INTRODUCTION

Insulin stimulates glucose uptake into muscle and adipose tissue through the translocation of a glucose transporter, GLUT4, from an intracellular compartment to the cell surface (reviewed by James et al., 1994). One of the defining features of GLUT4 is its very slow exocytic rate (kex) in basal adipocytes, which varies between 0.01 minutes⁻¹ and 0.024 minutes⁻¹ (Jhun et al., 1992; Satoh et al., 1993; Yang and Holman, 1993) and is considerably lower than that of GLUT1 (kex = 0.035 minutes⁻¹) (Yang and Holman, 1993) and the transferrin receptor (TfR) (kex = 0.111 minutes⁻¹) (Tanner and Lienhard, 1987). A major effect of insulin is to increase the rate of GLUT4 exocytosis such that it is now similar to that of GLUT1 and TfR (Jhun et al., 1992; Satoh et al., 1993; Tanner and Lienhard, 1987; Yang and Holman, 1993). These observations suggest that in the absence of insulin GLUT4 is actively sequestered at an intracellular location, and insulin overrides this sequestration allowing GLUT4 to recycle with the bulk of endocytic traffic.

Several models have been proposed to account for the unique trafficking of GLUT4. For example, GLUT4 may constantly shuttle between two intracellular organelles in basal cells in such a way that it is excluded from the cell surface recycling route (Fig. 1A). An adaptation of this model is the retention model (Fig. 1B) whereby GLUT4 may be actively retained within sub-domains of endosomes and/or TGN. Both of these mechanisms would result in a low rate of incorporation of GLUT4 into vesicles recycling to the cell surface. Insulin may either block endosomal retrieval or override the retention of GLUT4 and increase its rate of translocation to the cell surface.

SUMMARY

The facilitative glucose transporter, GLUT4 undergoes insulin-dependent movement to the cell surface in adipocytes. The magnitude of the insulin effect is much greater for GLUT4 than other recycling proteins such as the CD-MPR. In the present study we have studied the colocalisation of these proteins in adipocytes in an effort to explain this selective insulin-dependent recruitment of GLUT4. Using immunofluorescence microscopy or immuno-EM on 3T3-L1 adipocytes we find that there is considerable colocalisation between these proteins particularly within the area of the TGN. However, the distribution of CD-MPR was not significantly affected by insulin. The insulin-dependent recruitment of GLUT4 was concomitant with a selective decrease in GLUT4 labelling of cytoplasmic vesicles whereas the amount of GLUT4 in the TGN region (approx. 50% of total GLUT4) was relatively unaffected. To explore the possibility that the cytoplasmic GLUT4(+) vesicles represent an intracellular insulin-responsive storage compartment we performed quantitative immuno-EM on whole mounts of intracellular vesicles isolated from basal and insulin-stimulated adipocytes. These studies revealed that: (1) GLUT4 and CD-MPR were concentrated in small (30-200 nm) vesicles at a labelling density of 1-20+ gold particles/vesicle; (2) there was significant overlap between both proteins in that 70% of the total GLUT4 pool colocalised with CD-MPR; (3) a significant amount of GLUT4 (approx. 50% of total) was found in a subpopulation of vesicles that contained as little as 5% of the total CD-MPR pool; (4) the GLUT4(+)/CD-MPR(−) vesicles were highly insulin-responsive, and (5) the total number of GLUT4(+) vesicles, but not CD-MPR(+) vesicles, decreased by approx. 30% in response to insulin treatment. These data are consistent with a model in which GLUT4 is selectively sorted into a vesicular compartment in adipocytes that is recruited to the plasma membrane by insulin stimulation.

Key words: GLUT4, Mannose 6-phosphate receptor, Insulin, Protein trafficking
step effectively allowing GLUT4 to enter the cell surface recycling pathway. The potential of these types of mechanisms to exclude recycling proteins from the cell surface has been demonstrated for other molecules. Both furin (Molloy et al., 1994; Wan et al., 1998) and TGN38 (Ghosh et al., 1998) recycle via the cell surface, but at steady state they have a predominantly intracellular localisation conferred by their unique recycling kinetics. In support of this model, it has been shown that GLUT4 constitutively recycles via the cell surface in the absence of insulin stimulation although its basal recycling rate is very slow (Jhun et al., 1992). Insulin also regulates the recycling of other endosomal proteins such as the Tfr (Tanner and Lienhard, 1987) and MPR (Kandror and Pilch, 1996; Tanner and Lienhard, 1989), resulting in their partial redistribution to the cell surface, and suggesting a more general effect of insulin on the endosomal system.

The alternate model (Fig. 1C) suggests that GLUT4 is packaged into regulated storage vesicles which may be stored in the cytoplasm until insulin signals their exocytosis. This mechanism may be analogous to other regulated exocytic pathways, such as secretory granules and small synaptic vesicles. Exocytic secretory granules are formed from the trans-Golgi network (TGN) (reviewed by Tooze, 1998), whereas small synaptic vesicles are formed from the endosomal system (de Wit et al., 1999) or directly from the plasma membrane (Schmidt et al., 1997). Stimulation results in the direct fusion of these storage compartments with the cell surface. Fusion is mediated, at least in part, by an interaction between v-SNAREs, such as VAMP2, and t-SNAREs such as syntaxin (Pfeffer, 1999). In support of this model it has been shown that GLUT4 is localised to a compartment that is distinct from recycling endosomal markers (Livingstone et al., 1996; Malide et al., 1997; Martin et al., 1996), and that is capable of mediating the translocation of GLUT4 to the cell surface even following chemical ablation of the endosomal system (Martin et al., 1998). Furthermore, VAMP2 has recently been proposed to have a specific role in GLUT4 trafficking (Martin et al., 1998). There is evidence that the formation of this insulin-responsive compartment occurs early during adipocyte differentiation indicating that it might be a specialised feature of insulin-responsive cell types (El-Jack et al., 1999).

It is essential to distinguish between these different models of GLUT4 trafficking because the mechanism by which insulin triggers GLUT4 exocytosis will be fundamentally different in each case. This has been difficult to achieve for two reasons. Firstly, there is no obvious morphological feature that has permitted a clear distinction between insulin-responsive GLUT4 compartments and other GLUT4-containing elements of the endosomal/TGN system. Secondly, because intracellular protein sorting occurs on an iterative basis there is always considerable overlap between different proteins among a variety of organelles (Dunn et al., 1989; Geuze et al., 1987, 1988). In order to overcome these problems it is necessary to use quantitative approaches that can discern an accumulation of specific proteins in organelles, compared to other markers.

In this study we have chosen to compare the localisation and regulation of GLUT4 with the 46 kDa cation-dependent mannose 6-phosphate receptor (CD-MPR). The CD-MPR predominantly recycles between endosomes and the TGN, and only a small proportion recycles back to the TGN via the cell surface (Duncan and Kornfeld, 1988; Klumperman et al., 1993; Luzio et al., 1990; Press et al., 1998; Schulze-Garg et al., 1993). Thus, one can imagine that GLUT4 may follow a similar route in the basal state in order to avoid the cell surface recycling pathway. In support of this we have previously noted that there is considerable overlap in the intracellular distribution of these proteins in adipocytes (Martin et al., 1996; Hashimamoto and James, 2000). In the present study we show that unlike GLUT4, the intracellular distribution of CD-MPR is relatively unaffected by insulin. The major effect of insulin is on GLUT4-containing tubulo-vesicular elements distributed throughout the cytoplasm in 3T3-L1 adipocytes. Using whole mounts of isolated intracellular vesicles we have found that insulin preferentially effects a sub-population of vesicles which are enriched in GLUT4, but relatively depleted in endosomal proteins such as CD-MPR. These results indicate that insulin effects a specific compartment enriched in the GLUT4 protein.

MATERIALS AND METHODS

Materials

HRP, human apo-transferrin and all reagents for Tf/HRP synthesis were from Sigma (Poole, UK). Dulbecco’s modified Eagle medium (DMEM), Myocline-Plus foetal calf serum and antibiotics were from Gibco BRL (Paisley, UK). 125I-labelled goat anti-rabbit antibody was from Du Pont/NEN (UK). Normal sera were from Dako (Carpinteria, CA). All other reagents were as described (Livingstone et al., 1996). Protein A-gold was obtained from the Dept Cell Biology, University of Utrecht.

Cell culture

3T3-L1 fibroblasts were grown in 10% new born calf serum in DMEM at 37°C in 10% CO2 and passaged at about 70% confluence. Cells for use in experiments were grown in the same medium until two days post-confluence then differentiated into adipocytes as described previously (Frost and Lane, 1985). Cells were used between 8-12 days post-differentiation and between passages 4 and 12. Before use, cell monolayers were incubated in serum-free DMEM for 2 hours, or overnight (for immunofluorescence microscopy studies).

Preparation and use of HRP-conjugated transferrin

Tf/HRP was prepared, purified, iron-loaded and used as described previously (Livingstone et al., 1996). Briefly, after a 2 hour incubation in serum-free DMEM, adipocytes were incubated with 20 μg/ml Tf/HRP for 1 hour, chilled by washing in ice-cold isotonic citrate buffer (150 mM NaCl, 20 mM sodium citrate, pH 5.0) to remove cell surface-attached Tf/HRP and kept on ice to prevent vesicle trafficking during the DAB cytochemistry reactions. DAB was added at 100 μg/ml to all wells and H2O2 added to 0.02% (v/v) to one of each pair of wells. After a 1 hour incubation at 4°C in the dark the reaction was stopped using 5 mg/ml BSA in PBS, and samples prepared for immunoblotting.

Subcellular fractionation of adipocytes

Adipocytes were subjected to a differential centrifugation procedure as described previously (Piper et al., 1991) using HES buffer (20 mM HEPES, 1 mM EDTA, 255 mM sucrose, pH 7.4) containing protease inhibitors (1 μg/ml pepstatin A, 0.2 mM diisopropylfluorophosphate (DFP), 20 μM L-transpeptidase (leucylamido-4-guanidinobutane (E64) and 50 μM aprotinin) to yield fractions enriched in plasma membranes or low density microsomes. All fractions were resuspended in equal volumes of HES buffer and stored at –80°C prior to use.
Electrophoresis and immunoblotting

Proteins were electrophoresed on 7.5, 10 or 15% SDS-polyacrylamide gels and transferred onto nitrocellulose sheets as described previously (Martin et al., 1996). Immunolabelled proteins were visualised using either 125I-labelled goat anti-rabbit secondary antibody followed by autoradiography, or using HRP-conjugated secondary antibody and the ECL system (Amersham, Aylesbury, UK). Bands were quantitated either by using a p-counter or Lumi-Imager (Boehringer-Mannheim, Castle Hill, NSW, Australia). To quantify the degree of protein ablation the difference in immunoreactive signal between membranes obtained from cells incubated ± H2O2 was determined by densitometry using a Bio-Rad GS700 system (Livingstone et al., 1996; Martin et al., 1996).

Electron microscopy: isolated vesicles

Parafomaldehyde-fixed intracellular membrane vesicles were prepared from a post-nuclear/plasma membrane supernatant and allowed to adsorb to formvar-coated copper grids, prior to labelling for immuno-electron microscopy (immuno-EM) as described previously (Martin et al., 1996).

Electron microscopy: cryosections

3T3-L1 adipocytes were fixed using 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 1 hour, or 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 hours at room temperature. Fixed cells were embedded in 10% gelatin, and infused with 2.3 M sucrose overnight at 4°C, prior to snap freezing onto specimen holders in liquid N2. Ultracryomicrotomy was performed by a slight modification of the Tokuyasu technique (Tokuyasu, 1980) using a Leica Ultracut S/FCS cryomicrotome (Leica Instruments Pty Ltd, North Ryde, Australia) at −120°C and a diamond knife designed for ultracryotomy (Drukker, Cuijk, The Netherlands). Sections were picked up with a 1:1 mixture of 2.3 M sucrose: 2% methyl cellulose and transferred onto Formvar and carbon-coated copper grids (Liou et al., 1996). Immuno-EM was performed essentially as described previously (Martin et al., 1997) except that the sections were blocked using 1% cold water fish skin gelatin/0.2% acetylated BSA (Aurion, Wageningen, The Netherlands) in PBS, and the primary antibody was diluted in 0.5% cold-water fish skin gelatin/0.1% acetylated BSA in PBS. In order to increase the labelling efficiency, swine anti-rabbit IgG (Nordic, Tilburg, The Netherlands) was used at a dilution of 1:2000 in PBS/1% BSA as a bridge between the primary antibody and Protein A-gold, in the single-labelling experiments. Grids were viewed using a Jeol 1010 transmission electron microscope.

Quantitation of the distribution of GLUT4 and CD-MPR in cryosections was undertaken using prints of randomly selected cells. On the same prints random points were projected onto the cells, at a density of 400 points per 100 μm² and assigned to cell structures as if they were gold. The density of random points (400/100 μm²) is approximately equivalent to the density of gold particles/100 μm². The random point distribution, which is included in the results section, can be used to estimate the non-specific labelling of gold on cell structures. Gold particles located no more than 20 nm from a membrane were designated specific labelling and assigned to one of the following structures: the TGN region, plasma membrane, tubulo-vascular (TV)-elements within 100 nm of the plasma membrane, early endosomal vacuoles (EE), TV-elements within 100 nm of EE, late endosomal vacuoles (LE), TV-elements within 100 nm of LE, or cytoplasmic TV-elements. Early endosomes were defined as empty vacuoles enclosed by a limiting membrane. Late endosomes were defined as a limiting membrane surrounding a lumen that was partially or wholly filled with membranous or amorphous material.

Indirect immunofluorescence microscopy

Cells were incubated in the absence or presence of insulin (1 μM) in DMEM for 15 minutes and then fixed in acetone for 15 minutes, washed with PBS, washed again with PBS/0.15 M glycine and incubated in 5% normal swine serum/PBS for 30 minutes. Primary antibodies were diluted in PBS/1% normal swine serum and incubated for 1 hour. Cells were then washed with PBS/0.1% BSA and incubated for 30 minutes with a corresponding FITC- or Texas Red-conjugated secondary antibody (Molecular Probes, Eugene, OR) diluted in 2.5% normal rat serum/PBS. Normal rabbit serum was used as a negative control. The coverslips were washed with PBS, mounted onto glass microscope slides and viewed using a ×63/1.4 Zeiss oil immersion objective on a Zeiss Axiovert fluorescence microscope, equipped with a Bio-Rad MRC-600 laser confocal imaging system.

Antibodies

Polyclonal antibodies raised against a synthetic peptide corresponding to the C-terminal 12 amino acids of GLUT4, and the anti-GLUT4 monoclonal antibody (1F8), have been described previously (James et al., 1988, 1989). An anti-transferrin receptor monoclonal antibody was purchased from UBI (Lake Placid, New York). Anti-CD-MPR was a generous gift of Dr A. Hille-Rehfeld (Universität Göttingen, Germany) (Schulze-Garg et al., 1993) and anti-VAMP2 was a generous gift from Dr M. Takahashi (Mitsubishi Kasei Institute of Medical Research, Tokyo).

RESULTS

Subcellular distribution and insulin-regulation of GLUT4 and CD-MPR in 3T3-L1 adipocytes

The subcellular distribution and insulin regulation of GLUT4 and CD-MPR was compared in 3T3-L1 adipocytes using a number of techniques. Using immunofluorescence microscopy we found that both GLUT4 and CD-MPR were concentrated in the perinuclear region of basal 3T3-L1 adipocytes, with some labelling of punctate structures in the cell periphery (Fig. 2A). Insulin resulted in redistribution of GLUT4 to the cell surface, but had no detectable effect on the cell surface level of CD-MPR. However, there was a slight dispersal of GLUT4 and CD-MPR labelling in insulin-treated cells, although this may have been due to an overall effect of insulin on cell shape. VAMP2 was also found to have a perinuclear distribution similar to GLUT4 and CD-MPR, although the labelling was less dispersed. Insulin had no detectable effect on the distribution of VAMP2 using this technique. This is not surprising as we have previously shown by subcellular fractionation that insulin causes only a 2- to 3-fold translocation of VAMP2 to the plasma membrane fraction (Martin et al., 1996).

The absence of a significant effect of insulin on the distribution of CD-MPR was confirmed by subcellular fractionation of basal and insulin-stimulated cells. Both GLUT4 and CD-MPR were concentrated in the low density microsomal (LDM) fraction of 3T3-L1 adipocytes in the absence of insulin (Fig. 2B). Insulin treatment resulted in a marked (approx. 3.5-fold) increase in the amount of GLUT4 in the plasma membrane fraction, commensurate with a decrease (approx. 30%) in the LDM fraction. In contrast, insulin had a relatively modest effect on the distribution of CD-MPR, causing only a 50% increase in the plasma membrane fraction, which is presumably too low to be detected by immunofluorescence microscopy, and no detectable change in the LDM.

To determine the extent to which GLUT4 and CD-MPR were localised to the recycling endosomal system, we employed a procedure in which Tf/HRP is used to ‘ablate’ the transferrin receptor (TfR) recycling compartments of 3T3-L1 adipocytes
Consistent with previous studies, we observed substantial ablation of the TfR following a 1 hour incubation with Tf/HRP at 37°C (79±8%), but only modest ablation of GLUT4 (38±9%). Under the same conditions only approx. 35% of CD-MPR was ablated (Fig. 2C), indicating that substantial proportions of both GLUT4 and CD-MPR are excluded from the early and recycling endosomes. At 4°C no ablation of any protein was observed, consistent with inhibition of the internalisation of the Tf/HRP conjugate (results not shown). These results suggest that CD-MPR has a similar intracellular distribution to GLUT4 in basal 3T3-L1 adipocytes, but these two proteins are markedly different in their response to insulin.

Distribution of GLUT4 and CD-MPR in cryosections of basal and insulin-stimulated 3T3-L1 adipocytes

We have previously demonstrated co-localisation between the CD-MPR and GLUT4 in rat adipocytes, using both immuno-EM on isolated intracellular vesicles (Martin et al., 1996) and vesicle immunoabsorption (Hashiramoto and James, 2000). However, in these studies we did not perform an extensive quantitative analysis of this co-localisation, nor did we examine the overlap between these proteins isolated from insulin-treated cells. Furthermore, the nature of those experiments did not allow us to draw any conclusions concerning the intracellular location of the membranes involved.

In the present study we initially compared the localisation of GLUT4 and CD-MPR in cryosections of basal and insulin-stimulated 3T3-L1 adipocytes. In cryosections of basal 3T3-L1 adipocytes GLUT4 was distributed between TV-elements close to the Golgi region of the cells (54.0%) and TV-elements distributed throughout the cytoplasm (19.8%) (Fig. 3; Table 1). These two pools of GLUT4 were nominally designated as the
GLUT4 targeting in adipocytes

TGN region and cytoplasmic TV-elements respectively, in order to distinguish between them. There was little GLUT4 detected on, or close to, the cell surface or in/adjacent to early or late endosomal vacuoles under basal conditions (Table 1). The amount of GLUT4 labelling observed in the TGN region of 3T3-L1 adipocytes appeared to be higher than previously reported in other cell types, including brown adipocytes (Slot et al., 1991b) and cardiac muscle (Slot et al., 1997, 1991a). However, this is probably because many elements of the TGN/endosomal system are perinuclear in 3T3-L1 adipocytes.
where this is not the case in tissues such as cardiac muscle. Hence it is likely that this membrane does not correspond to TGN alone. For example, we have observed an accumulation of endocytic tracers, such as BSA-gold, in this region of the cell (data not shown). Similarly, in brown adipocytes a significant proportion (approx. 70%) of GLUT4 labelling in the area of the TGN can be positively identified as endosomal through the use of other markers (Slot et al., 1991b).

Following stimulation of the cells with insulin there was a >7-fold increase in GLUT4 labelling at the plasma membrane relative to basal cells (2.2±0.2 gold/μm plasma membrane vs. 0.3±0.0 gold/μm plasma membrane), concomitant with decreased labelling in the cytoplasmic TV-elements by approx. 50% and a slight decrease in labelling within the TGN region by approx. 20% (Table 1). These results suggest that one of the major insulin-responsive pools of GLUT4 in 3T3-L1 adipocytes is present in TV-elements distributed throughout the cytoplasm. It is likely that these elements are also found in the TGN but that here they exist together with other GLUT4(+) elements and this would explain the relatively small effect of insulin on GLUT4 labelling in the TGN region.

The CD-MPR had an overall distribution almost identical to that of GLUT4 in basal 3T3-L1 adipocytes (Fig. 3; Table 1). Labelling was predominantly distributed between TV-elements in the TGN region (53.7%) and in cytoplasmic TV-elements distributed throughout the cell (22.5%). Again there was low labelling of early and late endosomal vacuoles or TV-elements immediately adjacent to them. There was a slightly higher proportion of CD-MPR labelling on the surface of basal cells than was observed for GLUT4 (15.6% vs 10.5%, respectively).

![Fig. 4. Double-labelling for GLUT4 and CD-MPR in 3T3-L1 adipocyte cryosections.](image)

Cryosections of basal 3T3-L1 adipocytes were double-labelled using antibodies specific for GLUT4 (10 nm Protein A-gold) and CD-MPR (15 nm Protein A-gold). There was significant co-localisation between these two proteins, frequently observed in coated (arrows) vesicles in the region of the Golgi. There was only low labelling of the Golgi cisternae, and no labelling of the late endosomal compartments.

Table 1. Distribution of GLUT4 and CD-MPR in basal and insulin-stimulated 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>CD-MPR</th>
<th>Basal</th>
<th>Insulin</th>
<th>GLUT4</th>
<th>Basal</th>
<th>Insulin</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>5.1±1.0</td>
<td>5.8±1.5</td>
<td>3.8±0.6</td>
<td>18.9±1.9*</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Sub-PM TV-elements</td>
<td>6.0±2.6</td>
<td>9.1±2.2</td>
<td>6.3±0.7</td>
<td>18.3±2.3*</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>TGN region</td>
<td>53.7±4.6</td>
<td>50.0±3.0</td>
<td>54.0±6.0</td>
<td>43.8±5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early endosomes</td>
<td>1.9±0.7</td>
<td>1.3±0.4</td>
<td>4.5±1.1</td>
<td>2.7±0.5</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Adjacent TV-elements</td>
<td>1.2±0.3</td>
<td>0.9±0.3</td>
<td>5.0±1.6</td>
<td>3.9±1.3</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Late endosomes</td>
<td>2.0±0.5</td>
<td>2.8±1.1</td>
<td>2.1±0.6</td>
<td>0.4±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjacent TV-elements</td>
<td>3.2±1.1</td>
<td>4.4±0.9</td>
<td>4.1±1.3</td>
<td>2.9±0.8</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Cytoplasmic vesicles</td>
<td>22.5±4.1</td>
<td>27.3±4.1</td>
<td>19.8±4.3</td>
<td>9.3±1.8*</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Cryosections of 3T3-L1 adipocytes were single-labelled for GLUT4 or CD-MPR and the distribution of labelling quantified as described in Materials and Methods. The non-specific association of gold with cell structures can be estimated from the percentage of random points which, when projected onto the cells were assigned to cell structures as if they were gold (shown in the final column) and can be used to correct for potential background labelling. Results shown = mean distribution ± s.e.m., where n=7 cells except basal where n=8. For each condition between 5000-8000 gold particles were counted. *P<0.05, relative to basal.
However, unlike GLUT4, the amount of CD-MPR at the cell surface was unchanged following stimulation with insulin. Furthermore, there was no detectable effect of insulin on the distribution of CD-MPR between the TGN region and the cytoplasmic TV-elements (Table 1). These data suggest that GLUT4 and CD-MPR may be present in distinct populations of vesicles in the cytoplasm, of which only the former are affected by insulin stimulation. However, it does not exclude the possibility that GLUT4 and CD-MPR are co-localised in these structures, but that GLUT4 is selectively removed following insulin stimulation.

Co-localisation of GLUT4 and CD-MPR in 3T3-L1 adipocyte cryosections

In an attempt to resolve separate populations of GLUT4 and CD-MPR vesicles in 3T3-L1 adipocytes, we performed double-labelling immunoelectron microscopy (immuno-EM) on 3T3-L1 adipocyte cryosections. As the bridging antibody used for the single-labelling could not be used in the double-labelling experiments, it was necessary to omit glutaraldehyde during the fixation in order to optimise labelling efficiency. Unfortunately, this compromised the preservation of the ultrastructure, particularly outside the perinuclear region, thus limiting a detailed analysis of these two proteins at specific intracellular locations. Nevertheless, quantitation of the overall overlap between CD-MPR and GLUT4 revealed that approx. 47% of the CD-MPR(+) structures in 3T3-L1 adipocytes co-labelled for GLUT4. This co-localisation was predominantly observed in the perinuclear region (Fig. 4), although the disruption of peripheral structures precluded definitive localisation.

As a positive control for the co-localisation procedure we compared the distribution of GLUT4 and the insulin-responsive aminopeptidase (IRAP), also known as vpi165 (Kandror and Pilch, 1994; Keller et al., 1995; Mastick et al., 1994). IRAP is thought to translocate to the cell surface together with GLUT4 in response to insulin (Martin et al., 1997; Ross et al., 1996) and has a similar labelling efficiency as the CD-MPR antibody in 3T3-L1 adipocytes (results not shown). Consistent with previous studies we observed a high degree of overlap between GLUT4 and IRAP in adipocytes, in that approx. 62% of the IRAP(+) structures contained GLUT4. The higher overlap between GLUT4 and IRAP compared to that observed for CD-MPR is consistent with the generation of an intracellular compartment that may be enriched in GLUT4 and IRAP, but not CD-MPR.

Analysis of the distribution of GLUT4 and CD-MPR labelling in whole mount intracellular vesicle preparations

To account for the difference in insulin-regulation between GLUT4 and CD-MPR, we considered the possibility that GLUT4 may be selectively targeted to an insulin-responsive compartment, that may coincide with the cytoplasmic TV-elements identified in 3T3-L1 adipocyte cryosections. We used whole mount immunolabelling on vesicles isolated from 3T3-L1 adipocytes to more accurately quantify the co-localisation between GLUT4 and CD-MPR. The advantage of this method is that the labelling efficiency is much higher than that obtained in cryosections and so co-localisation between two different proteins can be analysed with high resolution. Furthermore, as indicated below, in addition to examining the degree of co-localisation between two markers, it is also feasible to compare labelling density in individual vesicles.

Intracellular vesicles from basal cells were initially single-labelled with antibodies specific for either GLUT4 or CD-MPR and the labelling profiles analysed (Table 1). The density of labelling was similar for both proteins, with a range of 1-20 gold particles/vesicle. The diameter of labelled vesicles varied from 30-200 nm. Occasionally labelled structures >200 nm in diameter were observed but these were excluded from the quantitation. Although there was no direct correlation between the vesicle size and the number of gold particles/vesicle, highly labelled vesicles (those with >10 gold particles for either GLUT4 or CD-MPR) were frequently larger (100 > 200 nm) and may represent endosomal vacuoles.

To quantify the degree of co-localisation between GLUT4 and CD-MPR, whole mounts of vesicles were double-labelled (Fig. 5). Using this protocol we observed significant overlap...
between GLUT4 and CD-MPR in intracellular 3T3-L1 adipocyte vesicles (Table 3), consistent with data obtained using cryosections. This high degree of co-localisation was surprising in view of the different effects of insulin on these two proteins (Fig. 2).

It was clear from the vesicle whole mount approach that there was considerable variation in the labelling density for different antigens among different vesicles. Thus, one possibility is that while two proteins such as GLUT4 and CD-MPR may co-localise this may not provide a true representation of the quantitative overlap. This may be important because different vesicle populations could represent discrete functional entities. To test this possibility we analysed the whole mount labelling data by quantifying the co-localisation between GLUT4 and CD-MPR within individual vesicle pools that were identified on the basis of labelling density. As shown in Fig. 6 the distribution of total label for both GLUT4 and CD-MPR among these different vesicle pools exhibited a Gaussian distribution with a peak at around 2-4 gold particles/vesicle for each antigen. However, when we examined the distribution of GLUT4 labelling in the CD-MPR(+) structures it became evident that the distribution patterns were non-overlapping. In fact, approx. 50% of the total GLUT4 labelling was associated with <5% of the total CD-MPR labelling. Conversely a large proportion of the total CD-MPR labelling (approx. 60%) was associated with GLUT4 vesicles that contained only 1-2 GLUT4 gold particles per vesicle, and which accounted for only approx. 25% of the total GLUT4 labelling. This is unlikely to be due to a random distribution of these two proteins among individual vesicles as we have previously observed a positive correlation between GLUT4 and IRAP labelling of vesicles, in which the labelling density of GLUT4 is mirrored by that of IRAP (Martin et al., 1997).

Effect of insulin on the distribution of GLUT4 and CD-MPR in intracellular vesicles

The significant correlation between IRAP and GLUT4 labelling in vesicles (Martin et al., 1997), compared to the poor correlation between GLUT4 and CD-MPR labelling, may be indicative of a sorting step that results in the formation of an insulin-responsive compartment, enriched in GLUT4 and IRAP. We hypothesised that if this were the case we should observe a net decrease in vesicles corresponding to this compartment after insulin treatment, whereas vesicles carrying other cargo, such as CD-MPR, should be unaffected. Initially we examined the effect of insulin on the total number of GLUT4(+) versus CD-MPR(+) vesicles in an intracellular
membrane fraction using single-labelling (Table 2). The labelling characteristics of vesicles isolated from insulin-stimulated cells were similar to those isolated from basal cells. The efficiency of labelling ranged from 1-20 gold particles/vesicle for both antibodies and the diameter of labelled vesicles varied from 30-200 nm. Most notably, there was a 30% decrease in the number of GLUT4(+) vesicles relative to the total number of vesicles after insulin treatment, whereas there was no change in the total number of CD-MPR vesicles (Table 2).

The most likely interpretation of the differential effects of insulin on GLUT4-containing vesicles relative to CD-MPR(+) vesicles is that insulin selectively stimulates the removal of vesicles that are enriched in GLUT4 but relatively depleted in CD-MPR, a result which is consistent with the vesicle sorting model (Fig. 7). One prediction, if this is the case, is that there should, in fact, be an increase in the degree of overlap between GLUT4 and CD-MPR in intracellular vesicles after insulin treatment. Consistent with this hypothesis, there was a detectable increase in co-localisation, with respect to both the percentage of GLUT4 vesicles co-labelled for CD-MPR (Table 3), and in the percentage of GLUT4 gold particles that overlapped with CD-MPR gold particles (results not shown), in vesicles prepared from insulin-stimulated cells.

As the total number of GLUT4 vesicles in the intracellular membrane preparations decreased with insulin (Table 2), but the total number of vesicles analysed from basal and insulin-stimulated cells was the same, a second prediction is that the proportion of GLUT4(+)CD-MPR(−) vesicles in the total labelled vesicle pool would decrease relative to the GLUT4(+)CD-MPR(+) vesicle population. To investigate this possibility we divided the GLUT4 vesicles, isolated from basal and insulin-stimulated cells, into GLUT4(+)CD-MPR(−) and GLUT4(+)CD-MPR(+) sub-populations and examined the distribution of GLUT4 labelling among these different populations. In support of this hypothesis, the percentage of GLUT4 gold particles labelling vesicles before insulin treatment, was compared between basal and insulin-stimulated insulin-responsive cells relative to the number of vesicles in unstimulated cells. The results show the percentage of GLUT4 gold particles labelling vesicles from insulin-stimulated cells relative to the vesicles from basal cells. Results shown are the percentage of vesicles relative to basal cells.

DISCUSSION

The intracellular sorting and trafficking route of GLUT4 in insulin-responsive cells remains poorly understood. While GLUT4 is localised to endosomes and the TGN (Ralston and Ploug, 1996; Slot et al., 1997, 1991a,b; Wang et al., 1996), a number of studies have questioned the contribution of these compartments to the insulin-stimulated translocation of GLUT4 to the cell surface (Bao et al., 1995; Chakrabarti et al., 1994; El-Jack et al., 1999; Kao et al., 1998; Kono-Sugita et al., 1996; Livingstone et al., 1996; Malide et al., 1997; Martin et al., 1996; Yang and Holman, 1993; Yeh et al., 1995). Despite numerous attempts, however, the identification of a separate insulin-responsive GLUT4 compartment has been difficult to achieve possibly due to the difficulty in separating such membranes from endosomes or TGN. To begin to dissect these compartments, we have compared the localisation and regulation of GLUT4 with that of the cation-dependent mannose 6-phosphate receptor, CD-MPR. The CD-MPR is an ideal marker for this purpose because it displays a similar intracellular distribution to GLUT4 in basal 3T3-L1 adipocytes and is highly expressed in these cells, unlike GLUT4, its distribution is largely unaffected by insulin stimulation.

In the present study we have unveiled key differences between GLUT4 and CD-MPR trafficking and we suggest these are primarily due to sorting of GLUT4 into a separate population of vesicles. Firstly, although GLUT4 and CD-MPR have a similar distribution in basal 3T3-L1 adipocytes, insulin does not elicit a significant translocation of CD-MPR to the cell surface. This is consistent with previous studies on the effects of insulin on numerous other recycling proteins in adipocytes (reviewed by James et al., 1994). Secondly, using cryosections of 3T3-L1 adipocytes we have found that GLUT4 is largely distributed between the TGN, endosomes and tubulo-vesicular elements in basal cells, but that the major effect of insulin is on the latter pool of tubulo-vesicular elements located in the peripheral cytoplasm. Importantly, this observation suggests that there are functional differences between the different compartments containing GLUT4, and that these compartments are differentially affected by insulin stimulation. Thirdly, using whole mount immuno-labelling of intracellular

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Basal</th>
<th>Insulin</th>
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<tbody>
<tr>
<td>GLUT4(+)</td>
<td>100</td>
<td>75.8±5.6</td>
</tr>
<tr>
<td>CD-MPR(+)</td>
<td>71.4±7.1</td>
<td>70.2±5.2</td>
</tr>
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Intracellular vesicles were prepared from basal 3T3-L1 adipocytes and double-labelled for GLUT4 and CD-MPR by immunoelectron microscopy. The percentage of either GLUT4(+) or CD-MPR(+) vesicles that could be co-labelled for the other protein was quantified. For each quantitation >200 labelled vesicles were counted from 3 individual vesicle preparations.

<table>
<thead>
<tr>
<th>vesicles</th>
<th>% Basal values</th>
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<tbody>
<tr>
<td>GLUT4 gold labelling</td>
<td>64±10 132±39</td>
</tr>
<tr>
<td>Number of vesicles</td>
<td>63±9 112±8</td>
</tr>
</tbody>
</table>

Intracellular vesicles were prepared from basal and insulin-stimulated 3T3-L1 adipocytes and double-labelled for GLUT4 and CD-MPR by immunoelectron microscopy. For both basal and insulin-stimulated cells >200 randomly chosen labelled vesicles were counted per preparation. The number of GLUT4 gold particles observed labelling vesicles which were labelled for CD-MPR (CD-MPR(+) or not (CD-MPR (−)), was compared between basal and insulin-stimulated cells. The results show the percentage of GLUT4 gold particles labelling vesicles from insulin-stimulated cells relative to the vesicles from basal cells. Results shown are the percentage of vesicles relative to basal cells.

n=3 ± s.e.m.

Table 3. Effect of insulin on the colocalisation between GLUT4 and CD-MPR in vesicles isolated from basal and insulin-stimulated 3T3-L1 adipocytes

Table 4. Effect of insulin on GLUT4 labelling in CD-MPR(+) and CD-MPR(−) vesicles

**Table 3. Effect of insulin on the colocalisation between GLUT4 and CD-MPR in vesicles isolated from basal and insulin-stimulated 3T3-L1 adipocytes**

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<td>Number of vesicles</td>
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**Table 4. Effect of insulin on GLUT4 labelling in CD-MPR(+) and CD-MPR(−) vesicles**
vesicles we found that GLUT4 and CD-MPR were highly co-localised, but that there was not a high degree of correspondence in the labelling density of individual vesicles, consistent with a differential sorting mechanism for these proteins. Thus, despite considerable co-localisation, there is a population of vesicles in the intracellular membrane fraction isolated from adipocytes that is highly enriched in GLUT4 compared to CD-MPR. This is not simply the result of random targeting of proteins to vesicles as previous studies have shown a clear correspondence in labelling density between GLUT4 and IRAP (Martin et al., 1997). In addition, using this technique we demonstrated that insulin stimulation caused a selective decrease of approx. 30% in the total number of GLUT4(+)/CD-MPR(+) vesicles in the intracellular membrane fraction, whereas the total number of CD-MPR(+) vesicles did not change (Table 2). Not surprisingly, it was the GLUT4-enriched vesicles that selectively decreased following insulin-stimulation (Table 4). While there is considerable evidence to support the generation of specific insulin-regulatable vesicles there is, as yet, no direct evidence for a secretory model. However, the alternative protein sorting model, where GLUT4 dynamically recycles through the endosomal system in the absence of insulin, seems less probable in view of the data presented here. Sequestration of GLUT4 as a result of its active sorting away from recycling vesicles within the endosomal system would have been more consistent with a reduction in the amount of GLUT4 labelling/vesicle which was not observed (Table 2; Fig. 7).

We believe our observations are consistent with a model in which GLUT4 is selectively targeted to a compartment under basal conditions that is specifically affected by insulin (Figs 1, 7). This is likely to be through either the generation of specific exocytic GLUT4 vesicles, similar to synaptic vesicles, or through concentration and retention in sub-domains of the endosomal system. The v-SNARE, VAMP2, has been implicated in the insulin dependent trafficking of GLUT4 (Martin et al., 1998) and VAMP2 is highly colocalised with GLUT4 in adipocytes (Martin et al., 1996). In order to determine the distribution of VAMP2 in the two sub-populations of GLUT4 vesicles described in this study we performed triple labelling experiments in vesicle whole mounts. While it was difficult to accurately quantify the distribution of VAMP2 due to low labelling (approx. 1-3 gold particles/vesicle) it was clear that, in contrast to CD-MPR, VAMP2 was distributed among the GLUT4(+)/CD-MPR(−) vesicles and the GLUT4(+)CD-MPR(+) vesicles with no obvious concentration in either population (data not shown). Hence, these data are consistent with the idea that the insulin-responsive GLUT4(+)CD-MPR(−) vesicles are competent to dock and fuse directly with the plasma membrane in an analogous manner to synaptic vesicles.

While our data point to the existence of a sub-population of GLUT4 vesicles that may represent a releasable pool we do not believe that this pool can account for the entire stimulation observed in response to insulin. Firstly, we assume that this pool corresponds to the cytoplasmic vesicles observed in adipocyte cryosections. However, this pool only represented approx. 20% of the total GLUT4 labelled in the cell (Table 1) and we know from biochemical experiments that as much as 30-50% of the entire intracellular pool shifts to the cell surface in response to insulin under steady state conditions (Robinson and James, 1992). Therefore, it seems likely that the endosomal/TGN pool must also contribute to the insulin effect either directly or by generating new releasable vesicles by repeated rounds of recycling. It is noteworthy that we did detect a slight decrease in GLUT4 labelling in the TGN region with insulin which, together with the decrease observed in the cytoplasmic vesicles, may account for the entire cell surface increase in GLUT4 levels. In agreement with these data the absolute amount of GLUT4 in the GLUT4(+)/CD-MPR(−) pool that was used to calculate the data shown in Table 4 was approx. 30%. Thus, while there was clearly a selective effect of insulin on this pool quantitatively it can not account for the insulin-dependent increase in cell surface levels of GLUT4 in which case it is likely that there is also a contribution of the CD-MPR(+) pool to the insulin effect. Hence, based on these data we suggest that adipocytes have the capacity to store GLUT4 in a releasable pool but that the size of this pool is limited, perhaps in the same way that the size of the ready releasable pool of synaptic vesicles is limited. This pool may constitute the initial burst of GLUT4 in response to an insulin challenge after which new releasable vesicles may need to be formed from the endosomal/TGN system, or GLUT4 traffic out of the latter system may be directly activated by insulin in a manner that is possibly unrelated to that used for the ready releasable pool. Evidence has recently been published to support the fact that insulin may stimulate exit of GLUT4 from multiple intracellular pools (Bogan and Lodish, 1999; Foran et al., 1999; Martin et al., 2000). It is interesting to note that in the very early stages of insulin stimulation of rat adipocytes a diminution in the number of small vesicles within the cytoplasm has been reported (Morre et al., 1996). While these vesicles were not characterised it is tempting to speculate that they may represent the same compartment identified here using the whole mount approach. Other evidence for the existence of a population of insulin-regulatable, GLUT4-enriched vesicles has come from biochemical analysis of the intracellular membranes from adipocytes. It is possible to separate two fractions enriched in GLUT4 one of which is enriched in endosomal proteins, endocytosed transferrin and TGN markers, and the other of which contains relatively few endosomal proteins, is highly insulin-responsive and is enriched in membranes from insulin-responsive cell types (Hashiramoto and James, 2000). Furthermore, studies comparing the recycling of endosomal and insulin-responsive proteins in basal and insulin-stimulated cells suggests that the insulin-responsive GLUT4 compartment is distinct from the endosomal system (Kandror, 1999).

Sequestration of insulin-responsive proteins such as IRAP is an early event in 3T3-L1 adipocyte differentiation, possible due to the inhibition of post-endosomal traffic and the generation of a new class of insulin-responsive vesicles (El-Jack et al., 1999). However, studies in non-insulin-responsive cell types such as CHO cells (Wei et al., 1998) and PC12 cells (Herman et al., 1994) have also suggested that GLUT4 sorts into a unique population of GLUT4-containing small vesicles. These also exclude the TIR suggesting that adipocytes may simply upregulate the generation of a class of secretory-type vesicle during differentiation. The studies in CHO cells have suggested that the GLUT4 vesicles are derived from either the endosomal system, or directly from the cell surface (Wei et al., 1998). The presence of GLUT4 in the TGN and endosomes is
consistent with the high degree of co-localisation with other proteins found in these compartments (Calderhead et al., 1990; Hanpeter and James, 1995; Tanner and Lienhard, 1989; Volchuk et al., 1995). Additional targeting to the highly enriched GLUT4 compartment likely provides the basis for selective insulin responsiveness. Hence, proteins such as GLUT4 and IRAP presumably contain unique targeting signals that facilitate efficient entry into this compartment. Other proteins, such as the CD-MPR or GLUT1, that are not translocated to the cell surface as efficiently as GLUT4, may also sort into this compartment but less efficiently. Importantly, this model implies that other factors, possibly independent of the cargo, may regulate insulin-dependent movement to the cell surface. It remains to be demonstrated that this compartment fuses directly with the cell surface following insulin stimulation. However, in view of the fact that in cryosections these elements appear to be vesicular and they contain the v-SNARE, VAMP2, that is known to interact with the cell surface t-SNARE, Syntaxin4, this seems probable.

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