RESEARCH COMMUNICATION

GLUT4 vesicle dynamics in living 3T3 L1 adipocytes visualized with green-fluorescent protein

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Insulin stimulates glucose uptake into its target cells by a process which involves the translocation of the GLUT4 isoform of glucose transporter from an intracellular vesicular compartment(s) to the plasma membrane. The step(s) at which insulin acts in the vesicle trafficking pathway (e.g. vesicle movement or fusion with the plasma membrane) is not known. We expressed a green-fluorescent protein–GLUT4 (GFP–GLUT4) chimera in 3T3 L1 adipocytes. The chimera was expressed in vesicles located throughout the cytoplasm and also close to the plasma membrane. Insulin promoted a substantial translocation of GFP–GLUT4 to the plasma membrane. Time-lapse confocal

microscopy demonstrated that the majority of GFP–GLUT4-containing vesicles in the basal state were relatively static, as if tethered (or attached) to an intracellular structure. A proportion (approx. 5%) of the vesicles spontaneously lost their tether, and were observed to move rapidly within the cell. Other vesicles appear to be tethered only on one edge and were observed in a rapid stretching motion. The data support a model in which GLUT4-containing vesicles are tightly tethered to an intracellular structure(s), and indicate that a primary site of insulin action must be to release these vesicles, allowing them to then translocate to and fuse with the plasma membrane.

INTRODUCTION

Insulin-stimulated glucose uptake into adipocytes and muscle cells involves translocation of the GLUT4 isoform of glucose transporter from an intracellular location to the plasma membrane. Immunofluorescence and electron-microscopic analysis of non-stimulated cells has revealed that GLUT4-containing vesicles are clustered adjacent to early or late endosomes, in the *trans*-Golgi reticulum, or in the cytoplasm often close to the plasma membrane [1–4]. A small proportion of GLUT4 is also localized within the constitutively recycling endocytic system, but mounting evidence favours the existence of a unique pool of insulin-regulatable GLUT4 vesicles [5,6].

The mechanism by which insulin causes GLUT4-containing vesicles to be recruited to, and fuse with, the plasma membrane has received much attention. There is good evidence that phosphoinositide 3-kinase (PtdIns 3-kinase) plays an important role, as insulin-stimulated GLUT4 translocation is blocked by wortmannin, a relatively specific inhibitor of PtdIns 3-kinase [7], and is mimicked by over-expression of a constitutively active PtdIns 3-kinase (iSH2.p110) [8,9]. One of the potential targets for PtdIns 3-kinase activation is protein kinase B, and there is indirect evidence that this enzyme may also be involved in the translocation response. In particular, a constitutively active membrane-targeted form of protein kinase B promotes GLUT4 translocation in the absence of insulin, when over-expressed in rat adipocytes or 3T3 L1 cells [10,11].

Other studies have focused on the molecular events by which the GLUT4-containing vesicles fuse with the plasma membrane

after insulin stimulation. These studies strongly suggest the possible involvement of cellubrevin, synaptobrevin and syntaxin-4 in the fusion of GLUT4 vesicles with the plasma membrane, a process which has considerable similarities to neuroexocytosis [12–15]. However, whether this fusion event is under direct regulation by insulin, or whether the primary site of action of insulin is to control the movements of vesicles from deep within the cell to the plasma membrane, is not known.

We have developed a novel system for monitoring GLUT4 trafficking in living single cells by taking advantage of the intrinsically fluorescent green-fluorescent protein (GFP) from *Aequoria victoria*. We have previously demonstrated that expression of a chimera between GLUT4 and GFP in Chinese hamster ovary cells allows us to follow insulin-stimulated GLUT4 translocation to the cell surface in living cells [16]. In the current study, we have continued the development of this system, such that we can follow GLUT4 movements in living 3T3 L1 adipocytes by time-lapse confocal microscopy. In the present paper we provide evidence that supports a model in which a primary site of insulin action must be to release GLUT4 vesicles from an intracellular ‘tethering’ (attachment) site.

MATERIALS AND METHODS

Materials

Murine 3T3 L1 fibroblasts were purchased from the American Tissue Culture Collection (CCL-92.1). Unless otherwise stated, all biochemical reagents and tissue-culture media were obtained

Abbreviations used: DMEM, Dulbecco's modified Eagles medium; FCS, fetal calf serum; GFP, green-fluorescent protein; sulfo-NHS-biotin, sulphosuccinimidyl-6-(biotinamido) hexanoate; PtdIns 3-kinase, phosphoinositide 3-kinase; TRITC, tetramethyl-Rhodamine isothiocyanate.

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from Sigma (Poole, Dorset, U.K.), except for myoclon plus fetal calf serum (FCS) which was obtained from Gibco (Paisley, U.K.). The plasmid pGFP-GLUT4 was generated as described previously [16], except that a mutant GFP[S65T/V163A] with improved fluorescence and stability characteristics was used (a gift from Dr. J. Pines, Wellcome/CRC Institute, Cambridge, U.K.) in place of wild-type GFP. All plasmids were purified by two sequential CsCl gradient centrifugations and were resuspended in 0.4 mM Tris/HCl/0.04 mM EDTA, pH 8.0.

Culture and microinjection of 3T3 L1 cells

3T3 L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn FCS/200 units/ml benzylpenicillin/100 µg/ml streptomycin and used between passages 5 and 10. Fibroblasts were seeded onto polylysine-coated coverslips and allowed to grow to confluence. Conversion into adipocytes was induced at confluence in DMEM containing 10% myoclon plus FCS, 0.25 µM dexamethasone, 0.5 mM isobutylmethylxanthine and 166 nM insulin for 2 days (time zero to day 2). This medium was replaced with DMEM containing 10% myoclon plus FCS and 166 nM insulin for a further 2 days (day 2 to day 4). From day 4 onwards, cells were fed with DMEM containing 10% myoclon plus FCS, and this medium was replaced every 2 days.

Cells were microinjected once the adipocyte phenotype was obtained, and this was usually from day 6 onwards. Plasmids at 20–100 µg/ml (in 0.4 mM Tris/HCl/ 0.04 mM EDTA, pH 8.0) were microinjected, using an Eppendorf semi-automatic microinjection system, into adipocytes in DMEM containing 10% FCS, 25 mM Hepes and 2 mM NaHCO₃. After microinjection, the cells were incubated in DMEM containing 10% (v/v) myoclon plus FCS for 16–24 h before treatments and fluorescence image analysis.

Analysis of GFP fluorescence in living cells

Microinjected adipocytes were serum starved for 2 h before fluorescence analysis in Krebs–Ringer phosphate buffer (136 mM NaCl/4.7 mM KCl/1.25 mM MgSO₄/1.25 mM CaCl₂/5 mM sodium phosphate) containing 2 mM NaHCO₃ and 25 mM Hepes, pH 7.4, at 37 °C. Alternatively, the cells were incubated in serum-free DMEM at 37 °C and 5% CO₂ for cell-surface labelling.

For cell-surface labelling, the cells were incubated with 0.5 mg/ml sulfo-NHS-biotin [sulphosuccinimidyl-6-(biotin-amido) hexanoate; Pierce Chemicals, Rockford, IL, U.S.A.] in PBS for 15 min at 15–20 °C, washed with PBS and then fixed in 4% paraformaldehyde in PBS for 15 min. This, and all subsequent steps, were performed at room temperature. The cells were blocked with 1% BSA in PBS (BSA/PBS) for 30 min and then incubated in a 1:100 dilution of Texas Red-avidin (Vector Laboratories, Peterborough, U.K.) in BSA/PBS for 30 min. After extensive washing in PBS, the coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA, U.S.A.) for confocal microscopy.

Immunofluorescence analysis

The cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 15 min, and permeabilized in PBS containing 1% Triton X-100 for 5 min. The cells were then blocked with BSA/PBS for 30 min, and incubated in a 1:1000 dilution of anti-tubulin monoclonal antibody (Serotec, Kidlington, Oxford, U.K.) in BSA/PBS for 30 min. This was followed by incubation in a 1:200 dilution of biotinylated anti-mouse IgG (Vector Labora-

tories) for 15 min, and finally a 1:100 dilution of Texas Red-avidin for 15 min. Alternatively, the fixed and permeabilized cells were incubated with 0.1 µg/ml tetramethyl-Rhodamine isothiocyanate (TRITC)-phalloidin (Molecular Probes). All coverslips were washed extensively between incubations in PBS, and finally mounted in Mowiol for confocal microscopy.

Confocal microscopy and image analysis

Confocal microscopy was performed with a Leica DM IRBE inverted confocal microscope controlled with TCS-NT4 software (Leica). Images were processed either with NIH Image 1.61 software (public domain software available from <ftp://zippp.nimh.nih.gov/pub/image/>) or with Adobe Photoshop 3.0. Time-lapse sequences were saved in 'animated GIF' format using GIF Builder 0.5 written by Dr. Yves Piguet, Ecole Polytechnique Fédérale de Lausanne, Switzerland (public domain software available from <http://iawwww.epfl.ch/Staff/Yves.Piguet/clip-2gif-home/GifBuilder.html>).

RESULTS AND DISCUSSION

Expression of GFP-GLUT4 in 3T3 L1 adipocytes

In our previous studies in Chinese hamster ovary cells we used a chimaera between GLUT4 and wild-type GFP. This construct suffered from several limitations, such as photoisomerization and lack of detectable fluorescence in 3T3 L1 adipocytes. For this reason we replaced the wild-type GFP sequence with a mutant GFP which exhibits improved fluorescence properties (increased brightness, no photoisomerization and improved thermal stability [17]). This chimaera, which places GFP at the N-terminus of GLUT4, was expressed in 3T3 L1 adipocytes by direct nuclear microinjection of plasmid DNA. GFP fluorescence was first detectable approx. 6 h post-injection, but was maximal after 24 h. Hence the experiments described below were all performed 24 h after injection.

In non-stimulated adipocytes, GFP-GLUT4-containing vesicles were distributed throughout the cytoplasm of the cell, both close to the nucleus as well as just beneath the plasma membrane (Figure 1a). Insulin caused a substantial re-distribution of GFP-GLUT4, such that an almost continuous line of fluorescence was observed around the plasma membrane after 60 min (Figure 1b). Insulin did not cause a complete translocation of GLUT4, as would be expected from studies on the re-distribution of native GLUT4 in these cells [3,4,6,18,19].

Careful inspection of a confocal section through the top of a 3T3 L1 adipocyte demonstrated that after a 1 h treatment with insulin, the distribution of GFP-GLUT4 in the membrane was still punctate in nature (Figure 1d). It remains a possibility therefore that GLUT4 does not evenly redistribute through the plasma membrane leaflet after insulin stimulation, and that it remains in discrete locations ('caps') ready for rapid re-internalization.

In non-insulin-stimulated cells we often see GFP-GLUT4 fluorescence in vesicles that are close to the plasma membrane. As it was difficult to distinguish whether these vesicles were within the plasma membrane leaflet, or were underlying the membrane, we surface labelled living cells with a membrane-impermeant reactive biotin (sulfo-NHS-biotin), followed by visualization with Texas Red-avidin. As shown in Figure 1(e), in the basal state there was little co-localization between GFP-GLUT4 and biotin. Most of the GFP-GLUT4 vesicles that reside in close proximity to the plasma membrane appeared to underly the membrane rather than be incorporated into the leaflet itself (see enlarged inset to Figure 1e). Potentially, these

Figure 1 Expression and translocation of GFP-GLUT4 in 3T3 L1 adipocytes

3T3 L1 adipocytes were microinjected with pGFP-GLUT4 and imaged 24 h later after incubation in the absence (a, c) or presence (b, d) of 200 nM insulin for 1 h. Panels (a–d) illustrate sections through the middle (a and b) or top (c and d) of two typical 3T3 L1 adipocytes. In (e) and (f), 3T3 L1 adipocytes were microinjected with pGFP-GLUT4; 24 h later the cells were incubated in the absence (e) or presence (f) of 200 nM insulin for 1 h, and then surface labelled with an impermeant amino-reactive biotin (sulfo-NHS-biotin). The cells were fixed and imaged for the distribution of GFP-GLUT4 (green) and biotin (red, visualized with Texas Red-avidin). Yellow indicates the areas of co-localization. The scale bars represent 10 μm .

vesicles are in a situation where they could rapidly fuse with the plasma membrane upon treatment of the cells with insulin. After the addition of insulin, a redistribution occurs, such that an almost continuous line of co-localization of GFP-GLUT4 and biotin occurs in the plasma membrane (Figure 1f). Indeed, in the enlarged inset of Figure 1(f), GFP-GLUT4 can be visualized in the membrane of the top cell (small arrowhead), but not in the membrane of the lower cell (large arrowhead), which does not express GFP-GLUT4.

Time-lapse confocal microscopy of GFP-GLUT4 movements

We next attempted to visualize the movements of GFP-GLUT4 vesicles by time-lapse confocal microscopy. A typical experiment on a non-stimulated 3T3 L1 adipocyte is shown in Figure 2. Here we followed GFP-GLUT4 vesicles over a period of 15 min, taking images every 30 s (i.e. a total of 30 images were collected). For convenience, we show sequences of this cell (3.5 min in length) in three different regions of interest (indicated b–d in Figure 2a). The phenomena we observed are best appreciated by looking at each of the sequences shown in Figures 2b–2d as a time-lapse movie sequence. These can be accessed via the World Wide Web as an on-line supplement (URL: <http://www.BiochemJ.org/bj/327/bj3270637add.htm>).

The majority of GFP-GLUT4-containing vesicles shown in Figure 2(a) were relatively immobile. This is clearly demonstrated in Figure 2(b). Indeed, during the entire 15 min period of observation these vesicles moved little more than two vesicle diameters (approx. 1–2 μm) from their starting position. However, we observed two other types of vesicle movement which are illustrated in the sequences shown in Figures 2(c) and 2(d). The vesicle indicated by an arrow in Figure 2(c), which is a more rarely observed type of vesicle where the lumen is clearly visible,

moves rapidly down the field of view, at approx. 0.1 $\mu\text{m/s}$ and then out of the plane of view of the confocal section (Figure 2c, panel 7). At the same time, the other vesicles in this field of view remained relatively static.

In Figure 2(d), again we can see the rapid movement of a vesicle (white arrow). This vesicle is static for at least 10 min (results not shown) until panel 3 of Figure 2(d), when it moves rapidly down and to the left at approx. 0.17 $\mu\text{m/s}$, before disappearing out of the focal plane (after Figure 2d, panel 6). The vesicle indicated with a yellow arrow appears to be tethered on one side, and stretches up, down and to the left, but does not escape its tethering.

Thus it appears that GFP-GLUT4-containing vesicles are tightly tethered to an intracellular structure. A small but significant proportion of these vesicles (approx. 5%) are released from this tether and are then free to move to alternative locations within the cell. We have observed that some vesicles become re-tethered after an initial rapid transit (results not shown). The data strongly suggest that a primary site of action of insulin must be to release these vesicles from this tethering site, such that they can then translocate to the plasma membrane. Interestingly, a similar phenomenon has been observed with GFP-labelled secretory granules in PC12 cells [20]. These vesicles also appeared to be either stationary or moved very slowly. Others travelled extensively, at rates of approx. 0.05 $\mu\text{m/s}$.

For technical reasons, we have found it difficult to observe an insulin-dependent translocation of GFP-GLUT4 to the plasma membrane on the microscope stage using laser scanning confocal microscopy. The reasons for this are not known. However, as the insulin effect on GFP-GLUT4 translocation appears to be slower than for native GLUT4 (results not shown), prolonged and continuous excitation with the lasers (for up to 60 min) may be phototoxic to 3T3 L1 cells. We are currently investigating

Figure 2 For legend see opposite page

Figure 3 Distribution of microtubules and actin microfilaments in 3T3 L1 fibroblasts and adipocytes

3T3 L1 fibroblasts (**a**, **b**, **d**, **e**) or adipocytes (**c**, **f**) were fixed, permeabilised and stained for tubulin (**a–c**) or actin microfilaments (**d–f**) with anti-tubulin antibodies (visualized with Texas Red–avidin) and TRITC–phalloidin respectively. The adipocytes in (**c**) and (**f**) also express GFP–GLUT4. In (**b**) and (**e**) the cells were treated for 2 h with 60 μ M nocodazole or 2 μ M cytochalasin D respectively. Note that the adipocyte in the centre of the field of view in (**f**) is surrounded by 3T3 L1 fibroblasts that have retained their actin microfilaments. Scale bar represents 10 μ m.

alternative methodologies to follow the insulin-dependent translocation of vesicles.

We investigated the possible nature of this tethering site. The rapid movement of some GLUT4 vesicles was reminiscent of the microtubule-based movements of peroxisomes in CV1 cells [21,22]. In these studies, peroxisomes were shown to move along microtubules at approx. 0.26–0.45 μ m/s, a process that was blocked by microtubule-destabilizing drugs such as nocodazole. We found that microtubules were present in 3T3 L1 fibroblasts, as visualized with anti-tubulin antibodies (Figure 3a), and collapsed in characteristic fashion upon treatment of these cells with nocodazole (Figure 3b). However, these microtubules appear to become depolymerized during the differentiation of 3T3 L1 fibroblasts into adipocytes (Figure 3c), and we see no close association of GFP–GLUT4 with the remaining tubulin in the adipocytes (Figure 3c). In addition, nocodazole had no effect on GFP–GLUT4 translocation to the cell surface in response to insulin, nor did vinblastine or colcemid, which also destabilize microtubules (results not shown).

In the apparent absence of a role for microtubules, we examined actin microfilaments, which were visualized with TRITC–phalloidin (Figures 3d–3f). Actin microfilaments were observed (Figure 3d) in 3T3 L1 fibroblasts, and these were destabilized in

the presence of cytochalasin D (Figure 3e). However, like microtubules, these actin microfilaments became depolymerized during 3T3 L1 adipocyte differentiation (Figure 3f). Consistent with this phenomenon, we found no effect of cytochalasin D on GFP–GLUT4 translocation to the cell surface in response to insulin (results not shown). These data rule out a role for both tubulin microtubules and actin microfilaments in the intracellular tethering of GFP–GLUT4 vesicles, and their translocation to the plasma membrane.

In conclusion, our data support a model in which GLUT4-containing vesicles are tightly tethered to an intracellular structure(s). This means that insulin may act at several points along the GLUT4 trafficking pathway, for example vesicle release, translocation and fusion with the plasma membrane. Our data suggest that the primary site of insulin action must be to release GLUT4 vesicles from their tether, but it remains to be established whether insulin also acts at the translocation and fusion steps. This hypothesis is consistent with recent data suggesting that insulin-stimulated GLUT4 translocation may require targeting of PtdIns 3-kinase to the high-density microsomal fraction [23–25], and that additional PtdIns 3-kinase-independent events are also required [26]. Future work must be directed at resolving the identity of the intracellular site of attachment of GLUT4

Figure 2 Time-lapse confocal microscopy of GFP–GLUT4 in 3T3 L1 adipocytes

3T3 L1 adipocytes were microinjected with GFP–pGLUT4 and a selected representative cell was imaged 24 h later. An image of the cell is shown in (**a**). Images of this cell, which was incubated at 37 °C, were taken at 30 s intervals over a 15 min period. Seven images (numbered sequentially) are shown from selected regions of interest (**b–d**) within this cell, and illustrate the three types of movement observed (see text for discussion). The eighth panel in each case plots the position of a selected vesicle during the time course shown. The scale bar represents 20 μ m. Panels (**b–d**) are best viewed as time-lapse movie sequences. These can be accessed through the World Wide Web by connecting to URL: <http://www.BiochemJ.org/bj/327/bj3270637add.htm>

vesicles, and determining whether PtdIns 3-kinase plays a role in the release of these vesicles, allowing them to translocate to the plasma membrane.

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