Compartment ablation analysis of the insulin-responsive glucose transporter (GLUT4) in 3T3-L1 adipocytes

Callum LIVINGSTONE*, David E. JAMES†, Jacqueline E. RICE*, David HANPETER‡ and Gwyn W. GOULD*§

*Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., †Centre for Molecular and Cellular Biology, University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia, and ‡Department of Surgery, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, U.S.A.

The translocation of a unique facilitative glucose transporter isoform (GLUT4) from an intracellular site to the plasma membrane accounts for the large insulin-dependent increase in glucose transport observed in muscle and adipose tissue. The intracellular location of GLUT4 in the basal state and the pathway by which it reaches the cell surface upon insulin stimulation are unclear. Here, we have examined the co-localization of GLUT4 with the transferrin receptor, a protein which is known to recycle through the endosomal system. Using an anti-GLUT4 monoclonal antibody we immunoisolated a vesicular fraction from an intracellular membrane fraction of 3T3-L1 adipocytes that contained $> 90\,\%$ of the immunoreactive GLUT4 found in this fraction, but only $40\,\%$ of the transferrin receptor (TfR). These results suggest only a limited degree of co-localization of these proteins. Using a technique to cross-link and render insoluble ('ablate') intracellular compartments containing the TfR by means of a transferrin–horseradish peroxidase conjugate (Tf–HRP), we further examined the relationship between the endosomal recycling pathway and the intracellular compartment containing GLUT4 in these cells. Incubation of non-stimulated cells with Tf–HRP for 3 h at 37 °C resulted in quantitative ablation of the intracellular TfR, GLUT1 and mannose-6-phosphate receptor and a shift in the density of Rab5-positive membranes. In contrast, only $40\,\%$ of intracellular GLUT4 was ablated under the same conditions. Ablation was specific for the endosomal system as there was no significant ablation of either TGN38 or lgp120, which are markers for the *trans* Golgi reticulum and lysosomes respectively. Subcellular fractionation analysis revealed that most of the ablated pools of GLUT4 and TfR were found in the intracellular membrane fraction. The extent of ablation of GLUT4 from the intracellular fraction was unchanged in cells which were insulin-stimulated prior to ablation, whereas GLUT1 exhibited increased ablation in insulin-stimulated cells. Pretreatment of adipocytes with okadaic acid, an inhibitor of Type-I and -IIa phosphatases, increased GLUT4 ablation in the presence of insulin, consistent with okadaic acid increasing the internalization of GLUT4 from the plasma membrane under these conditions. Using a combination of subcellular fractionation, vesicle immunoadsorption and compartment ablation using the Tf–HRP conjugate we have been able to resolve overlapping but distinct intracellular distributions of the TfR and GLUT4 in adipocytes. At least three separate compartments were identified: TfR-positive/GLUT4-negative, TfR-negative/GLUT4-positive, and TfR-positive/GLUT4-positive, as defined by the relative abundance of these two markers. We propose that the TfR-negative/GLUT4-positive compartment, which contains approximately $60\,\%$ of the intracellular GLUT4, represents a specialized intracellular compartment that is withdrawn from the endosomal system. The biosynthesis and characteristics of this compartment may be fundamental to the unique insulin regulation of GLUT4.

INTRODUCTION

Insulin stimulates glucose uptake into adipocytes and muscle, by virtue of the specific expression of an insulin-responsive glucose transporter, GLUT4. The translocation of this transporter from an intracellular site to the plasma membrane in insulin-exposed cells is largely responsible for the 20- to 30-fold increase in glucose transport observed in these cells (for a review see [1,2]). However, the exact intracellular location of GLUT4 in the basal state and the mechanism by which it reaches the cell surface upon insulin stimulation are unclear. Under basal or non-stimulated conditions $> 95\,\%$ of the GLUT4 is localized to tubular vesicular elements that are found clustered either adjacent to early or late endosomes, in the *trans* Golgi reticulum (TGR) or in the cytoplasm often close to the plasma membrane. The level of GLUT4 is decreased by 40–50 % at each of these locations by insulin, suggesting that all of these compartments participate in the insulin-regulated recycling of GLUT4 [3,4].

Numerous endosomal membrane proteins, including the mannose-6-phosphate receptor (M6PR) [5], the transferrin receptor (TfR) [6,7], GLUT1 [8] and the α2-macroglobulin receptor also translocate to the plasma membrane upon insulin stimulation in adipocytes. Moreover, Tanner and Lienhard [7] showed that, at least in adipocytes, GLUT1, TfR and M6PR are co-localized in an intracellular vesicle population. It is now well established that these membrane proteins recycle between an intracellular pool and the plasma membrane, and that this recycling involves the endosomal system (for a review, see [9]). Immunoelectron microscopy studies of GLUT4 in adipocytes have demonstrated that GLUT4 is also located, at least in part,

Abbreviations used: DAB, 3,3′-diaminobenzidine; deGlc, 2-deoxy-D-glucose; DMEM, Dulbecco's modified Eagle's medium; DFP, di-isopropylfluorophosphate; GLUT4, insulin-responsive glucose transporter; HRP, horseradish peroxidase; KRP, Krebs–Ringer phosphate; M6PR, mannose-6-phosphate receptor; SCAMP, secretory carrier membrane protein; TfR, transferrin receptor; Tf–HRP, transferrin–HRP conjugate; TGR, *trans* Golgi reticulum.

§To whom all correspondence should be addressed.

within the endocytic system as it undergoes constitutive recycling through clathrin-coated pits [4,10]. Using membrane-impermeant, glucose transporter-specific photolabels, it has been clearly demonstrated that GLUT4 constantly recycles between the plasma membrane and an intracellular site, both in the presence and absence of insulin [11,12]. This raises the possibility that the intracellular GLUT4 pool may simply comprise elements of the endosomal system.

Several lines of evidence suggest that GLUT4 trafficking is unlikely to be explained by such a model. First, GLUT4 is virtually excluded from the cell surface in non-stimulated cells whereas this is not the case for other membrane proteins such as GLUT1 or the TfR [13,14]. Secondly, the externalization rate of GLUT4 is lower than that of other recycling proteins such as TfR in adipocytes [6,15] and insulin results in a much larger fold increase in GLUT4 at the surface compared with other endosomal proteins (reviewed in [2,9]). Finally, proteins known to be involved in regulated secretory pathways in other cell types such as synaptobrevin and secretory carrier membrane proteins (SCAMPs) have been found in the intracellular GLUT4 vesicles in adipocytes [16]. These results, together with a mathematical analysis of GLUT4 translocation and recycling in adipocytes [17], point to the existence of a unique intracellular pool of GLUT4, which may be considered to be either a sub-endosomal compartment or a separate entity. The primary aim of this study was to determine the relationship between the intracellular GLUT4 compartment and the endosomal recycling pathway using a compartment ablation approach.

TfRs located at the cell surface rapidly exchange with an intracellular pool of receptors which comprises 50–70 % of the total receptor complement in adipocytes [6,7]. Horseradish peroxidase (HRP)-conjugated transferrin (Tf–HRP) binds specifically to the TfR and its recycling characteristics are identical to those of unconjugated transferrin [18]. Incubation of intact cells or cell homogenates exposed to Tf–HRP with DAB (3,3′-diaminobenzidine) and H_2O_2 allows HRP to transfer electrons from DAB to peroxide, causing the intracellular compartments to which the conjugate has been sequestered to be cross-linked into a high-molecular-mass complex and therefore ablated [19]. This DAB/H_2O_2-mediated cross-linking is dependent upon an adequate level of HRP in the microsomes and can be competitively inhibited by unconjugated transferrin. West et al. [20] have shown that when cells were pre-incubated with Tf–HRP, 80–90 % of labelled conjugate and transferrin could be removed by DAB/H_2O_2 treatment, while proteins belonging to other intracellular compartments (secretory pathways, late endosomes and lysosomes) were unaffected. This technique, termed compartment ablation analysis, has been used extensively to study trafficking of various receptors and antigen-processing events [18–22].

In this study we have adapted the use of Tf–HRP conjugate to achieve ablation of the recycling compartment in 3T3-L1 adipocytes. By immunoblotting fractions before and after ablation with antibodies specific for various markers, we have been able to identify an intracellular compartment of GLUT4 that appears to be distinct from the constitutive recycling pathway.

MATERIALS AND METHODS

Materials

Insulin, dexamethasone, isobutylmethylxanthine, HRP, human apotransferrin and all reagents for Tf–HRP synthesis were from Sigma (Poole, Dorset, U.K). Dulbecco's modified Eagle's medium (DMEM), Myoclone-Plus fetal-calf serum and antibiotics were from Gibco BRL (Paisley, U.K.). ^{125}I-labelled transferrin and ^{125}I-labelled goat anti-(rabbit IgG) antibody were from Du Pont. Okadaic acid was from Moana BioTech, Hawaii. All other reagents were as described elsewhere [14,23].

Cell culture

3T3-L1 fibroblasts were grown in 10 % newborn-calf serum in DMEM at 37 °C in 10 % CO_2 and passaged at about 70 % confluence. Cells for use in experiments were grown in the same medium until 2 days post-confluence, then differentiation into adipocytes was achieved as described elsewhere [24]. Cells were used for experiments between 8 and 12 days post-differentiation and between passages 4 and 12. Before use, cell monolayers were washed with serum-free DMEM in order to remove all serum-derived insulin and transferrin; they were then incubated with serum-free DMEM for 2 h.

Preparation of HRP-conjugated transferrin

This was by the carbodiimide method of Kishida et al. [25]. HRP (10 mg) was dissolved in 1 ml of 0.1 M NaCl/10 mM sodium phosphate, pH 7.2, at 4 °C and dialysed overnight against 1 litre of the same buffer. Disodium succinate (200 mg) and succinic anhydride (70 mg) were added to the protein solution and this was stirred for 30 min at 0 °C, then at room temperature for 30 min, and passed over an 8 ml G-50 Sephadex column. The solution was concentrated to 0.5 ml using an Amicon centriprep concentrator. *N*-Hydroxysuccinimide (25 mg) and *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride (40 mg) were added and the solution stirred for 3 h at 0 °C. This was again passed over an 8 ml G-50 Sephadex column in 0.1 M NaCl/1 mM sodium phosphate, pH 7.2, and the eluate collected. Activated HRP was immediately added to 1 ml of 10 mg/ml transferrin containing about 1×10^7 c.p.m. of ^{125}I-transferrin and stirred for 2 days at 2 °C. The conjugation reaction was quenched by addition of glycine and a sample of the reaction was run on a 10 % polyacrylamide gel to check, by autoradiography, for an increase in molecular mass of a proportion of the transferrin counts. Samples from three reactions were pooled and chromatographed on a 75 cm × 2 cm Sephacryl S-300 column in order to separate unconjugated transferrin from the Tf–HRP conjugate. Fractions were counted using an LKB 1275 Minigamma gamma counter and the appropriate fractions were pooled, concentrated and gel filtration was repeated as described above. Fractions were again pooled and concentrated and a sample again resolved on a polyacrylamide gel to ensure removal of unconjugated transferrin. Conjugate was divided into aliquots and stored at −80 °C until required for use. A sample was assayed for protein and iron-loaded exactly as described [20].

Use of Tf–HRP

After a 2 h incubation in serum-free DMEM, adipocytes were incubated with 20 μg/ml Tf–HRP for the times indicated to fill the transferrin itinerary. Cells were thereafter chilled by washing in ice-cold isotonic citrate buffer and kept on ice in order to prevent any further vesicle trafficking during the DAB cytochemistry reactions (see below). Cell-surface-attached Tf–HRP was removed by acid washing for 10 min in ice-cold isotonic citrate buffer (150 mM NaCl, 20 mM sodium citrate, pH 5.0) with three changes of buffer, then the monolayer was washed once in ice-cold PBS, pH 7.4.

DAB cytochemistry

DAB (freshly prepared as a 2 mg/ml stock and filtered through a 0.22 μm-pore-size filter) was added at 100 μg/ml to all cells and

H_2O_2 added (final concentration 0.02 %, v/v) to one of each pair of wells. After a 60 min incubation at 4 °C in the dark the reaction was stopped by washing in PBS containing 5 mg/ml BSA. This was then aspirated and samples prepared for immunoblotting. In all experiments, duplicate plates were used, one of which was exposed to DAB and H_2O_2, the other only to DAB as a negative control.

Analysis of the dose dependence of conjugate and DAB on ablation of TfR demonstrated optimum ablation at a concentration of 20 μg/ml of conjugate and 100 μg/ml DAB (results not shown). These conditions were routinely used in subsequent studies.

Cell-surface binding of transferrin to adipocytes

The binding of ^{125}I-transferrin to 3T3-L1 adipocytes was performed exactly as described in [6]. Briefly, after incubation in serum-free DMEM for 2 h, cells were washed three times in ice-cold Krebs–Ringer phosphate (KRP), then incubated in ice-cold KRP containing 1 mg/ml BSA with 250 ng/ml ^{125}I-transferrin (~200000 c.p.m./well) for 2 h. After this time, unbound ligand was aspirated and the monolayers washed three times with ice-cold KRP for 1 min. Cells were solubilized in 1 M NaOH, and radioactivity associated with each well was determined. Non-specific binding of radioligand was taken as that amount of ^{125}I-transferrin bound in the presence of 1 μM transferrin, and was found to vary between 20 and 40 % of the total counts per well.

Preparation of whole-cell homogenates

Whole-cell homogenates were prepared by scraping the cells into HES buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 255 mM sucrose) containing the protease inhibitors pepstatin A (1 μg/ml), di-isopropylfluorophosphate (DFP; 0.2 mM) and E64 (20 μM). Cells were homogenized by 20 strokes of a Teflon/glass homogenizer and protein precipitated using trichloroacetic acid in the presence of deoxycholate and 0.1 % SDS to facilitate protein recovery. After centrifugation, samples were solubilized in SDS/PAGE loading buffer [26].

Subcellular fractionation of adipocytes

Adipocytes were subjected to a differential centrifugation procedure as described previously [14]. Cells grown on 10-cm-diam. cell culture dishes were scraped into ice-cold HES buffer (5 ml per 10-cm-diam. plate) containing protease inhibitors (1 μg/ml pepstatin A, 0.2 mM DFP, 20 μM E64 and 50 μM aprotinin) and homogenized by 10 strokes of a Teflon/glass homogenizer. The homogenate was centrifuged at 19000 ***g*** for 20 min at 4 °C. The pellet from this spin was resuspended in 2 ml of HES buffer, layered on to 1 ml of 1.12 M sucrose in HES buffer and centrifuged at 100000 ***g*** for 60 min at 4 °C in a swing-out rotor. Plasma membranes were collected from the interface by careful aspiration, resuspended in HES buffer and collected by centrifugation at 41000 ***g*** for 20 min at 4 °C. The supernatant from the 19000 ***g*** spin was recentrifuged at 41000 ***g*** to yield a high-density microsomal pellet and the supernatant from this spin centrifuged at 180000 ***g*** for 75 min at 4 °C to collect low-density microsomes. All fractions were resuspended in equal volumes of HES buffer (cell equivalents), snap frozen in liquid nitrogen and stored at −80 °C prior to use.

Preparation of total internal membranes

Homogenates of adipocytes in HES buffer were centrifuged at 19000 ***g*** for 20 min at 4 °C to pellet the plasma membrane fraction [14,27]. The supernatant from this step was then centrifuged at 150000 ***g*** for 60 min at 4 °C to pellet the remaining membrane fraction. The pellet from this step was resuspended by gentle homogenization in 20 mM Hepes/1 mM EDTA, pH 7.4, containing protease inhibitors.

Sucrose gradient centrifugation

Samples of total internal membranes were loaded on to step gradients comprised of 1.5 M, 1.3 M, 1.1 M, 1.0 M, 0.9 M, 0.8 M, 0.7 M, 0.6 M and 0.5 M sucrose solutions. These were then centrifuged at 75000 ***g*** for 24 h at 4 °C. Samples were collected by tube puncture and the protein in each fraction precipitated. Samples were resuspended in SDS/PAGE buffer and stored at −80 °C prior to immunological analysis.

Immunoadsorption experiments

3T3-L1 adipocytes were fractionated as described above with minor modifications. For preparation of intracellular membranes (low-density microsomes) for immunoadsorption, six 10-cm-diam. plates of adipocytes were incubated with KRP buffer containing 1 % BSA for 2 h at 37 °C. All subsequent steps were performed at 4 °C. Plates were then rinsed with HES buffer, scraped with a rubber policeman into 6 ml of HES buffer and homogenized using a Yamato homogenizer [14]. The homogenate was centrifuged at 41000 ***g*** for 20 min to remove plasma membranes, mitochondria, nuclei and high-density microsomes [14]. The supernatant was layered on to a 1.5 M sucrose cushion in HES buffer and centrifuged at 120000 ***g*** in a Beckman SW-41 rotor for 60 min. The membrane layer above the sucrose layer (low-density microsomes) was carefully removed, adjusted to 100 mM NaCl, and incubated with the immunoadsorbent for 3 h at 4 °C with end-over-end rotation in PBS containing 0.1 % fish gelatin (Sigma) in a final volume of 750 μl. Samples were then centrifuged at 10000 ***g*** for 20 s to pellet the immunoadsorbent. The immunoadsorbent was resuspended in PBS (1 ml), incubated for 20 min at 4 °C, transferred to a new centrifuge tube and centrifuged again at 10000 ***g***. This procedure was repeated three times. All supernatants were recovered and centrifuged at 100000 ***g***. These membranes together with the immunoadsorbent were then prepared for SDS/PAGE. Cellulose fibres were used as the immunoadsorbent. Goat anti-(mouse IgG) or goat anti-(rabbit IgG) secondary antibodies, obtained from East Acres Biologicals, MA, U.S.A., were conjugated to cellulose fibres as described previously [28] at a ratio of 1 mg of antibody/mg of cellulose. Cellulose fibres were incubated with 0.5 % BSA in PBS for 1 h to block non-specific binding sites prior to incubation with membranes.

Electrophoresis and immunoblotting

Proteins were electrophoresed on 7.5, 10 or 15 % SDS/polyacrylamide gels and transblotted on to nitrocellulose sheets as described elsewhere [29]. Immunolabelled proteins were visualized using either ^{125}I-labelled goat anti-(rabbit IgG) second antibody followed by autoradiography, or using HRP-conjugated secondary antibody and the enhanced chemiluminescence system (Amersham). Bands were quantified either by using a γ-counter, or by densitometry. To quantify the degree of protein ablation we have measured the difference in immunoreactive signal between membranes obtained from cells incubated in the absence or presence of DAB/peroxide.

Antibodies

The anti-GLUT4 antibodies used were either a monoclonal antibody (1F8) [30], a rabbit polyclonal antibody raised against

a GLUT4 C-terminal synthetic peptide [31], or antibodies against the C-terminal 14-amino-acid residues of the human isoform of GLUT4 [32]. The monoclonal antibody was used for immunoadsorption and the polyclonal antibodies were used for immunoblotting. The anti-M6PR antibody was a kind gift from Dr. Gus Lienhard (Department of Biochemistry, Dartmouth Medical School, Hanover, NH, U.S.A.). The polyclonal antiserum against the C-terminal 21-amino-acid residues of TGN38 has been described [33], and was provided by Dr. George Banting (University of Bristol, U.K.), together with antiserum against lgp120. Anti-GLUT1 serum was from East Acres Biologicals (Southbridge, MA, U.S.A.). An anti-TfR monoclonal antibody was kindly provided by Dr. Ian Trowbridge (Salk Institute, San Diego, CA, U.S.A.) and Professor Colin Hopkins (University College, London). Anti-rab5 serum was the generous gift of Professor Reinhard Jahn (Yale University Medical School, U.S.A.).

Table 1 Tf–HRP binds to TfRs

Shown are data from a representative experiment in which the effects of unlabelled transferrin (80 μg/ml), HRP alone (50 μg/ml), or Tf–HRP (0.5 or 20 μg/ml) on the binding of 250 ng/ml ^{125}I-transferrin to cell-surface receptors was analysed. Each point is the mean of triplicate determinations ($\pm$S.D.). Transferrin binding to the surface of 3T3-L1 adipocytes was determined as described in the Materials and methods section.

Condition	Transferrin bound/well (% of control)
Control (basal)	100
+ HRP	95 $\pm$ 7
+ 80 μg/ml Transferrin	17 $\pm$ 4
+ 0.5 μg/ml Tf–HRP	72 $\pm$ 9
+ 20 μg/ml Tf–HRP	18 $\pm$ 3

RESULTS

Co-localization of TfR and GLUT4 in 3T3-L1 adipocytes

Previous attempts to characterize the intracellular GLUT4 compartment in adipocytes have utilized subcellular fractionation followed by immunoisolation. This approach relies on the use of antibodies specific for cytoplasmic domains in GLUT4 bound to a solid support, in order to selectively and specifically deplete the GLUT4-containing vesicles from the intracellular low-density microsomal fraction of adipocytes. Figure 1 shows an experiment in which vesicles enriched in either GLUT4 or TfR have been immunoadsorbed from 3T3-L1 adipocytes. Immunoadsorption using the GLUT4-specific antibody resulted in the purification of a fraction containing $> 90\,\%$ of the total intracellular GLUT4 and $40\,\%$ of total TfR. Conversely, using anti-TfR antibodies, a vesicle fraction was identified that contained $50\,\%$ of the TfR and only $40\,\%$ of GLUT4. While these studies clearly reveal an overlap in the intracellular targeting of these two proteins, the inability to completely deplete low-density microsomes of GLUT4 using anti-TfR antibodies (and vice versa) suggests that significant populations of these two proteins may reside in distinct internal compartments.

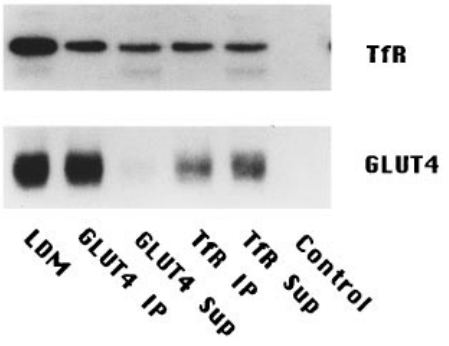

Figure 1 Immunoadsorption of intracellular vesicles containing GLUT4 or the TfR from 3T3-L1 adipocytes

Intracellular membranes (LDM) were isolated from non-stimulated 3T3-L1 adipocytes and incubated with anti-GLUT4 antibody (1F8) or anti-TfR antibody pre-coupled to goat anti-(mouse IgG)-conjugated cellulose fibres or goat anti-(mouse IgG)-conjugated cellulose fibres alone (Control). The cellulose fibres (IP) and the vesicle supernatants (sup) were recovered by centrifugation and prepared for SDS/PAGE. Immunoadsorbed fractions (GLUT4 IP, TfR IP, Control), the resultant supernatants (GLUT4 sup, TfR sup) and membrane starting material (LDM, 50 μg of protein) were immunoblotted with antibodies specific for either GLUT4 or the TfR, as indicated.

The disadvantage of the vesicle immunoadsorption technique is that it is often not possible to distinguish between different intracellular compartments containing a protein of interest. For instance, the most likely interpretation of the data in Figure 1 is that GLUT4 is distributed both in early endosomes containing the TfR and a distinct compartment. This interpretation is consistent with electron microscopy data [3,4]. In order to test this hypothesis more directly and to further characterize the intracellular GLUT4 compartment in adipocytes we have utilized a Tf–HRP conjugate to ablate the early endosomal/recycling system.

Ablation of TfR in 3T3-L1 adipocytes

Before studying the effect of compartment ablation on GLUT4 it was considered important to test the efficacy of the Tf–HRP conjugate in 3T3-L1 adipocytes. The conjugate, but not HRP, was effective in inhibiting ^{125}I-transferrin binding to adipocytes at 4 °C (Table 1). In a further control experiment, cells were incubated with ^{125}I-transferrin (3 nM) for 2 h at 37 °C, conditions previously shown to achieve a constant level of internalized transferrin [6]. After this time, cells were washed extensively, and incubated with either 20 μg/ml unlabelled transferrin, or an equivalent concentration of Tf–HRP for increasing times. The ability of unlabelled transferrin or Tf–HRP conjugate to chase-out the internalized radiolabelled transferrin was monitored. In both cases, the rate and extent of the loss of labelled transferrin were similar, indicating that the Tf–HRP was accessible to the internalized TfR pool (results not shown).

Non-stimulated adipocytes were incubated with Tf–HRP for different times between 20 and 180 min prior to ablation by exposure to DAB and H_2O_2. Whole-cell homogenates were then analysed for ablation of the TfR (Figure 2). Maximal ablation of the TfR was achieved after incubation of cells with Tf–HRP for about 2 h. These results demonstrate that the conjugate was effective and that ablation of TfR was observed. Note, however, that complete ablation was not achieved. We felt it likely that this was a consequence of a portion of TfR residing at the cell surface and thus being beyond the influence of the internalized conjugate. In an effort to address this point, cells were incubated with Tf–HRP for 1 h or 3 h at 37 °C, or alternatively for 3 h at 4 °C, and ablation performed as described. Cells were then subfractionated into plasma membrane or low-density

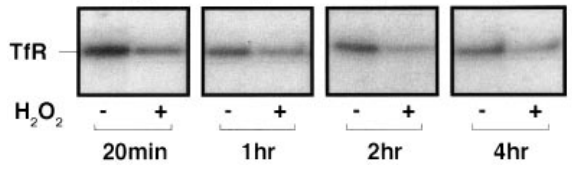

Figure 2 Time course of incubation with Tf–HRP conjugate on TfR ablation

An analysis of the effect of different times of incubation with Tf–HRP conjugate on the extent of ablation of the TfR was performed. Duplicate plates of cells were incubated with the Tf–HRP conjugate for either 20 min, 1, 2 or 4 h at 37 °C. After this time, DAB cytochemistry was performed on both of the duplicate plates, except that H_2O_2 was omitted from one but included in the other. Since peroxide is required for the ablation reaction, this allowed us to directly compare the effects of ablation on duplicate plates of cells otherwise treated identically. The presence or absence of peroxide is indicated. After completion of the ablation reaction, whole-cell homogenates were prepared and subjected to SDS/PAGE and immunoblotted with antibodies against the TfR. Shown is a representative immunoblot from three separate experiments.

microsomal fractions which were then subjected to SDS/PAGE and immunoblotted with an antibody against the TfR (Figure 3). Incubation of cells with conjugate at 4 °C did not result in significant loss of signal from the low-density microsomes ($97 \pm 7\%$ signal remaining, $n = 3$). In contrast, incubation with the conjugate for 1 h or 3 h at 37 °C resulted in $58 \pm 8\%$ and $85 \pm 7\%$ ablation respectively. There was no significant ablation from the plasma membrane fraction under any experimental condition.

Ablation of GLUT4 and other marker proteins

We examined the ability of the Tf–HRP conjugate to ablate other proteins localized to other intracellular compartments in 3T3-L1 adipocytes. Low-density microsomal and plasma membrane fractions were isolated from cells exposed to Tf–HRP for either 1 h or 3 h at 37 °C, and also cells exposed to Tf–HRP for 3 h at 4 °C. The levels of TGN38 or lgp120, markers for the TGR and lysosomal systems respectively, and also the two glucose transporters expressed in 3T3-L1 adipocytes, GLUT1 and GLUT4, were measured in each of these fractions by immunoblotting (Figure 4). No significant ablation of either TGN38 and lgp120 was observed in 3T3 L1 adipocytes, which is consistent with these proteins residing predominantly in intracellular compartments distinct from the recycling TfR pathway. Ablation of both GLUT4 and GLUT1 was reproducibly observed, implying some overlap of these proteins with the early endosomal/recycling pathway. The ablation of GLUTs 1 and 4

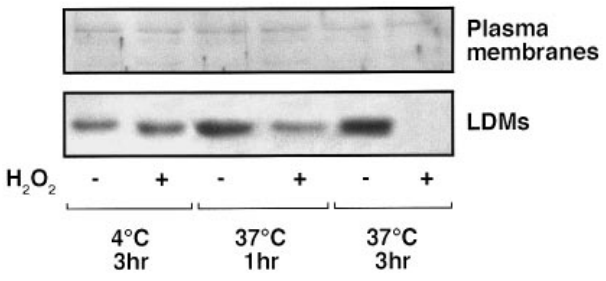

Figure 3 TfR is lost from an intracellular pool after ablation

Duplicate plates (10 cm diam.) of 3T3-L1 adipocytes were incubated with 20 μg/ml Tf–HRP for 1 h or 3 h at 37 °C, or for 3 h at 4 °C. DAB cytochemistry was then performed as described on one of the plates, while in the parallel plate, H_2O_2 was omitted (+ or − on the Figure). Cells were then fractionated into plasma membrane and low-density microsomal (LDM) fractions as described, and samples (50 μg of membrane protein) immunoblotted with antiserum against the mouse TfR.

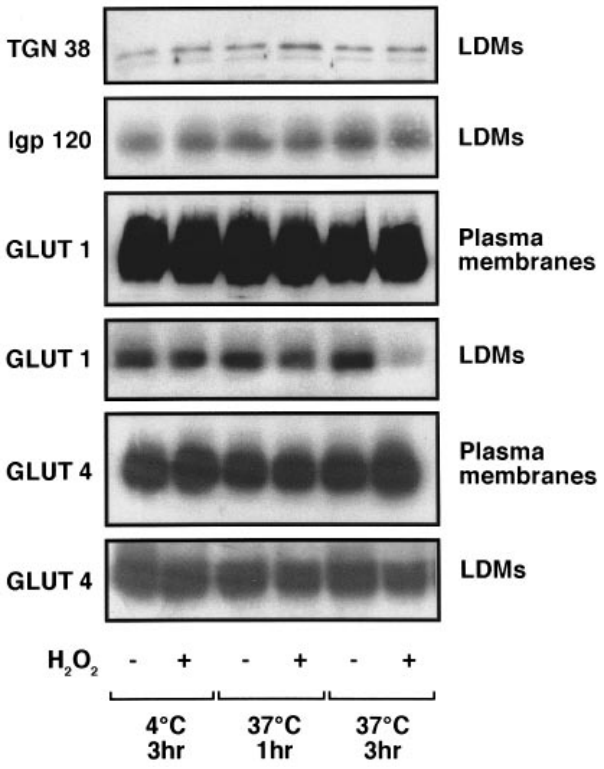

Figure 4 Effect of ablation on TGN38, lgp120, GLUT4 and GLUT1 in 3T3-L1 adipocytes

Identical samples to those shown in Figure 3 were immunoblotted with antibodies against TGN38, lgp120, GLUT4 or GLUT1 as described in the Materials and methods section. In the experiments presented, 25 μg of protein was loaded in each lane except plasma membranes blotted with anti-GLUT4 in which 100 μg of protein was loaded per lane.

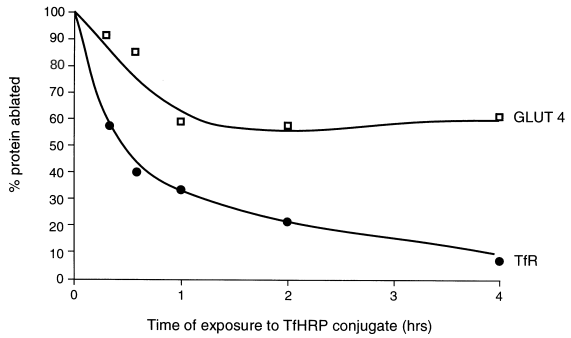

Figure 5 Comparison of the time course of ablation of TfR and GLUT4

Shown is a comparison of the time courses of Tf–HRP conjugate incubation on both TfR and GLUT4 ablation. The amount of each protein lost upon ablation, determined by immunoblot analysis, is expressed as a percentage of that measured in cells not exposed to peroxide at each time point. The mean values of three independent experiments are presented. The range of values was typically $\pm 15\%$ (S.D.).

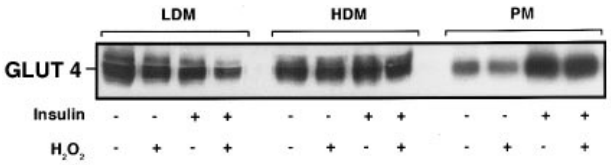

Figure 6 Subcellular fractionation indicates that ablated GLUT4 is lost from the low-density microsomal fraction

3T3-L1 adipocytes were incubated in serum-free medium for 1 h, and then for a further 1 h in the presence of Tf–HRP. Cells were washed in ice-cold buffer and exposed to DAB $\pm$ H_2O_2 for 1 h at 4 °C. Cells were then extensively washed, scraped into HES buffer and homogenized. Subcellular fractionation was performed as described in the Materials and methods section, and aliquots of plasma membrane (PM; 50 μg), low-density microsomes (LDM; 25 μg) and high-density microsomes (HDM; 25 μg) were subjected to SDS/PAGE and transferred to nitrocellulose. The blot was incubated with an anti-GLUT4 polyclonal antibody as described.

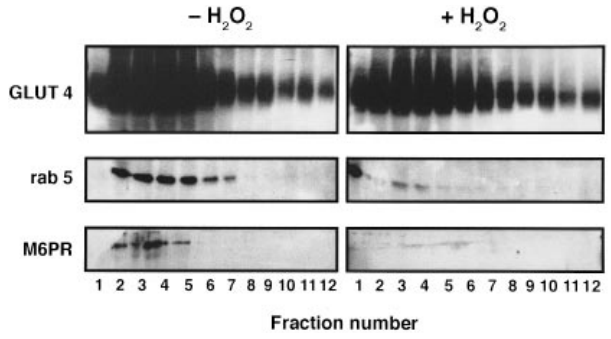

Figure 7 Distribution of rab5, M6PR and GLUT4

3T3-L1 adipocytes were incubated with Tf–HRP for 3 h and incubated with DAB $\pm$ H_2O_2 (as indicated on the Figure). Total internal membranes were prepared from cells as described and the membranes subfractionated on discontinuous sucrose gradients as described in the Materials and methods section. Samples were collected and subjected to SDS/PAGE and immunoblot analysis as described using antibodies to GLUT4, M6PR or rab5 as indicated. In the experiment shown, fraction 1 is the material which pelleted at the bottom of the gradient; samples 2 to 12 were isolated from the gradient such that sample 2 contains the highest density of sucrose and sample 12 the lowest. Quantification of the signals from the immunoblots indicated that some 40% of the GLUT4 signal was lost in the presence of peroxide, and that $\sim$80% of the rab5 signal was recovered in sample 1 in the presence of peroxide.

was restricted to the intracellular low-density microsomal fraction, and no ablation of cell-surface proteins was observed.

In marked contrast to the TfR, complete ablation of the intracellular GLUT4 pool could not be achieved; ablation reached a maximum when cells were exposed to conjugate for 60 min (40 ± 5 %, mean $\pm$ S.D., $n = 6$), and prolonged incubation with Tf–HRP did not increase the extent of ablation of GLUT4 (see for example, Figure 4). A comparison of the time course of ablation of these two proteins is presented in Figure 5.

Subcellular fractionation analysis indicated that the GLUT4 lost upon ablation was predominantly lost from the low-density microsomal fraction (Figure 6). Little if any GLUT4 was lost from high-density microsomal or plasma membrane fractions after ablation. The pattern and extent of GLUT1 ablation was similar to that of the TfR. Incubation of cells with Tf–HRP conjugate for 1 h resulted in partial ablation of GLUT1 ($\sim$35 %), but longer incubation times with Tf–HRP increased the amount of GLUT1 that was ablated (see Figure 4.)

In an attempt to further define the intracellular compartment(s) ablated using Tf–HRP, we have examined the effect of ablation on the cellular distribution of rab5, a marker for the early endosomal system [34], and M6PR, a marker for late endosomes. Cells were incubated with Tf–HRP for 3 h, then incubated with or without peroxide as described. Total internal membrane fractions were prepared and further subfractionated on discontinuous sucrose gradients as described. Fractions were collected from the gradients and analysed for the distribution of GLUT4, rab5 and M6PR by immunoblotting. The results of such an analysis are presented in Figure 7. The distribution of rab5 on sucrose gradients is markedly changed upon ablation. In control cells, rab5 exhibits a similar distribution to GLUT4 as determined by sucrose-density-gradient analysis. However, after ablation, essentially all of the rab5 protein is present in a single fraction corresponding to the small pellet present at the bottom of the tube. Since rab5 is not an integral membrane protein, this result is interpreted to imply that the vesicles to which rab5 is attached have acquired increased density as a result of DAB ablation, and now pass through the sucrose gradient. This observation supports the conclusion that early endosomal populations are ablated using this procedure, and further supports our contention that a large pool of GLUT4 is localized to non-endosomal compartments. In an effort to address the possibility that GLUT4 may be localized to late endosomal populations, we examined the effect of ablation on the distribution of M6PR in the same gradient fractions. The results clearly show that the ablation procedure effectively removed all of the immunodetectable M6PR from the intracellular membrane pool, suggesting that the remaining GLUT4 is not localized to late endosomal structures.

GLUT4-containing vesicles isolated after ablation contain a more defined polypeptide composition

Low-density microsomes were prepared from cells which had been ablated using the protocol described, and GLUT4-containing vesicles were isolated by immunoadsorption from this fraction. The polypeptide composition of these vesicles was compared with vesicles isolated using an identical procedure from control cells which were not exposed to peroxide, and the results are presented in Figure 8. The left-hand panel of Figure 8 shows that there were no gross changes to the polypeptide composition of the low-density microsomal fraction upon ablation. In contrast, comparison of the GLUT4-containing vesicles isolated before and after ablation indicated that the GLUT4 vesicles isolated after ablation contain a much more defined polypeptide composition than those isolated prior to ablation. The major component of the former is GLUT4 (broad band at $\sim$45 kDa), which is substantially enriched compared with vesicles isolated before ablation.

Effect of insulin treatment on GLUT4 and GLUT1 ablation

Insulin treatment of 3T3-L1 adipocytes resulted in a significant decrease in GLUT4 levels in the low-density microsomal fraction (by 51 ± 7 %, $n = 14$; see Figure 6), consistent with its recruitment from this fraction to the plasma membrane. The proportion of GLUT4 ablated from the low-density microsomal fraction isolated from cells exposed to insulin (1 μM, 30 min) was not significantly different from that lost from the low-density microsomal fractions prepared from resting cells (34.6 ± 11.7 % versus 40 ± 5 %; see Figure 6).

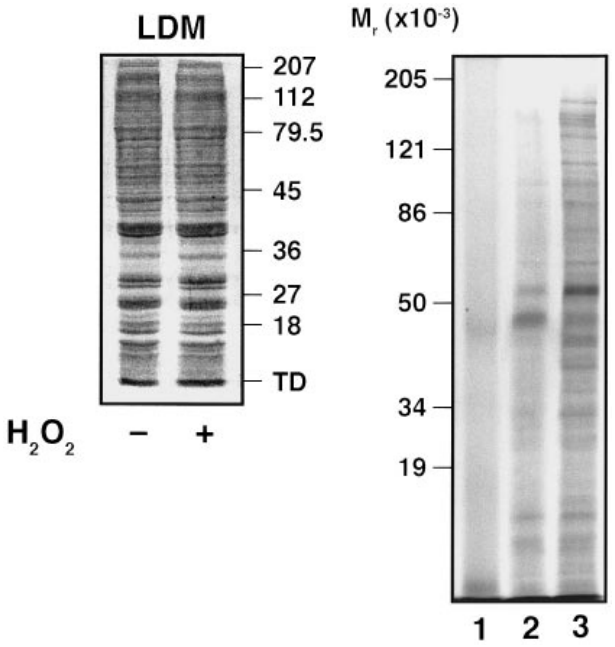

Figure 8 Polypeptide composition of the low-density microsomal (LDM) fraction and GLUT4-containing vesicles isolated before and after ablation

LDMs were prepared from plates of 3T3-L1 adipocytes exposed to Tf–HRP for 3 h, followed by DAB $\pm$ H_2O_2 as indicated. The LDMs were isolated as described and samples corresponding to 50 μg of protein were electrophoresed on 10% gels. Protein was subsequently visualized using Coomassie Brilliant Blue stain (left-hand panel). GLUT4-containing vesicles were isolated from the LDM fraction of cells which had been exposed to Tf–HRP for 3 h, followed by DAB $\pm$ H_2O_2. Samples (each containing the vesicles isolated from 35% of a single 10-cm-diam. plate of cells) were electrophoresed on 10% gels, and visualized using silver-staining (right-hand panel). In the experiment shown, lane 1 contains vesicles isolated using random IgG, lane 2 GLUT4-containing vesicles isolated after ablation, and lane 3 GLUT4-containing vesicles isolated before ablation. The position of molecular-mass standards is indicated.

Table 2 Effects of insulin on GLUT4 and GLUT1 ablation

3T3-L1 adipocytes were incubated in serum-free DMEM for 2 h at 37 °C. After 1 h Tf–HRP (20 μg/ml) was added to the medium, and subsequently insulin (1 μM) or vehicle was added for the final 30 min. After this time, cells were chilled in ice-cold buffer, and incubated with DAB $\pm$ H_2O_2 for 1 h at 4 °C in the dark. Subsequently, cells were extensively washed, total membranes were prepared, and the GLUT4 and GLUT1 content analysed by immunoblotting. Quantification of three (GLUT1) and four (GLUT4) independent experiments of this type was performed and the data presented as means $\pm$ S.D.

	Protein remaining (%)			
	Basal		Insulin	
	No H_2O_2	$+H_2O_2$	No H_2O_2	$+H_2O_2$
GLUT1	100	68 $\pm$ 7	100	34 $\pm$ 8*
GLUT4	100	60 $\pm$ 5	100	63 $\pm$ 12

* Indicates a statistically significant effect compared with the basal condition, $P < 0.05$.

In contrast, exposure of the cells to insulin prior to ablation increased the extent of GLUT1 ablation from this fraction (35% versus 65%; Table 2). In this experiment, cells were exposed to Tf–HRP for 1 h before ablation, conditions which result in partial ablation of GLUT1.

Effects of okadaic acid on GLUT4 ablation

Previous studies have shown that treatment of adipocytes with the phosphatase inhibitor okadaic acid stimulates glucose transport concomitant with a movement of GLUT4 to the cell surface [35,36]. However, exposure of adipocytes to insulin in the presence of okadaic acid partially inhibits both the stimulation of deGlc transport and GLUT4 translocation [36]. It has been suggested that this latter effect is a consequence of increased GLUT4 internalization from the plasma membrane in the presence of insulin and okadaic acid. We have used compartment ablation to address the mechanism of these effects, and the results of a series of experiments are presented in Table 3.

Okadaic acid stimulated 2-deoxy-D-glucose (deGlc) transport ~7-fold, compared with a ~20-fold increase with insulin. Under these conditions, the degree of GLUT4 ablation was decreased from ~40% to ~26%. Consistent with previous studies, okadaic acid inhibited insulin-stimulated glucose transport by 50%. In contrast to the decrease in GLUT4 ablation observed with okadaic acid alone, when added together with insulin there was an increase in the percentage of GLUT4 ablated from ~40% to ~50% (Table 3).

DISCUSSION

The unique property of GLUT4 that distinguishes it from other transporter isoforms is its absolute exclusion from the cell surface in the absence of insulin. Despite considerable efforts to characterize the intracellular GLUT4 compartment in muscle and fat cells it is still unclear whether GLUT4 is targeted to a unique secretory compartment or to a subcompartment within the pre-existing endosomal system [3,4,9,37,38]. In the present study we have utilized the TfR as a prototypic marker of the endosomal/recycling system in an effort to distinguish between each of these possibilities. Using both vesicle immunoadsorption and compartment ablation following internalization of a Tf–HRP conjugate we have been able to resolve two distinct intracellular GLUT4 compartments: one which is TfR-positive and the other which is relatively devoid of TfR.

GLUT4 is localized to recycling endosomes

Quantitative ablation of the intracellular TfR was achieved after a ~2 h incubation with the Tf–HRP conjugate (Figures 2, 3 and 4). Under the same conditions, ~40% of the intracellular GLUT4 was ablated (Figures 4 and 5). This result is consistent with the immunoadsorption studies (Figure 1) and also with previous electron microscopy studies [3,4]. Hence, in non-stimulated adipocytes as much as 40% of the intracellular GLUT4 is in the recycling compartment. This recycling apparently occurs via the clathrin-coated pit pathway as GLUT4 labelling of cell-surface clathrin-coated pits and lattices has been observed in both non-stimulated and insulin-stimulated adipocytes [10]. It is also not surprising that an investigation of the targeting motifs that regulate the intracellular sequestration of GLUT4 has revealed motifs, one in the N-terminus [39] and one in the C-terminus [40], that appear to modulate internalization from the cell surface.

A large pool of GLUT4 is not ablated

At least 60% of the intracellular GLUT4 was not ablated using the Tf–HRP conjugate, consistent with the vesicle immunoadsorption studies. This situation was unique to GLUT4 in that

Table 3 Effects of okadaic acid on deGlc transport and GLUT4 ablation in 3T3-L1 adipocytes

Plates of 3T3-L1 adipocytes were incubated in serum-free medium for 2 h prior to use. For all experiments, insulin and/or okadaic acid when present were added at 1 μM for 30 min prior to the measurement of deGlc transport as described. In experiments to determine GLUT4 ablation, duplicate plates of adipocytes were incubated with Tf–HRP for 1 h at 37 °C prior to challenge with insulin and/or okadaic acid for 30 min. After this time cells were chilled and processed for ablation as described. One of the plates was exposed to peroxide, the other was not. The extent of ablation is therefore expressed as a percentage decrease in low-density microsomal GLUT4 content comparing the plate treated with peroxide with that without for each condition. Values presented are the means of either six independent experiments (deGlc transport) or three independent experiments (ablation) and are $\pm$S.D.

Condition	DeGlc transport (fold increase over basal)	GLUT4 ablation (% decrease compared with non-ablated controls)
Basal	1.0	40 ± 4
Okadaic acid	7 ± 0.4*	26 ± 6**
Insulin	22 ± 3*	35 ± 12
Insulin + okadaic acid	12 ± 2*	52 ± 6*

* Indicates a statistically significant change compared with basal cells ($P < 0.05$); ** indicates $P \sim 0.05$.

GLUT1, a distinct glucose transporter isoform that is expressed in 3T3-L1 adipocytes, was, like the TfR, quantitatively ablated (Figure 4). This is consistent with the substantial degree of co-localization of GLUT1 and TfR observed by others [7].

What does this TfR-negative pool of GLUT4 represent? The TfR is considered to be the best marker of early recycling endosomes and so this would tend to exclude this compartment as being a candidate. This is consistent with previous electron microscopy studies [3,4], and also supported by our observation that cellubrevin is quantitatively ablated using this methodology (S. Martin, C. Livingstone, G. W. Gould and D. E. James, unpublished work). This conclusion is further strengthened by our demonstration that the distribution of rab5 is markedly changed on sucrose gradients. We have shown that in control cells, rab5, a marker for early endosomes [34,41], exhibits a similar distribution to GLUT4 following sucrose-density-gradient analysis of intracellular membranes (Figure 8). However, after ablation, rab5 but not GLUT4 shifts into a more dense region of the gradient. The data presented in Figure 7 again confirm that a large pool of GLUT4 has not been affected by the ablation reaction. It is noteworthy that a transferrin-negative intracellular pool of GLUT4 has also been identified following expression of this transporter in PC12 cells by stable transfection [38], suggesting that this compartment may not be unique to insulin-sensitive cells.

One possibility that cannot be directly excluded from any of these studies is that the non-ablated GLUT4 compartment represents the TGR. Slot et al. [3,4] assigned approximately 13% of intracellular GLUT4 to the TGR in brown adipocytes and a similar amount in cardiac muscle so there is little doubt that GLUT4 does populate this compartment. However, it has recently been shown by vesicle immunoadsorption that TGN38, a putative TGR marker, is not found in GLUT4 vesicles isolated from 3T3-L1 adipocytes [42]. The nature of the TGR is, however, somewhat ill-defined. In fact the use of the Tf–HRP ablation technique has revealed what appears to be a clear heterogeneity in the composition of the TGR. We find that following a 3 h incubation with Tf–HRP we see almost complete loss of the M6PR but no significant loss of TGN38 (see Figures 4 and 7). This is curious because a significant proportion of both of these proteins has been shown to reside in the TGR by electron microscopy. One possibility which is supported by both morphological studies and other data relating to GLUT4 localization is that the TGR is a heterogeneous compartment made up of discrete elements, some of which may be GLUT4-positive and others that may be TGN38-positive. Until this complexity has been resolved we cannot exclude a subdomain of the TGR as being a major site for intracellular GLUT4 storage. Similarly, we cannot exclude the possibility that this compartment is derived from late endosomes. However, this does not seem likely as other late endosomal proteins such as the M6PR do not translocate to the cell surface with insulin to the same extent as GLUT4, and furthermore we have observed complete ablation of the M6PR under conditions which result in only a $\sim 40\%$ ablation of GLUT4 (see Figure 7).

An alternative proposal which is supported by a growing body of evidence is that this non-ablatable GLUT4 pool may be a specialized secretory compartment, analogous to small synaptic vesicles. Evidence in favour of this has been provided from several studies. For example, membrane proteins which are found in regulated secretory compartments in other cell types have been found in the GLUT4-containing compartment in adipocytes [9,16,43,44]. Cain et al. [16] showed that VAMP, a neuronal synaptobrevin homologue, may be found in the GLUT4 compartment in adipocytes. We have recently extended these observations to show that VAMP2, which is also found in synaptic vesicles [44,45], co-localizes with GLUT4 but that the more ubiquitous endosomal synaptobrevin homologue, cellubrevin [44], is found in a distinct intracellular compartment (S. Martin, C. Livingstone, G. W. Gould and D. E. James, unpublished work). In addition, SCAMPs, proteins found in all regulated secretory carrier membranes, have also been co-localized with GLUT4 in intracellular membranes [16,43]. GLUT4 has also been shown to target to the regulated secretory pathway in atrial cardiomyocytes (J. W. Slot, S. Martin and D. E. James, unpublished work) and PC12 cells [46]. These results, together with the studies described herein, lend support to the concept of a GLUT4-containing pool which may represent a discrete compartment that has a specialized function to regulate the polypeptide composition of the plasma membrane transiently.

We have undertaken a preliminary analysis of the polypeptide composition of the GLUT4-containing pool(s) isolated by immunoadsorption before and after ablation (Figure 8). Silver-stained patterns of these vesicles clearly demonstrate that the GLUT4-containing vesicles isolated after ablation contain significantly fewer resident proteins than vesicles isolated before ablation, and are enriched for a protein of molecular mass ~ 45 kDa which immunological analysis has demonstrated to be GLUT4 (results not shown). It will be of interest to further characterize the other resident proteins in these vesicles.

The compartment ablation approach has also been applied to a functional analysis of GLUT4 trafficking in the presence and

absence of okadaic acid, an inhibitor of protein phosphatases [47]. It is well established that okadaic acid treatment of adipocytes inhibits insulin-stimulated glucose transport and GLUT4 translocation [36]. Here we show that in the presence of okadaic acid, the extent of ablation of GLUT4 in insulin-treated 3T3-L1 adipocytes is markedly increased (Table 1). This is consistent with the inhibitory action of okadaic acid being explained by an increased rate of GLUT4 internalization from the plasma membrane under these conditions. In contrast, okadaic acid treatment in the absence of insulin results in a decrease in GLUT4 ablation. This is consistent with okadaic acid inhibiting GLUT4 recycling in the basal state resulting in increased GLUT4 levels at the cell surface and an increase in deGlc transport (Table 3). The compartment ablation approach will be a useful tool in the analysis of the effects of other reagents proposed to interfere with GLUT4 trafficking.

In conclusion, we suggest that a proportion of the intracellular GLUT4 is localized to the early endosomal/recycling system in resting adipocytes, but a larger proportion exists in a separate, TfR-negative compartment which is devoid of other marker proteins such as rab5, or the M6PR. The use of the TfR ablation technique should enable us to address a number of questions relevant to this compartment in adipocytes. What is the function of this compartment and how does GLUT4 find its way from the endosomal system into this organelle? What other proteins are in this location and what are the effects of insulin on this compartment versus the endosomal system? These types of questions are currently under investigation.

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