated with muscle contraction.

Key words: clathrin, endocytosis, glucose uptake, GLUT4, internalization, muscle cell

INTRODUCTION

Insulin promotes glucose disposal into skeletal muscle, and this is the major mechanism removing dietary glucose from the circulation. Glucose uptake into skeletal muscle fibres occurs primarily through the glucose transporter GLUT4 [1]. GLUT4 is a member of the SLC2 (solute carrier 2) transporter family of facilitated hexose transporters, and it is rather selectively expressed in muscle and fat cells. Extensive study for nearly 30 years of the traffic of GLUT4 in these two cell types has revealed that the transporter is a constitutively recycling membrane protein, and defects in its cycling are associated with insulin resistance [2,3]. Such cycling consists of exocytic movement of the transporter within post-biosynthetic vesicles of endosomal origin towards the plasma membrane, and of endocytic movement from the membrane back to the sorting endosomal system. Therefore it is crucial to understand both the exocytosis and endocytosis of this transporter in order to explore the nature of defects in insulin resistance, and to design strategies to bypass or overcome them.

Inside the cell, GLUT4 is found in endosomal membranes that contain the TfR (transferrin receptor), as well as in an insulin-

responsive TfR-negative GLUT4 storage compartment (reviewed in [3,4]). At steady-state, only 5–10% of the total cellular GLUT4 content is exposed at the cell surface in either adipocytes or muscle cells. Insulin changes the net distribution of GLUT4 within minutes, attaining a new steady-state whereby 20–50% of the cellular GLUT4 content becomes exposed at the surface of muscle or fat cells respectively. Insulin-derived signals have an impact on several stages of GLUT4 exocytosis, including mobilization to the cellular periphery, vesicle tethering, docking and fusion, thereby increasing the cell-surface amount of GLUT4 and the rate of glucose uptake [3,5,6] without the need for on-going protein synthesis. Muscle contraction also increases cell surface GLUT4, and this response is independent of the signals elicited by insulin [7]. Instead, the gain in surface GLUT4 elicited by exercise requires input from AMPK (AMP-activated protein kinase) and Ca²⁺-dependent signals [7]. GLUT4 content at the cell surface is also increased by distinct stressors, notably those associated with reduced energy availability inside the cell [8–10].

Although GLUT4 exocytosis has been studied extensively, GLUT4 endocytosis and its possible regulation had remained less scrutinized. Recent studies have begun to shed light on GLUT4 endocytic mechanisms and their metabolic regulation. The aim

Abbreviations used: AMPK, AMP-activated protein kinase; AP, adaptor protein; BAR, Bin/ amphiphysin/Rvs; CCL-dyn, clathrin- and caveolae-independent, dynamin-dependent endocytosis; CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; CHC, clathrin heavy chain; CHO, Chinese-hamster ovary; CLC, clathrin light chain; CME, clathrin-mediated endocytosis; γ cR, γ -c receptor; CtxB, cholera toxin B; DNP, dinitrophenol; GPI, glycosylphosphatidylinositol; GEEC, GPI-anchored-protein-enriched early endosome; IL2R β , interleukin 2 receptor β -subunit; IRAP, insulin-responsive aminopeptidase; LDLR, low-density lipoprotein receptor; LOX-1, lectin-like oxidized LDLR-1; siRNA, small interfering RNA; TfR, transferrin receptor.

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of the present paper is to examine the current knowledge of GLUT4 endocytosis in muscle and fat cells and its regulation by physiological stimuli.

DIVERSITY OF ENDOCYTIC ROUTES

Several mechanisms of internalization from the plasma membrane exist in mammalian cells that display distinct commonalities and differences (reviewed in [11–13]). In all instances, cargo molecules get ‘ready’ by concentrating within a small region of the plasma membrane, a process that has entropic cost. Secondly, they get ‘set’ by interacting with specific membrane coating proteins or lipids, resulting in membrane bending and invagination. Finally, scission occurs at significant enthalpic cost, occluding cargo molecules from the extracellular milieu and allowing them to ‘go’ into the cell interior. Internalized vesicular cargos are then targeted to intracellular compartments. The different internalization processes have been classified here based on the coat proteins decorating the endocytic vesicles.

CME (clathrin-mediated endocytosis)

CME is the best understood mechanism for the internalization of cargo from the cell surface and a large number of proteins contribute to this process (reviewed in [11,14,15]). CME leads to the creation of 100–150 nm-diameter CCVs (clathrin-coated vesicles) which are decorated by CHC (clathrin heavy chain) and CLC (clathrin light chain). CHC and CLC assemble into triskelia which, in turn, self-assemble into a polyhedral structure that forms the essential spherical protein cage enveloping the cargo-containing endocytic membrane. The clathrin-containing protein coat begins to assemble on a shallow membrane invagination [CCP (clathrin coated pit)] that, in turn, deepens and progresses to the final budded CCV. Additional noteworthy proteins involved in CME are those that harbour ENTH (epsin N-terminal homology), ANTH (AP180 N-terminal homology), BAR (Bin/amphiphysin/Rvs) or F-BAR [FCH (Fes and CIP4 homology)-BAR] domains and can either bind to curved membranes or are able to generate membrane curvature [16,17]. These CME ancillary proteins contribute to the protein-coated membrane invagination characteristic of a CCP. In addition, APs (adaptor proteins), such as AP-2, simultaneously bind to cargo molecules, the plasma membrane, and to the clathrin complex to ensure that the appropriate cargo is concentrated into CCPs [18]. The best characterized molecules which are internalized by CME are the TfR and the LDLR (low-density lipoprotein receptor). Each clathrin-dependent cargo contains a short motif, such as [DE]XXXL[L]I and YXX ϕ , recognized by AP-2, or other motifs, such as FXNXY, recognized by the accessory clathrin adaptors Dab2 (Disabled 2), ARH (autosomal recessive hypercholesterolaemia), epsin and β -arrestin [18]. Resolution of a CCP requires deepening invagination, followed by dissociation of the budding membrane from the plasma membrane, in order to generate a CCV. Final membrane scission occurs by constriction of the

bud neck effected by the mechanochemical GTPase dynamin. This step requires the action of actin and its binding partners. Aside from its mechanochemical function, dynamin may also recruit actin to the newborn vesicle by interacting with proteins that regulate actin polymerization, such as syndapin, intersectin, cortactin and mAbp1 (mouse actin binding protein 1) [19].

Caveolae

Caveolae (often described as cave-like invaginations of the plasma membrane) constitute the best-studied clathrin-independent endocytic mechanism [12]. Recent advances in fixation techniques [20] reveal that caveolae are spherical membrane invaginations with a spiky protein coat and specialized neck region [20,21]. Caveolae are stable structures constituted by the protein caveolin, which assemble in the endoplasmic reticulum and subsequently traffic to the cell surface [22]. Once at the plasma membrane, caveolae function as endocytic structures, as well as platforms for organization of cellular signalling networks [22]. Three caveolin isoforms exist in mammals (Cav1–3), which make up at least part of the caveolar protein coat and form stable oligomeric ensembles required to generate caveolae [20,23–25]. Also required for caveolae stability is the recently identified component PTRF (polymerase I and transcript-releasing factor)-Cavin [26]. Several cargo molecules are internalized through the cell surface by caveolae, including the glutamate transporter EAAC1 (excitatory amino-acid carrier 1) [27], TGF β R (transforming growth factor β receptor) [28], ubiquitinated EGFR (epidermal growth factor receptor) [29], β 1 integrins in human fibroblasts [30] and A1 adenosine receptors [31]. However, neither cargo-recognition motifs nor APs have been identified to date for caveolar internalization. Since caveolae are exquisitely dependent on the clustering of membrane lipids, such as cholesterol and the ganglioside GM1 [32], the partitioning of plasma membrane components into lipid-ordered domains may contribute to cargo concentration for caveolar internalization. Indeed, lactosylceramide and other glycosphingolipids are specifically internalized via caveolae [32], suggesting that lipids themselves may represent specific endocytic cargo of caveolae. In spite of these marked differences between caveolae-mediated endocytosis and CME, caveolar internalization also requires dynamin [33–35] and the dynamin-binding protein intersectin [36]. Consistent with a limited common mechanism of action, dynamin phosphorylation by Src on Tyr²³¹ and Tyr⁵⁹⁷, which increases dynamin self-assembly [37] and potentiates both caveolar [33,38] and clathrin [39] internalization. Moreover, dynamin is found near the bud neck of caveolae [34], as it is in CCPs.

Other routes

Aside from CME and caveolae, other modes of internalization exist, as reviewed previously [11–13], which can be subclassified further based on their requirement for dynamin. Internalization of the IL2R β (interleukin 2 receptor β -subunit) is CME- and caveolae-independent, but requires dynamin, and IL2R β can be isolated in detergent-resistant membranes [40]. Interestingly, IL2R β -positive endocytic patches on the plasma membrane

have no apparent protein coat when compared with CCPs [40], possibly reflecting a more prominent contribution of membrane lipids in pit formation. For the purpose of the present paper, this route of internalization has been termed CCI-dyn (clathrin- and caveolae-independent, dynamin-dependent endocytosis). Several other cargo molecules also exhibit similar internalization, notably the γ cR (γ -c receptor) [19], the high affinity IgE receptor [41], and LOX-1 (lectin-like oxidized LDLR-1) [42]. CCI-dyn cargo internalization requires distinct cytoplasmic sequences, such as the first 27 amino acids in IL2R β [43] and D⁴DL⁶ in LOX-1 [42]. This suggests that protein adaptors must recognize cargo destined for CCI-dyn. However, without more specific markers, it is not possible to conclude at present whether all CCI-dyn cargo internalizes via the same mechanism. The internalization of IL2R β [44] and γ cR [19] is reduced upon disruption of actin filaments by latrunculin B. Consistent with this observation, IL2R β internalization is sensitive to expression of dominant-negative mutants or siRNA (small interfering RNA) gene silencing of Rac1, Pak (p21-activated kinase) 1/2 and cortactin [44]. The precise molecular mode of action of these proteins as well as that of dynamin in CCI-dyn remains to be determined.

Aside from CME, caveolar endocytosis and CCI-dyn, dynamin-independent internalization occurs by at least two distinct routes. The first one requires flotillin and internalizes GPI (glycosylphosphatidylinositol)-anchored proteins [45], targeting cargo to GEECs (GPI-anchored-protein-enriched early endosomes). This GEEC-directed pathway of internalization requires the regulatory GTPases Cdc42 (cell division cycle 42) [46,47] and Arf1 (ADP-ribosylation factor 1) [48]. The second one is macropinocytosis, consisting of large actin-dependent membrane protrusions that fold over the plasma membrane [49]. Macropinocytosis is a major mechanism for fluid-phase uptake, but fluid can also enter via the GEEC-directed pathway [47,49], and by a process involving specific dynamin splice variants that is neither clathrin- nor caveolin-mediated [50]. CtBP3 (C-terminal binding protein 3)/BARS (brefeldin A-ribosylated substrate) proteins function in vesicle scission in the process of dynamin-independent fluid-phase uptake [51].

GLUT4 INTERNALIZATION

CME

Numerous lines of evidence suggest that GLUT4 undergoes CME. First, GLUT4 is found in isolated CCVs [52] and can be detected by immunoelectron microscopy within clathrin lattices on plasma-membrane lawns (shells) of 3T3-L1 adipocytes [53]. Secondly, hypertonic sucrose and low extracellular K⁺, which perturb CME, reduce GLUT4 internalization in L6 myoblasts [54,55] and myotubes [54] (measured by a direct assay, see below) and increase steady-state GLUT4 levels in 3T3-L1 [56] and rat adipocytes [57]. Thirdly, cytosolic acidification, which also perturbs CME, reduces internalization of IRAP (insulin-responsive aminopeptidase), a protein that shares its intracellular itinerary with GLUT4 [58].

Table 1 GLUT4 endocytic motifs observed by measurement of steady-state cell surface GLUT4

Bold indicates cellular models of physiological targets of insulin: muscle cells and adipocytes.

Motif	Cell type	References
F ⁵ QQI ⁸	CHO	[63]
	Rat adipocytes	[59]
L ⁴⁸⁸ L ⁴⁸⁹	NIH 3T3 fibroblasts	[91]*
	3T3-L1 fibroblasts	[92]*
	L6 myoblasts	[93]*

*Fused cytosolic portions of GLUT4 with GLUT1 to use as a reporter construct.

Table 2 GLUT4 endocytic motifs observed by measurement of GLUT4 'return-to-basal'

All cell types are cellular models of physiological targets of insulin: muscle cells and adipocytes.

Motif	Cell type	References
F ⁵ QQI ⁸	Rat adipocytes	[59]
	3T3-L1 adipocytes	[60,94]
L ⁴⁸⁸ L ⁴⁸⁹	3T3-L1 adipocytes	[92]*, [60,94]
C-terminal 37 amino acids	Rat adipocytes	[59]

*Fused cytosolic portions of GLUT4 with GLUT1 to use as a reporter construct.

Several studies suggest that AP-2 is the endocytic adaptor that targets GLUT4 for CME. GLUT4 contains two sequences, F⁵QQI⁸ and L⁴⁸⁸L⁴⁸⁹, that resemble the AP-2-binding motifs YXX ϕ and [DE]XXXL[L] respectively. By yeast two-hybrid analysis, a peptide corresponding to the GLUT4 N-terminus (containing the F⁵QQI⁸ motif) was found to bind the μ -adaptin chain of AP-2, and this interaction was lost when Phe⁵ was mutated to an alanine residue in the GLUT4 peptide [59]. Moreover, mutation of the F⁵QQI⁸ and L⁴⁸⁸L⁴⁸⁹ motifs within the full-length transporter increased the steady-state content of GLUT4 at the cell surface (Table 1), consistent with reduced internalization as a result of decreased binding to AP-2. One way to monitor GLUT4 internalization is by determining the rate of decline of cell surface GLUT4 from the insulin-stimulated steady-state level upon cessation of insulin-stimulated exocytosis (termed 'return-to-basal', see Table 2). This is accomplished by measuring the loss of GLUT4 from the cell surface of insulin-stimulated cells upon insulin removal and addition of the phosphatidylinositol 3-kinase inhibitor wortmannin to block insulin signalling promoting GLUT4 exocytosis [59]. Both F⁵QQI⁸ and L⁴⁸⁸L⁴⁸⁹ sequences were implicated in GLUT4 internalization under such return-to-basal conditions, supporting the participation of AP-2-dependent internalization. However, Blot and McGraw [60] demonstrated that although the L⁴⁸⁸L⁴⁸⁹ motif participates in GLUT4 clearance from the cell surface under return-to-basal conditions, this occurs as a result of recognition by the intracellular clathrin adaptor AP-1, which results in the sorting of GLUT4 away from a rapidly recycling membrane compartment [61]. Moreover, mutation of a similar dileucine motif within IRAP (L⁷⁶L⁷⁷), a protein with an intracellular itinerary similar to that of GLUT4, did not alter its internalization yet perturbed its biosynthetic membrane traffic [62]. Therefore only the F⁵QQI⁸ sequence may be required

**Table 3 GLUT4 endocytic motifs observed by direct measurement of GLUT4 internalization**

Bold indicates cellular models of physiological targets of insulin: muscle cells and adipocytes.

Motif	Cell type	References
F ⁵ QQI ⁸	CHO	[95], [96]*
	3T3-L1 adipocytes	[60]
L ⁴⁸⁸ L ⁴⁸⁹	CHO	[95], [97]†, [98]*
	COS-7	[97]†

*Fused cytosolic portion of GLUT4 to luminal portion of transferrin receptor to use as a reporter construct.

†Fused cytosolic portions of GLUT4 with GLUT1 to use as a reporter construct.

for the proper internalization of GLUT4, whereas L⁴⁸⁸L⁴⁸⁹ in GLUT4 (L⁷⁶L⁷⁷ within IRAP) does not participate directly in this process.

The above-described studies were performed using assays that measure changes in cell surface GLUT4 levels that also reflect changes in the basal recycling of the transporter. As such, these measurements can be influenced by alterations in GLUT4 membrane traffic other than internalization itself. In contrast, two direct measurements of GLUT4 internalization have been described that are not subject to complications imparted by subsequent intracellular sorting. The first one involves labelling exofacial epitopes on GLUT4 under conditions that preclude vesicle movement, such as cooling the cells to 4°C, followed by re-warming the cells to allow internalization and determination of the remaining label exposed to the extracellular milieu [54]. The second method calculates GLUT4 internalization as the ratio of anti-GLUT4 antibody uptake to steady-state cell surface GLUT4 labelling, all at 37°C [60]. When performed over short time periods (<10 min), these measurements allow reliable determination of the rate of internalization of the transporter, without any contribution from other stages of GLUT4 traffic. When measured by either of these methods, both the F⁵QQI⁸ and L⁴⁸⁸L⁴⁸⁹ sequences were required for GLUT4 internalization in CHO (Chinese-hamster ovary) or COS-7 cells (Table 3). However, in mature 3T3-L1 adipocytes (cells that express GLUT4 endogenously, in contrast with CHO and COS-7 cells), only the F⁵QQI⁸ motif was required for GLUT4 internalization. These results suggest that although L⁴⁸⁸L⁴⁸⁹ can function as a GLUT4 endocytic signal in non-insulin target cells, this does not occur in mature 3T3-L1 adipocytes. Perhaps the most compelling evidence that GLUT4 utilizes F⁵QQI⁸ to internalize via CME is that an F5A mutation within the F⁵QQI⁸ motif causes a loss of GLUT4 from CCPs [63]. Taken together, these results suggest that F⁵QQI⁸ is essential for recognition by AP-2 and sorting of GLUT4 to CCPs.

Further support for the participation of AP-2 and CME in GLUT4 internalization stems from the effect of agents that perturb CME components. Gene silencing of AP-2 by siRNA in 3T3-L1 adipocytes slowed down GLUT4 internalization in the presence of insulin, but not in the basal state [60]. In the latter, it remains possible that adaptors other than AP-2 may recruit GLUT4 to CCPs in the basal state, given that under those conditions GLUT4 co-localizes with clathrin puncta in the TIRF (total internal reflection fluorescence) zone [64]. Together, these res-

ults suggest that AP-2 is an adaptor that gets GLUT4 'ready' (i.e. committed) for internalization via CME in 3T3-L1 adipocytes under distinct physiological conditions. However, since GLUT4 is capable of clathrin-independent internalization (see below), depleting AP-2 may result in its default re-routing to internalization through other mechanisms.

Unlike depletion of AP-2, depleting CHC should not alter the amount of AP-2-containing endocytic pits forming at the cell surface [65]. Therefore cargo would be ready for CME, even though it would not proceed to the 'set and internalize' stages. This would ensure that cargo destined for CME is not redirected to other endocytic routes. Accordingly, we recently used siRNA gene silencing to deplete CHC in L6 myoblasts. By this approach, TfR internalization was largely blocked, and GLUT4 internalization was reduced, albeit only by 50% [54]. Consistent with these results, GLUT4 exhibited partial co-localization with clathrin puncta at the cell surface ([54] and Figure 1). Thus in unstimulated muscle cells, GLUT4 internalization occurs at least in part via CME.

Taken together, inhibition of CME by hyperosmolarity, K⁺-free media or siRNA gene silencing of CHC demonstrates that GLUT4 internalizes in part via CME in both adipocytes and muscle cells. Yet CME does not account for all of the internalization of GLUT4 in these cells, and several lines of evidence suggest that clathrin-independent internalization of GLUT4 also occurs, as reviewed below.

Clathrin-independent endocytosis

Reduction or sequestration of membrane cholesterol by methyl-β-cyclodextrin, nystatin or filipin reduced GLUT4 internalization in 3T3-L1 adipocytes [60,66] and L6 muscle cells [54]. In the latter study, low concentrations of methyl-β-cyclodextrin that do not affect CME were used, as demonstrated by unperturbed TfR internalization. Consistent with its sensitivity to cholesterol depletion, GLUT4 has been detected in caveolae by biochemical purification or by immunoelectron microscopy in 3T3-L1 and rat adipocytes [66–69]. Moreover, expression of the caveolin-1 S80E mutant, which sequesters endogenous caveolin isoforms in the ER, reduced GLUT4 internalization in 3T3-L1 adipocytes [70]. However, other studies have failed to observe GLUT4 in caveolae in rat adipocytes [71,72]. It is unclear what can account for these discrepancies, although the differential sensitivity of caveolar integrity to sample preparation [20] may provide an explanation. At present, the bulk of the evidence supports the possibility that CME-independent internalization of GLUT4 in adipocytes is via caveolae.

In contrast to 3T3-L1 adipocytes, internalization of GLUT4 in L6 muscle cells is not perturbed upon disruption of caveolae by expression of caveolin-1 S80E [54]. Furthermore, siRNA gene silencing of syntaxin-6, which is required for the delivery of GM-1 along the secretory pathway to the plasma membrane [32], and reduced caveolar internalization in L6 muscle cells, but did not affect the internalization of GLUT4 [54]. Consistent with these results, caveolin-1 is not found in immuno-isolated GLUT4 vesicles from rat skeletal muscle [73], and GLUT4 exhibits virtually

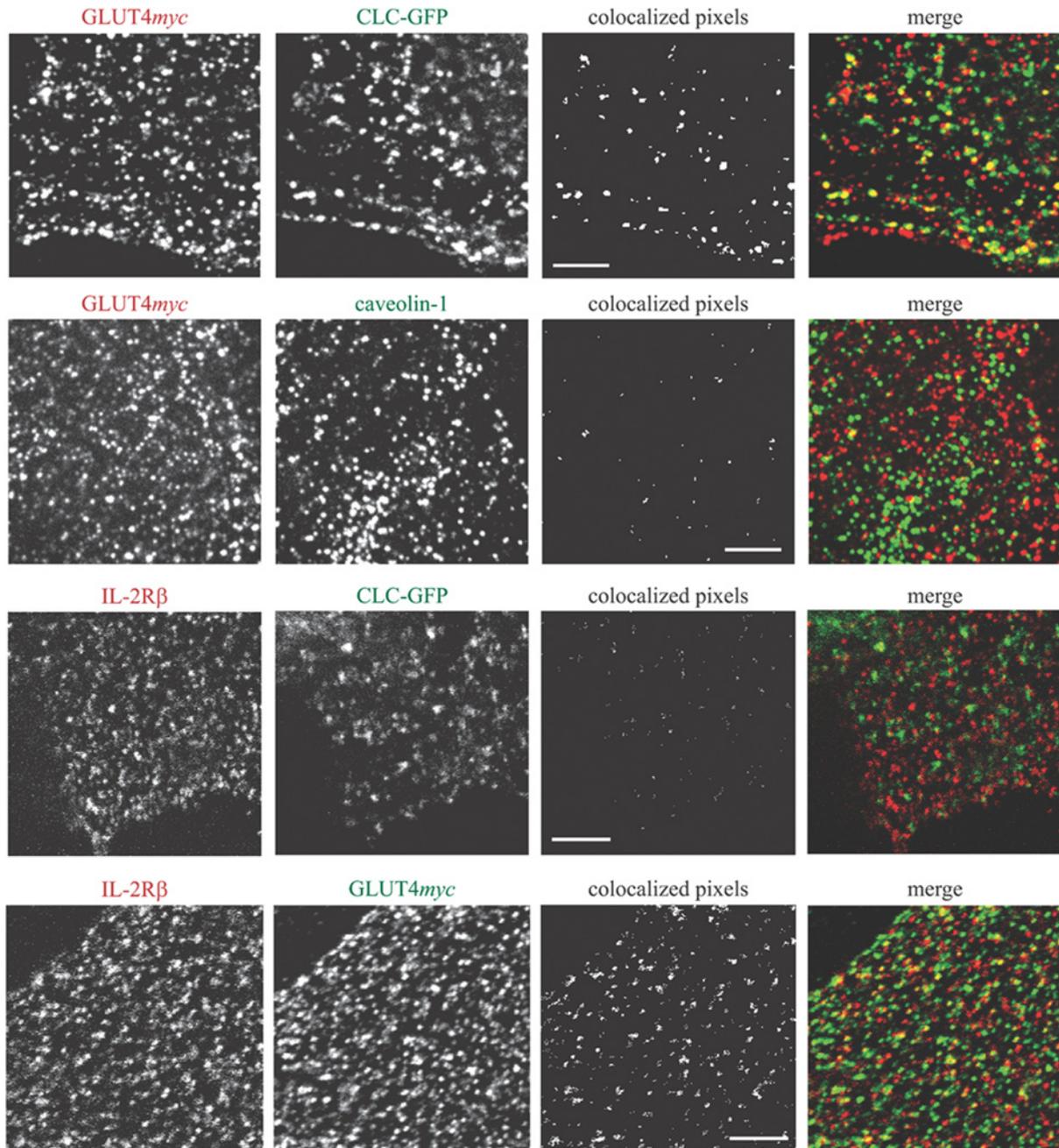


Figure 1 GLUT4 exhibits partial co-localization with IL2R β and CLC but not caveolin-1 at the cell surface

L6 myoblasts stably expressing GLUT4 harbouring an exofacial Myc epitope (L6-GLUT4-Myc) were transiently transfected with cDNA encoding IL2R β or CLC fused to GFP (green fluorescent protein) (CLC-GFP) as described previously [54]. Cell surface GLUT4-Myc and/or IL2R β were labelled in intact cells with appropriate antibodies, followed by fixation and permeabilization and labelling with an anti-caveolin-1 antibody, as indicated. Cell surface or intracellular antibodies were then visualized by labelling with the corresponding Cy3 (indocarbocyanine)- or Cy2-conjugated secondary antibodies. Images were obtained by confocal microscopy and are representative images of a single z-section focusing at the coverslip-adhered surface of the cell. A panel depicting pixels that exhibited both Cy3 and Cy2 or GFP fluorescence (co-localized pixels) is also shown for each image. Scale bars, 5 μ m.

no co-localization with caveolin-1 by immunofluorescence microscopy (Figure 1 and [54]). Therefore, in muscle cells, the CME-independent internalization of GLUT4 does not occur via caveolae.

The above results illustrate the need for a more precise identification of the characteristics of CME-independent GLUT4 internalization. Abrogation of this route upon expression of the dominant-negative dynamin 2 K44A mutant provided an

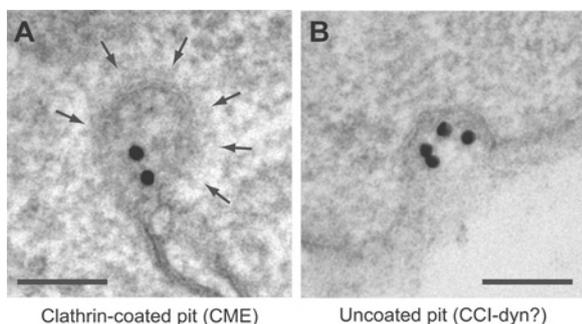


Figure 2 GLUT4 is found in both CCPs and non-coated pits at the plasma membrane

L6-GLUT4-Myc myotubes were stimulated with 100 nM insulin for 20 min at 37°C. Cells were then transferred to 4°C, washed and labelled with an anti-Myc antibody, followed by a 10 nm-gold-conjugated secondary antibody. Internalization of surface proteins was then allowed to resume by re-warming of the cells to 37°C for 5 min prior to fixation. The cells were then embedded in a plastic (Epon) resin and prepared for electron microscopy. Representative images are shown of gold particles observed within CCPs and uncoated pits, as indicated. Arrows indicate the clathrin coat. Scale bars, 100 nm.

interesting clue [54], suggesting that GLUT4 internalizes via CCI-dyn, akin to the route of IL2R β internalization. Supporting this interpretation, both proteins, duly labelled through exofacial epitopes, co-localize at the cell surface in L6 myoblasts (Figure 1). Moreover, cell surface IL2R β (exogenously expressed) did not co-localize with clathrin in L6 myoblasts, consistent with its internalization via CCI-dyn and not by CME (Figure 1).

In summary, the above results lead to the conclusion that GLUT4 internalizes via both CME and by CCI-dyn-mediated internalization in muscle cells. Such a notion is supported by the detection of GLUT4 within both CCPs and clathrin-uncoated pits at the plasma membrane by immunoelectron microscopy (Figure 2). In this case, GLUT4-Myc was loaded with an anti-Myc antibody and a secondary antibody conjugated to 10-nm gold particles at the surface of live L6 myotubes, at 4°C, and then allowed to internalize for 5 min before processing for electron microscopy. The illustrated image shows recruitment of GLUT4-associated gold particles into a CCP (CME) and into a non-coated pit (possibly representing GLUT4 being internalized via CCI-dyn) forming at the plasma membrane. However, more rigorous analysis is required to identify the origin of non-coated GLUT4-containing pits/vesicles.

The results summarized above suggest that GLUT4 is internalized via clathrin as well as non-clathrin routes in adipocytes and muscle cells (Figure 3). Of note, the endocytic routes employed by GLUT4 were not dependent on GLUT4 expression levels in L6 muscle cells [54] or 3T3-L1 adipocytes [60]. As adipocytes are sensitive to AP-2 depletion only upon insulin treatment [60], the reliance on CME in these cells may be stimulus-dependent, as discussed below. The relationship between the clathrin-independent routes employed by GLUT4 in muscle and adipocytes remains to be determined. One major difference is the abundance of caveolae in adipocytes [74], where they appear as rosettes [75] or caves [76]; in contrast, such structures have not been observed in

muscle cells. Alternatively, cell-type specific adaptors may direct GLUT4 to either caveolae or CCI-dyn in adipocytes and muscle cells respectively.

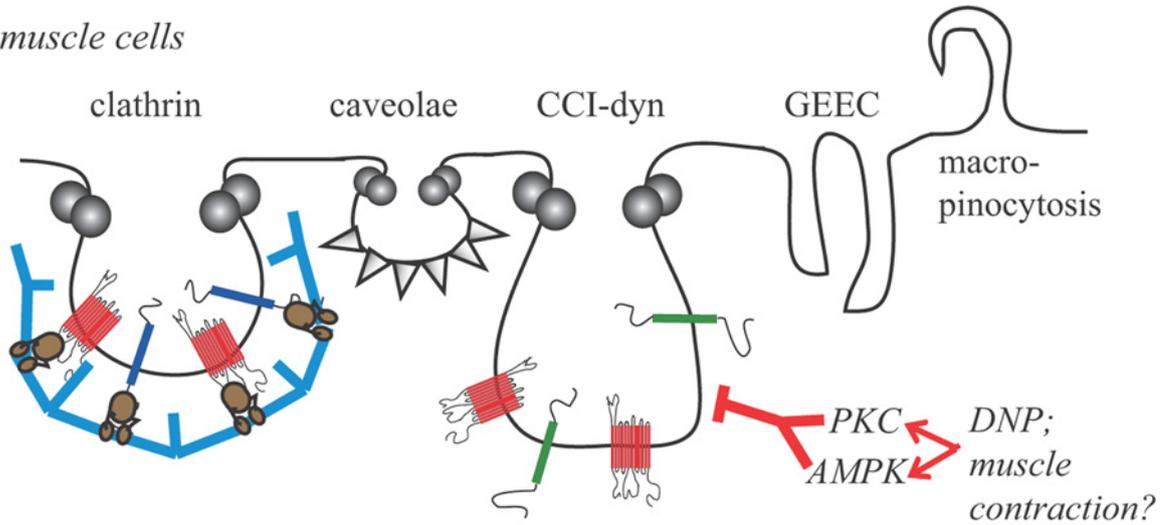
REGULATION OF GLUT4 ENDOCYTOSIS

The continuous recycling of GLUT4 offers the flexibility to regulate both its exocytic and endocytic rates. It is well documented that insulin rapidly stimulates the rate of exocytosis of the transporter in adipose and muscle cells in culture [3,5,6]. However, there is scant information on which mechanisms regulate its endocytosis. Although insulin exerts a small and sometimes debated reduction in GLUT4 endocytosis in adipose cells [77–82], the hormone does not affect the rate of GLUT4 internalization in rat cardiomyocytes [10] and L6 muscle cells [54,83]. It is conceivable that insulin slows down GLUT4 internalization only in mature fat and not in skeletal muscle tissues, although this has not been documented directly. Consistent with this interpretation, the stimulation of glucose uptake by insulin in adipocytes is larger than that typically observed in muscle [84].

The insulin-dependent reduction in the rate of GLUT4 internalization in 3T3-L1 adipocytes is due to re-routing from rapid clathrin-independent internalization to a slower CME [60]. To achieve this switch in endocytic routes, insulin stimulation drastically reduces the internalization of clathrin-independent cargo, as seen by slower internalization of CtxB (cholera toxin B) upon treatment with the hormone. The preferential use of CME by GLUT4 upon treatment with insulin, as seen by the sensitivity of GLUT4 internalization to AP-2 depletion only in the stimulated state, may thus result from an inability of the transporter to engage the inhibited clathrin-independent endocytic route demarked by CtxB. As a result, upon insulin stimulation, the overall rate of GLUT4 internalization in 3T3-L1 adipocytes is limited by the rate of its CME. Consistent with these observations, Czech and colleagues showed that insulin causes increased residency of GLUT4 within clathrin puncta at the plasma membrane in 3T3-L1 adipocytes [64]. The insulin-signalling intermediates and the endocytic components regulated by the hormone are currently not known. Importantly, Akt, which regulates GLUT4 exocytosis, is not required for insulin regulation of GLUT4 endocytosis [85]. In contrast to 3T3-L1 adipocytes, in L6 muscle cells, insulin does not affect the rate of internalization of GLUT4 as mentioned above, or that of TfR [54]. The elucidation of the signalling intermediates targeting GLUT4 endocytosis in 3T3-L1 adipocytes will aid greatly in understanding the possible differences between insulin target tissues.

Other than insulin, muscle contraction is a major physiological stimulus that also increases the amount of cell surface GLUT4. Muscle contraction is triggered by membrane depolarization, evoking a rise in intracellular Ca²⁺ and reducing intracellular [ATP], which, in turn, results in the activation of AMPK.

A) muscle cells



B) adipocytes

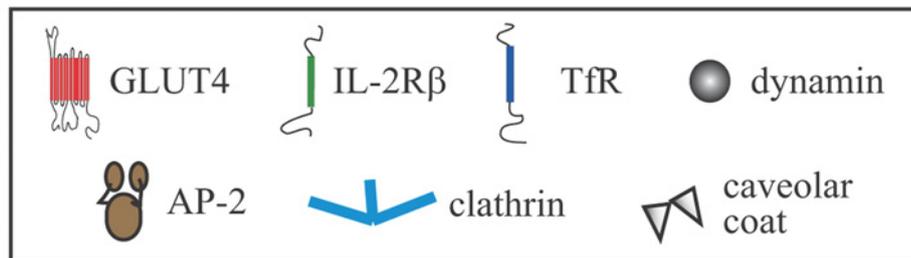
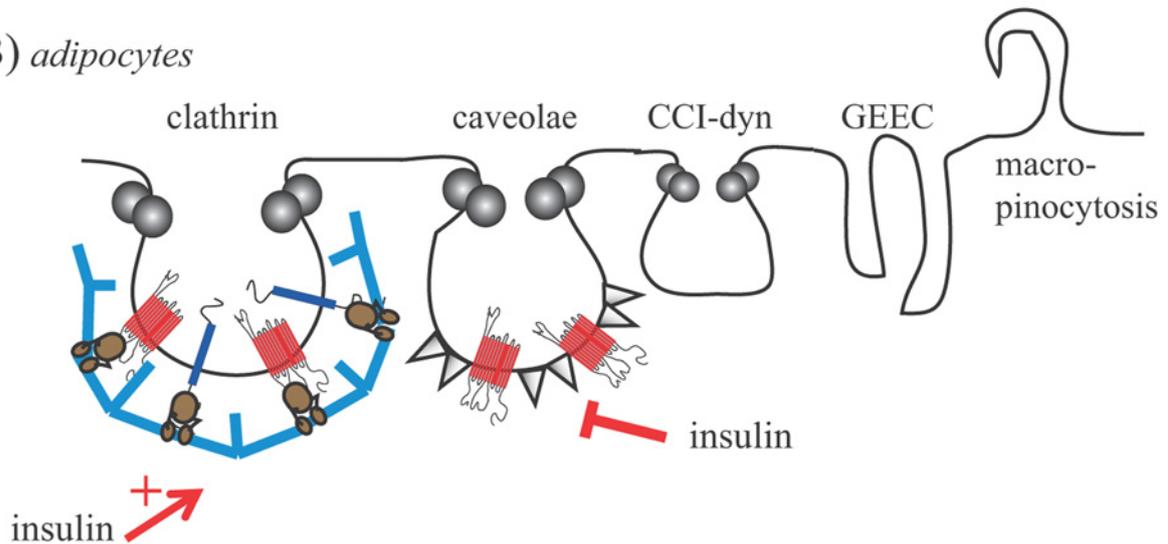


Figure 3 Diagrammatic representation of GLUT4 internalization routes in myoblasts (A) and adipocytes (B)

Endocytic pits representing each known internalization mechanisms are shown. Also illustrated is the proposed distribution of cargo molecules (GLUT4, TfR and IL2Rβ) within these endocytic structures. Regulation by insulin (A) and energy stress (B) is indicated, and is described in the main text. PKC, protein kinase C.

Each of these individual signals has been proposed to contribute to the gain in cell surface GLUT4 upon muscle contraction (reviewed in [7]). In addition to differences in the participating

signals, muscle contraction and insulin recruit GLUT4 to the cell surface from different intracellular locations [86]. Several findings suggest that the signals elicited by muscle contraction may



lead to a reduction in GLUT4 internalization. First, exposing L6 myotubes to medium with high $[K^+]$ to reduce the membrane potential and raise intracellular Ca^{2+} , resulted in a marked reduction in GLUT4 internalization [87]. A short exposure to oligomycin or DNP (dinitrophenol), agents that perturb mitochondrial oxidative respiration and transiently reduce cellular [ATP], slowed down GLUT4 internalization in cardiomyocytes [10] and L6 muscle cells [54]. In the search for a molecular mechanism regulating GLUT4 endocytosis, we found that activation of AMPK is necessary [8,87] to increase cell surface GLUT4 in response to mitochondrial uncoupling. Moreover, direct activation of AMPK simultaneously with activation of protein kinase C was sufficient to slow down GLUT4 internalization [54]. Interestingly, exposure to the uncoupler DNP retards the internalization not only of GLUT4 but also of IL2R β , the prototypical cargo internalized via CCI-dyn. In contrast, DNP did not affect CME, as seen by unaffected internalization of TfR upon treatment with the uncoupler [54]. These results suggest that energy stress halts the internalization of multiple cargo via CCI-dyn in muscle cells. The diverse modalities of regulation of GLUT4 endocytosis are illustrated in Figure 3.

The reduction in GLUT4 internalization caused by signals involved in muscle contraction raises the hypothesis that this physiological stimulus may act primarily at the cell surface, drawing GLUT4 from the continuously recycling pool rather than from a specialized compartment. This scenario is consistent with the demonstration that contraction increases surface GLUT4 and TfR in mature muscle [88], and that insulin and contraction have different effects on the morphology of GLUT4 intracellular depots observed by fluorescence microscopy [89]. Future studies with primary skeletal muscle will help to reveal the contribution of GLUT4 internalization to the increased glucose uptake following muscle contraction and exercise. This may be tested in the newly-generated transgenic mice expressing Myc-tagged GLUT4 in its target tissues [90].

CONCLUSION AND PERSPECTIVES

We have examined recent studies showing that GLUT4 internalization occurs through both CME-dependent and CME-independent mechanisms in adipocytes and muscle cells. The CME-independent routes appear to differ between adipocytes and muscle cells, possibly involving caveolae in the former and CCI-dyn in the latter. Insulin stimulation reduces GLUT4 internalization exclusively in adipocytes by slowing down the rate of entry via the clathrin-independent route, redirecting GLUT4 to slower CME. Although this action of the hormone is not observed in muscle cells, signals that emulate those elicited during muscle contraction retard GLUT4 internalization via CCI-dyn. As such, the internalization of GLUT4 emerges as an important target in order to up-regulate the amount of transporters at the muscle cell surface, with the potential function to overcome insulin resistance through increased glucose uptake into skeletal muscle.

FUNDING

This work was supported by the Canadian Institutes of Health Research [grant number MOP-7307] to A.K. C.N.A. was supported by a fellowship from the Canadian Diabetes Association. M.F. was supported by the Swiss National Science Foundation [grant number 310000-120280]; the Reuters Foundation; and the Sir Jules Thorn Charitable Overseas Trust Regulatory Foundation.

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Received 7 August 2008/5 September 2008; accepted 22 September 2008

Published on the Internet 10 December 2008, doi 10.1042/BSR20080105
