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Article

Characterization of GLUT4-containing vesicles in 3T3-L1 adipocytes by total internal reflection fluorescence microscopy

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Insulin-responsive GLUT4 (glucose transporter 4) translocation plays a major role in regulating glucose uptake in adipose tissue and muscle. Whether or not there is a specialized secretory GSV (GLUT4 storage vesicle) pool, and more importantly how GSVs are translocated to the PM (plasma membrane) under insulin stimulation is still under debate. In the present study, we systematically analyzed the dynamics of a large number of single GLUT4-containing vesicles in 3T3-L1 adipocytes by TIRFM (total internal reflection fluorescence microscopy). We found that GLUT4-containing vesicles can be classified into three groups according to their mobility, namely vertical, stable, and lateral GLUT4-containing vesicles. Among these groups, vertical GLUT4-containing vesicles exclude transferrin receptors and move towards the PM specifically in response to insulin stimulation, while stable and lateral GLUT4-containing vesicles contain transferrin receptors and show no insulin responsiveness. These data demonstrate that vertical GLUT4-containing vesicles correspond to specialized secretory GSVs, which approach the PM directly and bypass the constitutive recycling pathway.

GLUT4, IRAP-pHluorin, GSV, transferring, TIRFM

Insulin plays a major role in regulating glucose uptake in muscle and adipocytes by inducing the redistribution of GLUT4 from intracellular pools to the PM[1–3]. Under the basal state, more than 95% of GLUT4 is sequestered intracellularly because of fast internalization and slow externalization[4]. Insulin increases the externalization and inhibits the internalization of GLUT4, resulting in redistribution of GLUT4 to the PM[5]. Although GLUT4 is distributed throughout the cell[6,7], accumulating evidence from immunoelectron microscopy, compartment isolation and compartment ablation experiments suggests the existence of a group of insulin-responsive GLUT4 storage vesicles which respond specifically to insulin stimulation[8–16]. Two models have been proposed to depict the mechanism of GSV translocation. One model suggests that the net gain of GLUT4 in the PM is achieved by recruiting GLUT4 from the GSV pool into the constitutive recycling compartment, and then translocating it to the PM via the constitutive recycling pathway, while the other model suggests that GSVs are translocated to the PM directly in response to insulin stimulation[17].

EGFP-tagged GLUT4 has been used widely to visualize single GSVs in adipocytes using TIRFM[18–21]. Because of the heterozygosity of GLUT4 distribution[6,7], it is assumed that GLUT4-EGFP-labelled vesicles should

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include GSVs, endosomes, and other intracellular compartments. In the present study, we used dual-color TIRFM to systematically analyze the mobility of GLUT4-containing vesicles in 3T3-L1 adipocytes. We found that GLUT4-containing vesicles can be classified into three groups according to their mobility: one group approaches the PM vertically, another group stays immobile beneath the PM for a long time, and the third group makes long-range lateral movements beneath the PM. We named them as vertical, stable and lateral GLUT4-containing vesicles accordingly. Of these, only vertical GLUT4-containing vesicles exclude transferrin receptors and respond specifically to insulin stimulation, and thus correspond to the previously defined insulin-responsive GSVs. The existence of specialized insulin-responsive GSVs that exclude transferrin receptors provides further support for the second model which proposes that GSVs are directly translocated to the PM in response to insulin stimulation.

1 Materials and methods

1.1 Cell Culture and transfection

3T3-L1 cells were cultured and electroporated as described previously[18]. Adipocytes were serum-starved for at least 2 h and then transferred to a home-made closed perfusion chamber prior to imaging experiments. To label transferrin receptors, Alexa Fluor 568-conjugated transferrin (40 μg/mL) (transferrin-Alexa568) (Invitrogen) was added to serum-free DMEM during the last hour of serum starvation. All the experiments were performed at 30℃. Insulin stimulation was applied at a final concentration of 100 nmol/mL throughout the study. Unless otherwise stated, all drugs were purchased from Sigma.

1.2 TIRFM setup and image collection

TIRFM was conducted using an Olympus OX71 based on the prismless and through-the-lens configuration[20]. The penetration depth of the evanescent field was estimated to be 113 nm by measuring the incidence angle with a prism (n=1.518) using a 488 nm laser beam. Dual-color images were captured by PCO EMCCD (PCO, Kelheim, Germany) connected to a dual-view microimager (Optical Insights, Tucson, AZ, USA). A total of 500 images were acquired for each cell at a frequency of 5 frames per second beginning either before or 3 min after insulin stimulation.

1.3 Data Analysis

Image analysis was conducted as described previously[18]. Briefly, raw images were filtered with KNN (K-Nearest Neighbor, K=9 from 25 neighbors) and low-pass (cut-off spatial frequency 0.5) filters to remove hotspot noise from the CCD and high frequency noise. Secondly, the 'opening-by-reconstruction' algorithm in Matlab was employed to remove the diffused background. Individual vesicles were then separated from the background by an intensity-based threshold. Vesicles were assessed manually in Image J (NIH) and classified into corresponding groups. Stable vesicles were defined as those that were immobile (displacement <300 nm in the x-y direction) for more than 40 s; lateral vesicles were defined as those making long-range movements of more than 10 pixels (about 670 nm) in the x-y direction; and vertical vesicles were defined as those that traveled vertically from the deep cytosome, staying immobile beneath the PM for several seconds, and then disappearing into the cytosome or fusing with the PM.

To identify fusion events involving GLUT4-EGFP vesicles, two concentric circles (normally ~1.0 μm and ~1.3 μm in diameter) were centered on the vesicle being analyzed[18]. The fluorescence within the inner circle and between the two circles (annulus) was plotted. If there was diffusion of fluorescence after fusion, it was expected that there should also be a subsequent increase in fluorescence in the annulus area. When fluorescence intensity in the annulus area exceeded 5 times the standard deviation of the background fluorescence, it was assumed that a fusion event had occurred.

PHluorin is a green fluorescence protein whose fluorescence is very sensitive to environmental pH. IRAP-pHluorin has been shown to be a very useful marker for identifying fusion events[22]. To identify fusion of IRAP-pHluorin-marked vesicles, only one circle (normally ~1.0 μm) was centered on the vesicle being analyzed. If the pHluorin fluorescence intensity exceeded 5 times the standard deviation of the background[22] it was considered that a fusion event had occurred.

For normally distributed data, population averages are given as the mean and standard error of the mean (SEM), and statistical significance was tested with the Student's t-test.

2 Results

2.1 Identification of three groups of GLUT4-containing vesicles in 3T3-L1 adipocytes

To track GLUT4-containing vesicles in 3T3-L1 adipocytes, we selected EGFP-tagged GLUT4 as a vesicle marker. It was found that approximately half of GLUT4-containing vesicles approached the PM vertically, stayed immobile beneath the PM for several seconds, and then disappeared suddenly (Figure 1A upper panel, supplementary movie 1). We named these vesicles as vertical GLUT4-containing vesicles. Approximately another 40% of the GLUT4-containing vesicles stayed immobile underneath the PM for a long time, usually throughout image acquisition which lasted for 100 s. Thus we name them as stable GLUT4-containing vesicles (Figure 1A middle panel, supplementary movie 2). The remaining approximately 10% GSVs displayed lateral movement beneath the PM. They traveled for long distances in the lateral direction, so we named them as lateral GLUT4-containing vesicles (Figure 1A bottom panel, supplementary movie 3). After defining these groups of GLUT4-containing vesicles, we characterized the duration of the period they were beneath the PM, their velocity in the lateral direction and their displacement between appearance and disappearance sites. As shown in Figure 1B, C and D, kinetic characteristics of these groups of GLUT4-containing vesicles were significantly different from each other.

2.2 Stable and lateral GLUT4-containing vesicles are GLUT4-containing endosomes

Previous studies have indicated that there is some GLUT4 present in the recycling endosome compartment, which is adjacent to the PM. This kind of GLUT4-containing vesicle has different characteristics from insulin-responsive GSVs[11,14,16]. The transferrin receptor is a marker protein for the recycling endosome compartment[11,14,16]. To further clarify the identities of the three groups of GLUT4-containing vesicles observed here, recycling endosome compartments were labeled with transferrin-Alexa568 in GLUT4-EGFP transfected 3T3-L1 adipocytes. Using dual-color TIRFM, we found that more than 70% of the stable GLUT4-containing vesicles and at least 80% of the lateral GLUT4-containing vesicles contained transferrin receptors, while more than 90% of the vertical GLUT4-containing vesicles excluded transferrin receptors (Figure 2C). These results showed that stable and lateral GLUT4-containing vesicles are GLUT4-containing recycling endosome compartments. It should also be noted that vertical, stable and lateral GLUT4-containing vesicles accounted for approximately 50%, 40% and 10% of total GLUT4-containing vesicles, respectively (Figure 1E). Thus, GLUT4-containing recycling endosome compartments account for approximately 50% of all GLUT4-containing vesicles beneath the PM, a finding that is consistent with previous reports[11,16,23].

2.3 Vertical GLUT4-containing vesicles correspond to the previously defined insulin-responsive GLUT4 storage vesicles

To determine whether vertical GLUT4-containing vesicles correspond to the previously defined insulin-responsive GLUT4 storage vesicles, we analyzed a large number of GLUT4-containing vesicle fusion events. Since fusion events are rare under the basal state, only fusion events happening after insulin stimulation were included. Statistical analysis showed that 84.5% of all fusion vesicles excluded transferrin receptors (Figure 3B). Of these transferrin receptor-negative fusion vesicles, 85.7% approached the PM vertically, while more than 70% of transferrin receptor-positive fusion vesicles showed lateral movement underneath the PM (Figure 3C).

IRAP-pHluorin is considered more sensitive than GLUT4-EGFP for identifying vesicle fusion events[22], and so we subsequently analyzed the fusion events of GLUT4-containing vesicles under both the basal state and insulin stimulation using IRAP-pHluorin. As shown in Figure 4B, transferrin receptors were excluded from 86.7% and 88.5% of fusion events under the basal state and insulin stimulation respectively. Since the vesicle lumen has a low pH, the fluorescence of IRAP-containing vesicles is weak and cannot be detected before fusion. However, once IRAP-containing vesicles colocalize with transferrin, tranferrin-Alexa568 fluorescence acts as a label for IRAP-containing vesicles, allowing analysis of their movements. As shown in Figure 4C, under insulin stimulation more than two thirds of the transferrin receptor-positive vesicles underwent lateral movement before fusion. These quantitative data con-

firm our GLUT4-EGFP findings (Figure 3C) discussed above.

3 Discussion

In muscle and adipocytes, insulin-stimulated glucose uptake is mediated by the redistribution of GLUT4 from intracellular compartments to the PM[1–3]. Previous reports have demonstrated that GLUT4 is distributed between constitutive recycling compartments and special insulin-responsive GSVs[8–17]. However, the identity of specialized insulin-responsive GSVs and the route they take in approaching the PM has been elusive. In the present study, intracellular GLUT4-containing compartments were characterized using TIRFM in 3T3-L1 adipocytes. Three groups of GLUT4-containing vesicles were identified, namely vertical, stable and lateral GLUT4-containing vesicles.

We have demonstrated that vertical GLUT4-containing vesicles correspond to the previously described insulin-responsive GSVs, while stable and lateral GLUT4-containing vesicles correspond to vesicles that exist in constitutive recycling compartments. More importantly, our observation that vertical GLUT4-containing vesicles exclude transferrin receptors provides direct evidence for the hypothesis that GSVs are translocated to the PM directly, not via the constitutive recycling compartments, as suggested in the second model described above[17].

In summary, our results provide further evidence for the existence of specialized insulin-responsive GSVs and reveal that their translocation route to the PM is direct and not via the constitutive recycling compartment.

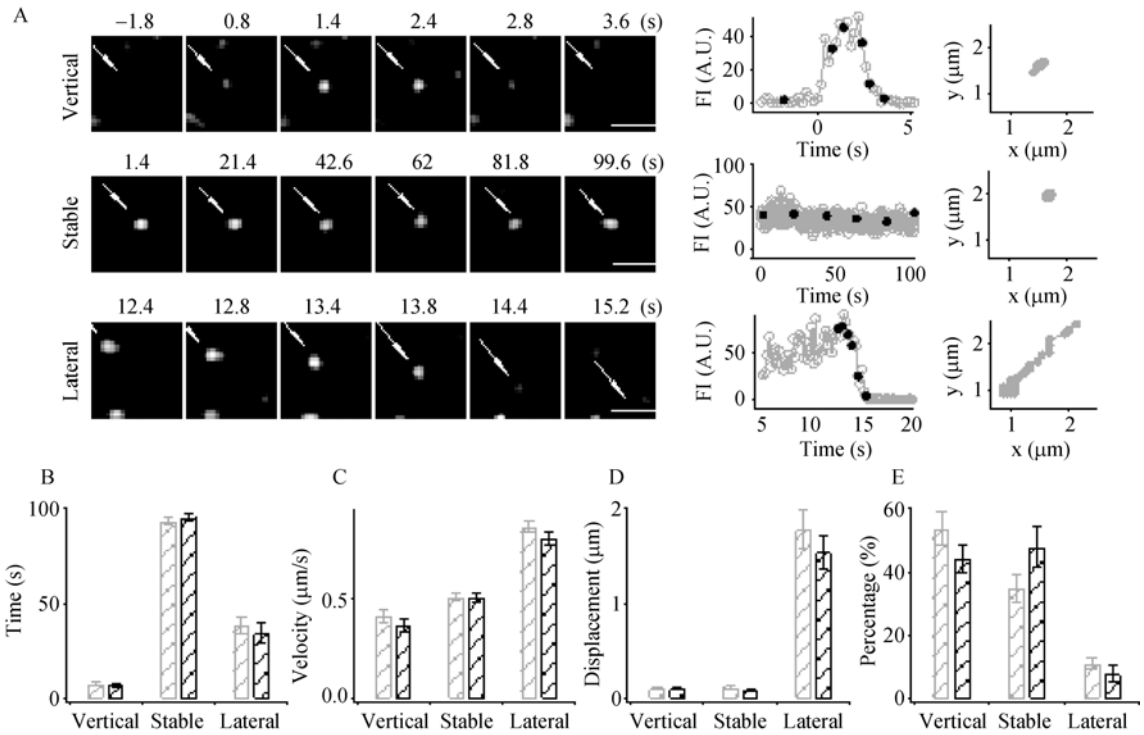

Figure 1 Mobility of the three groups of GLUT4-containing vesicles in 3T3-L1 adipocytes. A, TIRFM images of vertical, stable and lateral GLUT4-containing vesicles are shown in the left panel. Vertical GLUT4-containing vesicles moved vertically up from the deep cytoplasm, stayed immobile beneath the PM for several seconds, and then moved back into the cytoplasm or fused with the PM. Stable GLUT4-containing vesicles stayed immobile beneath the PM for a long time, usually throughout image acquisition which lasted for a period of 100 s. Lateral GLUT4-containing vesicles made long-range lateral movements beneath the PM. Times indicated are relative to the appearance of vesicles beneath the PM. Fluorescence intensity (FI) is in arbitrary units (AU) and *x*-*y* trajectories of vesicles are shown in the middle and right panels, respectively. Filled circles in the middle panel correspond to the image points displayed in the left panel. Bar: 1 μm. B, The duration of observations. C, Vesicle velocity. D, Distance between vesicle appearance and disappearance sites in the *x*-*y* direction. E, percentages of the three groups of GLUT4-containing vesicles beneath the PM under the basal state (gray) and after insulin stimulation (black).

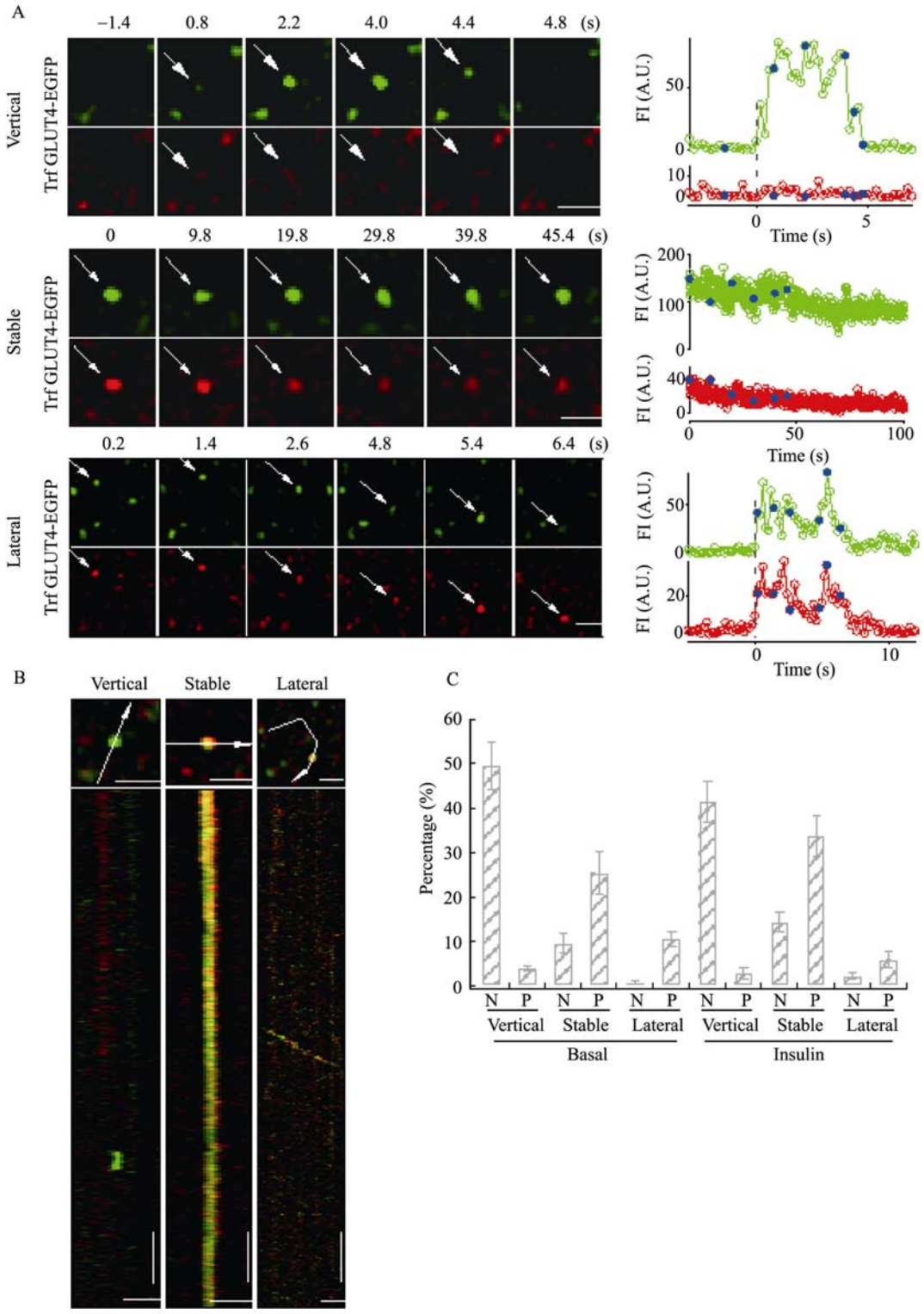

Figure 2 Stable and lateral GLUT4-containing vesicles are GLUT4-containing endosomes. A, the left panel shows images of three types of GLUT4-containing vesicles labeled simultaneously with GLUT4-EGFP and transferrin-Alexa568. Fluorescence intensities of vesicles are shown in the right panel as in Figure 1A. The dashed line indicates the appearance of vesicles. See Supplementary Movies 4, 5 and 6. Bar, 1 μm. B, Kymograph of GLUT4-containing vesicles shown in Figure 2A. The arrow indicates the direction in the kymograph (left to right). Horizontal bar, 1 μm. Vertical bar, 10 seconds. C, Percentages of transferrin receptor positive ('P') and negative ('N') vesicles in vertical, stable and lateral groups. Data are normalized to the total number of vesicles per cell. Figure 2C and Figure 1E come from the same batch of data. N=5 cells. There are normally about 100 vesicles in each cell.

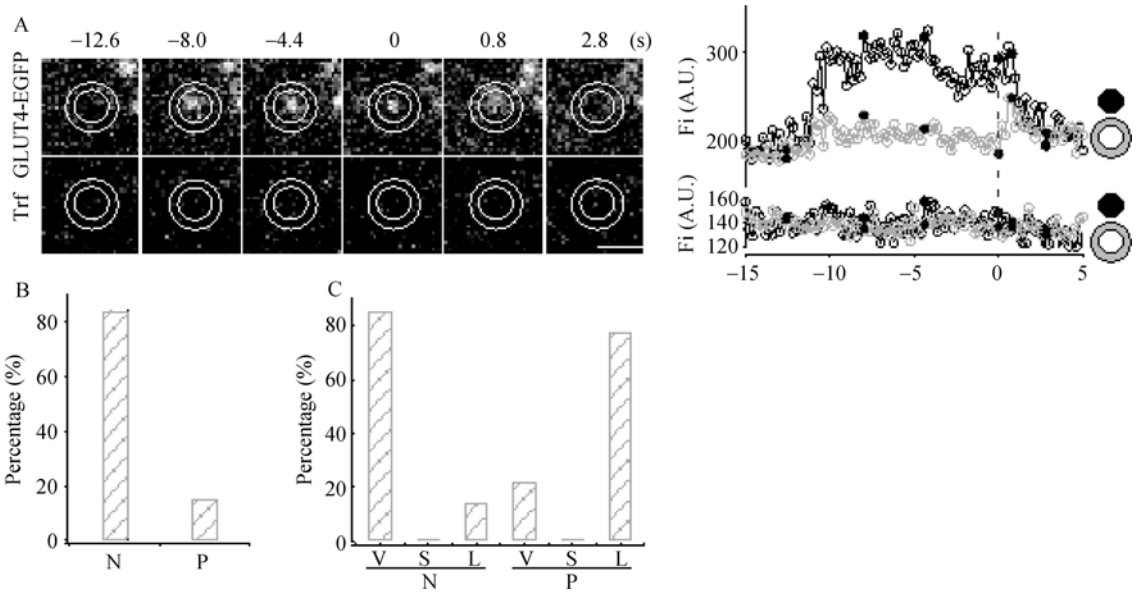

Figure 3 The majority of GLUT4-containing vesicles undergoing fusion exclude transferrin receptors and approach the PM vertically. A, The left panel shows images of a GLUT4-containing vesicle undergoing fusion. The vesicle approached the PM vertically, stayed immobile beneath the PM for approximately 10 sec, and then fused with the PM. As indicated, no transferrin-related fluorescence was detected in the vesicle. Times indicated are relative to the start of fusion. Bar, 1 μm. Fluorescence intensities of the inner circle and the annulus centered on the vesicle are shown in the right panel. The inner circle and the annulus are depicted with a diameter of ~1.0 and ~1.3 μm, respectively in the left panel images. A significant increase in annulus fluorescence was observed after fusion and was followed by an exponential decay, indicating diffusion of GLUT4-EGFP from the fusion site. The dashed line indicates the start of fusion. See Supplementary Movie 7. B, Percentages of transferrin receptor positive ('P') and negative ('N') fusion events under insulin stimulation. C, origin of transferrin receptor positive ('P') and negative ('N') fusion events for the different groups of GLUT4-containing vesicles. "V" stands for Vertical, "S" stands for Stable, and "L" stands for Lateral.

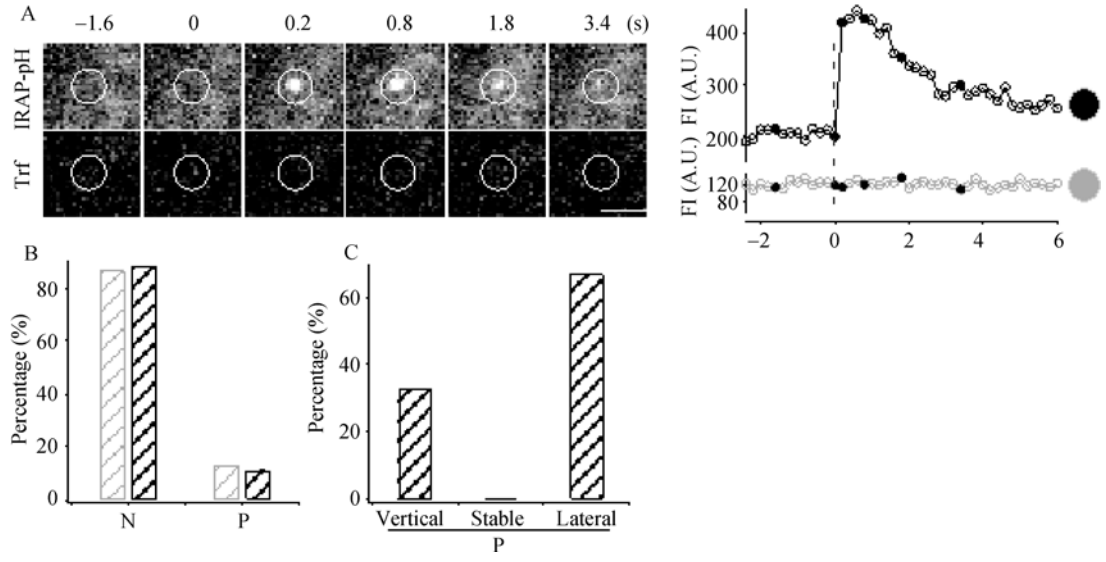

Figure 4 Transferrin was excluded from most IRAP-pHluorin fusion vesicles. A, The left panel show images of the fusion process of one IRAP-pHluorin-containing vesicle. No signal was detected for the transferrin receptors of this vesicle. Times indicated are relative to the start of fusion. Bar, 1 μm. The right panel shows fluorescence intensity in the circle centered on the vesicle. The circle is depicted with a diameter of ~0.8 μm in images in the left panel. An increase in fluorescence intensity indicates the start of fusion. See Supplementary Movie 8. B, Transferrin was excluded from nearly 90% of IRAP-pHluorin fusion events under both the basal state (gray) and after insulin stimulation (black). "P" stands for transferrin receptor positive and "N" stands for transferrin receptor negative. C, Most transferrin receptor positive ('P') vesicles undergoing fusion made long-range lateral movements before fusion. Only transferrin-positive fusion events were analyzed under insulin stimulation as vesicles which excluded transferrin receptors were not visible before fusion. Fusion events are very rare under the basal state and transferrin-positive fusion events are rarer still.

Table 1 Percentages of different types of vesicles under the basal state and after insulin stimulation

			Percentage±SEM (%)	'N+P'±SEM (%)
Basal	vertical	N	49.7±5.26	53.5±5.30
		P	3.85±0.453	
	stable	N	9.49±2.06	34.9±4.44
		P	25.4±4.95	
	lateral	N	0.682±0.462	11.2±1.56
		P	10.5±1.80	
Insulin	vertical	N	41.5±4.71	44.2±4.66
		P	2.68±1.22	
	stable	N	14.2±2.20	47.9±4.44
		P	33.7±4.71	
	lateral	N	2.06±0.811	7.97±1.56
		P	5.91±1.82	

Data were normalized separately to the total number of vesicles per cell under the basal state and after insulin stimulation. "N" stands for transferrin negative and "P" stands for transferrin positive. N=5 cells (there are normally about 100 vesicles in each cell). Both basal and insulin-stimulated data were obtained for each cell.

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1 Bryant N J, Govers R, James D E. Regulated transport of the glucose transporter GLUT4. Nat Rev Mol Cell Biol, 2002, 3: 267—277
2 Czech M P, Corvera S. Signaling mechanisms that regulate glucose transport. J Biol Chem, 1999, 274: 1865—1868
3 Huang S, Czech M P. The GLUT4 glucose transporter. Cell Metab, 2007, 5: 237—252
4 Rea S, James D E. Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. Diabetes, 1997, 46: 1667—1677
5 Holman G D, Cushman S W. Subcellular localization and trafficking of the GLUT4 glucose transporter isoform in insulin-responsive cells. Bioessays, 1994, 16: 753—759
6 Slot J W, Garruti G, Martin S, et al. Glucose transporter (GLUT-4) is targeted to secretory granules in rat atrial cardiomyocytes. J Cell Biol, 1997, 137: 1243—1254
7 Slot J W, Geuze H J, Gigengack S, et al. Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J Cell Biol, 1991, 113: 123—135
8 Hashiramoto M, James D E. Characterization of insulin-responsive GLUT4 storage vesicles isolated from 3T3-L1 adipocytes. Mol Cell Biol, 2000, 20: 416—427
9 Herman G A, Bonzelius F, Cieutat A M, et al. A distinct class of intracellular storage vesicles, identified by expression of the glucose transporter GLUT4. Proc Natl Acad Sci USA, 1994, 91: 12750—12754
10 Kupriyanova T A, Kandror V, Kandror K V. Isolation and characterization of the two major intracellular Glut4 storage compartments. J Biol Chem, 2002, 277: 9133—9138
11 Livingstone C, James D E, Rice J E, et al. Compartment ablation analysis of the insulin-responsive glucose transporter (GLUT4) in 3T3-L1 adipocytes. Biochem J, 1996, 315(Pt 2): 487—495
12 Malide D, Ramm G, Cushman S W, et al. Immunoelectron microscopic evidence that GLUT4 translocation explains the stimulation of glucose transport in isolated rat white adipose cells. J Cell Sci, 2000, 113 Pt 23: 4203—4210
13 Martin S S, Haruta T, Morris A J, et al. Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. J Biol Chem, 1996, 271: 17605—17608
14 Millar C A, Shewan A, Hickson G R, et al. Differential regulation of secretory compartments containing the insulin-responsive glucose transporter 4 in 3T3-L1 adipocytes. Mol Biol Cell, 1999, 10: 3675—3688
15 Ramm G, Slot J W, James D E, et al. Insulin recruits GLUT4 from specialized VAMP2-carrying vesicles as well as from the dynamic endosomal/trans-Golgi network in rat adipocytes. Mol Biol Cell, 2000, 11: 4079—4091
16 Zeigerer A, Lampson M A, Karylowski O, et al. GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. Mol Biol Cell, 2003, 13: 2421—2435
17 Dugani C B, Klip A. Glucose transporter 4: cycling, compartments and controversies. EMBOJ, 2005, Rep 6: 1137—1142
18 Bai L, Wang Y, Fan J, et al. Dissecting multiple steps of GLUT4 trafficking and identifying the sites of insulin action. Cell Metab, 2007, 5: 47—57
19 Huang S, Lifshitz L M, Jones C, et al. Insulin stimulates membrane fusion and GLUT4 accumulation in clathrin coats on adipocyte plasma membranes. Mol Cell Biol, 2007, 27: 3456—3469
20 Li C H, Bai L, Li D D, et al. Dynamic tracking and mobility analysis of single GLUT4 storage vesicle in live 3T3-L1 cells. Cell Res, 2004, 14: 480—486
21 Lizunov V A, Matsumoto H, Zimmerberg J, et al. Insulin stimulates the halting, tethering, and fusion of mobile GLUT4 vesicles in rat adipose cells. J Cell Biol, 2005, 169: 481—489
22 Jiang L, Fan J, Bai L, et al. Direct quantification of fusion rate reveals a distal role for AS160 in insulin-stimulated fusion of GLUT4 storage vesicles. J Biol Chem, 2008: 283: 8508—8516
23 Blot V, McGraw T E. Molecular mechanisms controlling GLUT4 intracellular retention. Mol Biol Cell, 2008, 19: 3477—3487