Analysis of the co-localization of the insulin-responsive glucose transporter (GLUT4) and the *trans* Golgi network marker TGN38 within 3T3-L1 adipocytes

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The exposure of isolated adipocytes to insulin results in an ~ 20-fold increase in the rate of glucose transport into the cell. This increase is mediated by the movement of a pool of intracellular vesicles containing the so-called insulin-responsive glucose transporter (GLUT4) to the cell surface. In the resting state, most of the GLUT4 molecules are sequestered inside the adipocyte in an as yet unidentified intracellular compartment. TGN38 is an integral membrane protein which has been shown to be predominantly localized to the *trans* Golgi network [Luzio, Brake, Banting, Howell, Braghetta and Stanley (1990) *Biochem. J.* **270**, 97–102]. Here we investigate whether GLUT4 and TGN38 are co-localized in the murine 3T3-L1 adipocyte cell line. Immunoadsorption of intracellular vesicles containing GLUT4 with an anti-peptide antibody specific for this isoform did not deplete the low-density microsomal fraction of TGN38 in these cells; more-

over, no TGN38 was detected in the GLUT4-containing vesicles by immunoblotting with a TGN38-specific antiserum. Immunoadsorption of TGN38-containing vesicles and subsequent analysis of the proteins in these vesicles revealed that a detectable amount of GLUT4 (5–10%) did co-localise with TGN38. The amount of GLUT4 in the TGN38-containing vesicles did not change in response to insulin. Immunofluorescence analysis of TGN38 and GLUT4 in these cells revealed markedly different staining patterns. Reversal of insulin-stimulated glucose transport and subsequent analysis of the TGN38-containing vesicles demonstrated that during the re-cycling of GLUT4 to the intracellular storage site there was no increase in the amount of GLUT4 co-localized with TGN38. Taken together, these results suggest that the *trans* Golgi network is not the major site of the intracellular GLUT4 pool within 3T3-L1 adipocytes.

INTRODUCTION

Insulin stimulates glucose disposal into peripheral tissues (muscle and fat). Acute insulin treatment of isolated adipocytes results in a 20–30-fold increase in the rate of glucose transport into these cells. The mechanism underlying this effect has been extensively studied, and it is now well established that most, if not all, of the increase in glucose transport elicited by insulin can be accounted for by an increase in the number of glucose transporters at the plasma membrane following the rapid movement (translocation) of a pool of intracellular vesicles containing transport proteins to the cell surface (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; James et al., 1989; Zorzano et al., 1989; Holman et al., 1990; Calderhead et al., 1990; Slot et al., 1991a,b). Adipocytes and muscle express a unique glucose transporter isoform termed GLUT4, or the insulin-responsive glucose transporter (for review see Bell et al., 1990; Gould and Bell, 1990). In the resting state only about 5% of the total cellular GLUT4 is at the surface of isolated rat adipocytes; upon exposure to insulin, 40–50% of the GLUT4 is found at the cell surface, and there is a corresponding decrease in the amount of intracellular GLUT4 (Holman et al., 1990; Calderhead et al., 1990; Slot et al., 1991a,b). Upon removal of insulin, there is a rapid reversal of this translocation and GLUT4 re-appears inside the cell. This recycling of GLUT4 is similar to that observed with other continually recycling proteins. Levels of the bifunctional mannose 6-phosphate/insulin-like growth factor-II receptor (M6PR/IGFIIIR) also increase at the cell surface after insulin stimulation, and this

protein recycles between the plasma membrane, prelysosomal intracellular compartments and the *trans* Golgi network (TGN) (Oka et al., 1984). The transferrin receptor also recycles between the plasma membrane and endosomal compartments (Tanner and Lienhard, 1987; Davis et al., 1986). In adipocytes and muscle, the translocation of GLUT4 has been demonstrated by several techniques, including specific labelling with a cell-impermeant photoactivatable glucose analogue (Holman et al., 1990; Calderhead et al., 1990), and by immuno-electron-microscopy with GLUT4-specific antibodies (Slot et al., 1991a,b). However, despite these advances, the definitive identification of a specific intracellular compartment to which GLUT4 is sequestered in the absence of insulin remains to be achieved. Recent evidence has shown that GLUT4 undergoes a continual re-cycling process between the intracellular compartment and the plasma membrane, and that this pathway involves clathrin-coated pits and endosomes (Slot et al., 1991a,b; Piper et al., 1992; Jhun et al., 1992; Yang et al., 1992; Yang and Holman, 1993). Immuno-electron-microscopy has identified GLUT4 in tubulo-vesicular structures located *trans* to the Golgi stack, suggesting that the compartment to which GLUT4 is localized may be the TGN; however, GLUT4 has also been localized to vesicular structures located in regions near the plasma membrane and in clusters throughout the cytoplasm (Blok et al., 1988; Slot et al., 1991a,b; Smith et al., 1991).

Recent work from several groups has shown that the TGN is an organelle independent of the Golgi stacks (Cheque and Pfeffer, 1990; Lippincott-Schwartz et al., 1991; Reaves and Banting,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GLUT4, insulin-responsive glucose transporter; IGFIIIR, insulin-like growth factor-II receptor; KRP, Krebs–Ringer phosphate buffer; KRM, Krebs–Ringer Mes buffer; LDM, low-density microsomes; M6PR, mannose 6-phosphate receptor; *Staph. a.*, formaldehyde-fixed *Staphylococcus aureus*; TGN, *trans* Golgi network.

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1992) and that, since certain integral membrane proteins can recycle between the TGN and the plasma membrane (Breitfeld et al., 1985; Duncan and Kornfeld, 1986; Stoorvogel et al., 1989; Ladinsky and Howell, 1992; Reaves et al., 1993; Meresse and Hoflack, 1993), it might well be considered part of an endosomal membrane system (Luzio and Banting, 1993). Thus the TGN would appear to be a prime candidate for the location of the intracellular pool of GLUT4 in non-insulin-stimulated adipocytes.

A procedure for the isolation of the intracellular glucose-transporter-containing vesicles, based on the specific adsorption of these vesicles to *Staphylococcus aureus* (*Staph. a*) cells coated with anti-transporter antibodies, has been described (Biber and Lienhard, 1986; Brown et al., 1988; Cain et al., 1992). Analysis of these vesicles has demonstrated the presence of proteins related to the synaptic-vesicle VAMP (vesicle-associated microtubule proteins) protein family (Cain et al., 1992), low-molecular-mass GTP-binding proteins (Cormont et al., 1991) and has also shown that these vesicles are not enriched in the Golgi marker galactosyltransferase (Brown et al., 1988), suggesting that, although the intracellular pool of GLUT4 might be in an endosomally related compartment, it is probably not in the Golgi stack itself.

One of the proteins which recycles between the TGN and the plasma membrane is a monotopic integral membrane protein designated TGN38 (Luzio et al., 1990; Ladinsky and Howell, 1992; Reaves et al., 1993). Although this molecule recycles between the TGN and the plasma membrane, at steady state the vast majority is in the TGN. It is thus a convenient marker for this organelle. The availability of epitope-mapped monoclonal and polyclonal antibodies against TGN38 prompted us to determine whether GLUT4-containing vesicles are enriched for this protein, and to evaluate the role of the TGN in the recycling of this metabolically important glucose transporter. We demonstrate that the majority of the insulin-responsive glucose transporter, GLUT4, and the TGN marker protein, TGN38, do not co-localize within the intracellular vesicles of 3T3-L1 adipocytes. It is further shown that, after insulin stimulation of GLUT4 translocation, GLUT4 levels do not change in this compartment, suggesting that GLUT4 does not translocate through the TGN. Upon reversal of insulin-stimulated GLUT4 translocation, we see no evidence of GLUT4 re-sequestration involving the TGN.

MATERIALS AND METHODS

Materials

Formaldehyde-fixed *Staphylococcus aureus* cells were obtained from Calbiochem (Pansorbin) or from Sigma (Poole, Dorset, U.K.). Insulin, dexamethasone and 3-isobutyl-1-methylxanthine were from Sigma. Dulbecco's modified Eagle's medium (DMEM), Myoclon plus fetal-calf serum and all antibiotics were from Gibco (Paisley, Scotland, U.K.). Newborn-calf serum was from Flow Laboratories (Irvine, Scotland, U.K.). All other reagents were as described by Gould et al. (1992).

Cell culture

3T3-L1 fibroblasts (American Tissue Culture Collection) were grown in 10% newborn-calf serum in DMEM at 37 °C in an atmosphere of air/CO₂ (9:1). Cells were passaged at sub-confluency and then grown in the same media until confluent. Differentiation into the adipocyte phenotype was exactly as described by Frost and Lane (1985). Before use, cells were

incubated in serum-free DMEM for 2 h. Cells were used between days 9 and 12 post-differentiation, and at passages between 3 and 10.

Deoxyglucose transport and insulin reversal

Deoxyglucose transport was assayed by the uptake of [1,2-³H]-deoxyglucose (Gibbs et al., 1988). Monolayers were washed with 3 × 3 ml of Krebs-Ringer phosphate buffer (KRP: 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄, 1.25 mM CaCl₂, 5 mM sodium phosphate, pH 7.4) at 37 °C and then covered with 1 ml of KRP. Insulin (100 nM) or carrier was present for the times indicated in the Figure legends, generally 30 min. Uptake was initiated by addition of [³H]deoxyglucose (final concn. 100 µM; 0.25 µCi/ml). After incubation for 2 min, uptake was terminated by aspirating the medium and washing the monolayers three times with ice-cold PBS (150 mM NaCl, 10 mM NaH₂PO₄, pH 7.4). The cells were solubilized with 1.0 ml of 1% Triton X-100, and the radioactivity in a sample was measured by liquid-scintillation spectrometry. Uptake values were corrected for the non-specific association of [³H]deoxyglucose with the cells by subtracting the uptake in the presence of 10 µM cytochalasin B, a potent inhibitor of transport (Bloch, 1973). This correction typically amounted to about 30% of the uptake by basal cells. In all experiments described herein, the increase in deoxyglucose transport measured in response to treatment with 100 nM insulin for 30 min was between 10 and 22-fold, depending on the plating of the cells.

Insulin-stimulated glucose transport was reversed by the low-pH washing procedure described by Gibbs et al. (1986). Monolayers of adipocytes were stimulated with insulin for 30 min, then washed three times with Krebs-Ringer Mes buffer (KRM: 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄, 1.25 mM CaCl₂, 5 mM Mes, pH 6.0) containing 1 mg/ml BSA at 37 °C. After these washes, cells were incubated in KRM for the times indicated, washed once with KRM, twice with KRP, then assayed for deoxyglucose transport as described above.

Preparation of plasma membrane and low-density microsomal (LDM) membrane fractions

Subcellular fractionation of 3T3-L1 adipocytes was performed exactly as described by Piper et al. (1991) after treatment for 30 min with either 1 µM insulin or carrier. For the immuno-adsorption experiments, the LDM membranes were prepared as follows: 10 cm plates of 3T3-L1 adipocytes were incubated in serum-free DMEM for 2 h, then washed with 3 × 5 ml of KRP and covered with 10 ml of the same buffer. Cells were stimulated with insulin (100 nM) for the times indicated in the Figure legends, or treated with vehicle as a control. Cells were subsequently washed with 3 × 8 ml of ice-cold Buffer A (20 mM Hepes/KOH, 150 mM KCl, 2 mM MgCl₂, pH 7.2), then scraped off the plates into 10 ml of the same buffer, but containing 1 µg/ml pepstatin A, 200 µM di-isopropyl fluorophosphate and 10 µM L-trans-epoxysuccinyl-leucylamido-4-guanidinobutane. Cells were homogenized by 20 hand-driven strokes of a Teflon Dounce homogenizer, and the homogenates were centrifuged at 16000 g_{av} for 20 min at 4 °C. The fat cake was carefully aspirated, and the supernatant containing the low-density microsomes was decanted into fresh tubes before immunoadsorptions.

Immunoadsorptions

Formaldehyde-fixed *Staph. a.* cells were extracted and washed exactly as described by Brown et al. (1988). The cells were then

washed twice in Buffer A containing 1% BSA, and loaded as described below with either anti-GLUT4 antibodies or serum, anti-TGN38 serum or irrelevant serum as a control (Brown et al., 1988).

Before immunoadsorption of a specific vesicle population, the low-density microsomal fractions were 'pre-cleared' by incubation for 30 min at 4 °C with irrelevant-IgG-coated *Staph. a.* cells, as this has been shown to decrease non-specific binding (Brown et al., 1988). The *Staph. a.* cells were removed by centrifugation, and immunoadsorption of either GLUT4- or TGN38-containing vesicles was performed by slow rotation of a portion of the low-density microsomes with antibody-coated *Staph. a.* cells for 2 h at 4 °C (~20 µg of affinity-purified anti-GLUT4 antibodies, 10 µl of anti-GLUT4 serum, or 10 µl or 25 µl of anti-TGN38 serum was used routinely for these adsorptions; corresponding amounts of irrelevant IgG or serum were used as controls). After 2 h, the *Staph. a.* cells were recovered and washed as described (Brown et al., 1988), and the vesicle proteins were solubilized by incubation with 1% Triton X-100 in PBS. After incubation with occasional agitation for 20 min, the *Staph. a.* cells were pelleted and the supernatant was carefully removed. Samples for electrophoresis were prepared by addition of 4 × SDS/PAGE sample buffer (Gibbs et al., 1986) directly to this supernatant. Note that most of the GLUT4 molecules present in the vesicles remained bound to the pelleted *Staph. a.* cells, and were thus not detectable in the solubilized vesicle fraction.

The supernatants from the immunoadsorptions were centrifuged at 100 000 g_{av} for 1 h at 4 °C to pellet all membranes. The pellets were directly resuspended in SDS/PAGE sample buffer.

Electrophoresis and immunoblotting

Proteins were separated on 10% Laemmli gels and transferred to nitrocellulose as previously described (Gould et al., 1992). Immunoblotting was performed exactly as described by Brant et al. (1992).

Antibodies

The antibodies against the C-terminal 14 amino acids of the human isoform of GLUT4 have been previously described (Brant et al., 1992), as has the rabbit antiserum against the C-terminal 21 amino acids of TGN38 (Wilde et al., 1992). The anti-M6PR antibody was kindly given by Dr. Suzanne Pfeffer (Department of Biochemistry, Stanford University Medical Center, Stanford, CA, U.S.A.). Rhodamine-conjugated antibodies were obtained from Sigma.

Immunofluorescence

3T3-L1 fibroblasts were cultured and differentiated on 22 mm glass coverslips as described above, and processed for immunofluorescence analysis as previously described (Reaves et al., 1993).

RESULTS

Subcellular fractionation of adipocytes

Fractions corresponding to plasma membranes, LDM membranes, high-density microsomes and mitochondria/nuclei were analysed by SDS/PAGE and immunoblotting using antibodies specific to GLUT4 or the TGN marker, TGN38. The results (Figure 1) showed that GLUT4 is predominantly localized to the

LDM membrane fraction, and that in response to insulin there is a re-distribution of GLUT4 from this fraction to the plasma membrane; quantification of immunoblots such as that shown in Figure 1 revealed that the amount of GLUT4 in the LDM fraction decreased from $72.5 \pm 9\%$ of the total GLUT4 to $38.4 \pm 3.8\%$ in response to insulin; the plasma-membrane GLUT4 content increased from 15.5 ± 4.3 to $46.5 \pm 4.6\%$ in response to insulin (values are means $\pm$ S.D. of five independent fractionations). TGN38 was present only in the LDM, and did not appear to translocate to the plasma membrane in response to insulin in any of the fractionation experiments performed.

Immunoadsorption of GLUT4-containing vesicles

Vesicles were immunoadsorbed from the LDM fraction of 3T3-L1 adipocytes, lysed, subjected to SDS/PAGE, and the separated proteins were transferred to nitrocellulose. Figure 2(a) shows an immunoblot, prepared by using an anti-GLUT4 antibody to probe the LDM membranes remaining after immunoadsorption of vesicles with either irrelevant IgG (lane 1) or anti-GLUT4 (lane 2) antibodies. As shown, the use of *Staph. a.* cells coated with anti-GLUT4 antibodies efficiently and specifically removed GLUT4 from the LDM fraction, compared with that remaining after immunoadsorption with an irrelevant IgG. In contrast, immunoblotting of the same fractions with an anti-TGN38 antibody demonstrated that the amount of TGN38 remaining in the LDM after removal of the GLUT4-containing vesicles (Figure 2b, lane 4) was the same as that remaining after immunoadsorption with an irrelevant IgG (Figure 2b, lane 3). In three independent experiments, we have seen no decrease in the TGN38 signal obtained from LDM after immunoadsorption with anti-GLUT4 antibodies. Densitometric analysis from these three experiments showed that the TGN38 signal after such immunoadsorption was $96 \pm 10\%$ of the signal obtained by using random IgG (results not shown; mean $\pm$ S.D., $n = 3$).

Immunoadsorption of TGN38-containing vesicles

We next performed a series of immunoadsorption experiments using an antiserum against the C-terminus of TGN38, the results of which are presented in Figure 3. Immunoblot analysis, using

Figure 1 Subcellular distribution of GLUT4 and TGN38

Subcellular fractions corresponding to plasma membranes (PM), LDM, high-density microsomes (HDM) and mitochondria/nuclei (m/n) were prepared from either basal cells (—) or cells treated for 30 min with 1 µM insulin (+). Samples were immunoblotted with either anti-GLUT4 antibodies or anti-TGN38 antisera. Subcellular fractionation was performed on 10^7 cells, and the amounts of samples loaded corresponded to 2.5% of the PM fraction, 6% of the LDM membrane fraction, 3% of the HDM and 2% of the m/n fraction. Samples were matched for protein between basal and insulin-treated samples. For the GLUT4 blot, the radioactivity (c.p.m.) associated with the LDM fractions was 6253 (basal) and 3643 (+ insulin); that associated with the PM fractions was 833 (basal) and 1868 (+ insulin). The anti-GLUT4 immunoblot was developed with 125 I-labelled goat anti-rabbit IgG, and the anti-TGN38 blot was developed by using enhanced chemiluminescence. A representative immunoblot is presented; 49.5 kDa indicates the position of a marker protein.

Figure 2 GLUT4 and TGN38 reside in distinct subcellular compartments

(a) LDM membranes were depleted of GLUT4-containing vesicles by immunoabsorption as described in the Materials and methods section. After immunoabsorption with either random IgG (lane 1) or anti-GLUT4 (lane 2) antibodies, the LDM membranes were pelleted by centrifugation and immunoblotted with antibodies against GLUT4. Membranes from 10^6 cells were loaded on each lane. Abbreviation: TD, tracking dye. (b) LDM membranes were depleted of GLUT4-containing vesicles by immunoabsorption as described in the Materials and methods section. After immunoabsorption with either random IgG or anti-GLUT4 antibodies, the LDM membranes were pelleted by centrifugation. The vesicles isolated by immunoabsorption were released from the *Staph. a.* cells by Triton X-100 extraction, and run in parallel; the immunoblot was developed with antiserum against TGN38 at a dilution of 1:250. Lanes: 1, vesicle isolated with random IgG; 2, vesicles isolated by immunoabsorption with anti-GLUT4 antibodies; 3, LDM membranes after immunoabsorption with random IgG; 4, LDM membranes after adsorption with anti-GLUT4. Lanes 1 and 2 represent 75% of the vesicles isolated from 10^7 cells; lanes 3 and 4 contain membranes from 10^6 cells per lane. Positions of molecular-mass markers (kDa) are indicated.

Figure 3 Analysis of TGN38-containing vesicles

LDM membranes from either basal (lanes 2–4) or insulin-treated (lanes 5–7) 3T3-L1 adipocytes were prepared, and immunoabsorptions performed by using *Staph. a.* cells coated with random IgG, anti-GLUT4 or anti-TGN38 as described in the Materials and methods section. Lanes: 1, sample of LDM membranes, before immunoabsorption; 2 and 5, vesicles isolated with random IgG; 3 and 6, vesicles isolated with anti-GLUT4 antibodies; 4 and 7, vesicles isolated with anti-TGN38 antiserum. Each lane contains 16% of the vesicles isolated from 10^7 cells; the immunoblot was developed with anti-GLUT4 antibodies. The entire running gel is shown, and the positions of molecular-mass markers (kDa) are indicated.

antibodies raised against GLUT4, of anti-TGN38-adsorbed vesicles from basal (lane 4) or insulin-treated (lane 7) cells demonstrated a small but consistently observed amount of GLUT4 co-localized with TGN38. Quantification of the amount of GLUT4 in the TGN38-containing vesicles revealed that

Figure 4 Immunofluorescence analysis of 3T3-L1 adipocytes with anti-GLUT4, anti-TGN38 and anti-M6PR antibodies

Methanol-fixed 3T3-L1 adipocytes were processed for immunofluorescence analysis as described in the Materials and methods section. Cells were initially incubated with a rabbit polyclonal anti-GLUT4 antiserum (a), a rabbit polyclonal anti-TGN38 antiserum (b) or a rabbit polyclonal anti-M6PR/IGF1R antiserum (c). Bound antibody was detected with a Rhodamine-labelled goat anti-rabbit IgG. Magnification $\times 960$.

between 5 and 10% of the total LDM GLUT4 was present in these vesicles, and that this value did not change in response to acute insulin treatment of the cells (i.e. 5–10% of the GLUT4 present in the LDM at each condition). Analysis of the LDM fractions after adsorption of TGN38-containing vesicles showed no significant decrease in the amount of GLUT4 after this treatment (results not shown).

Immunofluorescence analysis of methanol-fixed 3T3-L1 adipocytes

To assess further the degree of co-localization between TGN38 and GLUT4 in basal cells, we have also performed immuno-

Figure 5 M6PR is present in the GLUT4-containing vesicles

Vesicles were isolated by using either anti-GLUT4 or random IgG and run on SDS/PAGE (7% gel). After transfer to nitrocellulose, the blot was probed with antibodies against the M6PR. Lanes: 1, vesicles isolated from resting (basal) cells by using anti-GLUT4; 2, the corresponding sample obtained with random IgG.

Table 1 Reversal of insulin-stimulated glucose transport in 3T3-L1 adipocytes

Deoxyglucose transport was assayed as described in the Materials and methods section. Reversal of insulin-stimulated glucose transport was achieved by washing the cells in low-pH buffer for the times indicated. Each point is the mean of triplicate determinations, and has been corrected for the non-specific association of deoxyglucose with the cells by subtraction of the uptake measured in the presence of 10 μ M cytochalasin B.

	Transport (pmol/min per 10 ⁶ cells)
Maximally insulin-stimulated rate	384 $\pm$ 50
5 min reversed	188 $\pm$ 24
30 min reversed	68 $\pm$ 5.3
Basal rate	38 $\pm$ 5.9

fluorescence analysis of 3T3-L1 adipocytes. Cells were grown on cover-slips and processed for immunofluorescence analysis using either a rabbit polyclonal anti-GLUT4 antiserum or a rabbit polyclonal anti-TGN38 antiserum. Binding of the primary antibodies was detected with a Rhodamine-labelled second antibody. The patterns of staining obtained with either anti-GLUT4 antibodies (Figure 4a) or anti-TGN38 antibodies (Figure 4b) are both juxtanuclear, but are clearly different; the anti-GLUT4 antibody gives a distinct focus of staining, whereas that produced by the anti-TGN38 antibody is more reticular. This suggests that the two proteins largely reside in different intracellular compartments.

The bifunctional M6PR/IGFIIR (Morgan et al., 1987) has previously been shown to migrate from an intracellular membrane pool to the plasma membrane in response to insulin

Figure 6 Effects of insulin reversal on co-localization of GLUT4 and TGN38

TGN38-containing vesicles were isolated from the LDM of insulin-treated cells which had undergone reversal treatment for 5 min (lane 3) or 30 min (lane 4), and compared with vesicles isolated from fully insulin-stimulated cells (lane 2). For comparison, the TGN38-containing vesicles from basal cells are shown in lane 1. The vesicle proteins were run on SDS/PAGE, transferred to nitrocellulose and probed with antibodies against GLUT4. Each lane contains 25% of the intracellular GLUT4-containing vesicles isolated from 10⁷ adipocytes. The LDM fraction contains 12% of the LDM from 10⁷ cells. The entire running gel is shown, and the positions of molecular-mass markers are indicated. The radioactivity (c.p.m.) associated with lanes 1, 2, 3, 4 and 5 was 420, 241, 266, 229 and 2680 respectively.

treatment in both rat adipocytes and H-35 hepatoma cells (Oppenheimer et al., 1983; Wardzala et al., 1984; Oka et al., 1984; Appell et al., 1988). It therefore seemed appropriate to compare the pattern of immunofluorescence staining in 3T3-L1 adipocytes observed with a rabbit polyclonal antiserum against GLUT4 with that obtained with a rabbit polyclonal antiserum against M6PR/IGFIIR. As shown in Figure 4(c), there is some apparent overlap between the two in the juxtanuclear region; however, the anti-M6PR/IGFIIR antiserum (Figure 4c) additionally recognizes a plethora of dispersed small vesicular structures not detected by the anti-GLUT4 antiserum (Figure 4a). Further evidence for an at least partial overlap of GLUT4 and M6PR/IGFIIR is provided by the demonstration of M6PR immunoreactivity in GLUT4-containing vesicles isolated by immunoadsorption (Figure 5).

GLUT4 content of TGN38 vesicles after insulin reversal

Cells were stimulated with 100 nM insulin for 30 min at 37 °C, and then washed three times in KRM buffer, pH 6.0, conditions which have previously been shown effectively to reverse insulin-stimulated glucose transport and result in the re-sequestration of GLUT4 to its intracellular pool (Gibbs et al., 1986). Analysis of the uptake of deoxyglucose under reversal conditions revealed that insulin-stimulated glucose transport was reversed with a $t_{1/2}$ of about 7–8 min (Table 1). A similar $t_{1/2}$ has been reported by others (Yang and Holman, 1993). TGN38-containing vesicles were isolated from the LDM fractions of basal cells, cells exposed to insulin for 30 min, and cells exposed to insulin and then 'reversed' by washing in KRM for 5 or 30 min. These vesicles were then analysed for the presence of GLUT4 by immunoblotting, the result of which is shown in Figure 6. Quantification of the amount of GLUT4 in the TGN38 vesicles indicated that the levels did not significantly change during insulin reversal.

DISCUSSION

It has previously been shown that, after insulin exposure, the levels of GLUT4 at the surface of 3T3-L1 adipocytes increase ~20-fold (Calderhead et al., 1990; Holman et al., 1990). This

increase correlates with a loss of GLUT4 from an as yet unidentified intracellular pool. Given the recent observations that GLUT4 undergoes continuous recycling from this pool to the plasma membrane and back (Jhun et al., 1992; Yang and Holman, 1993), it is clearly relevant to define biochemically and morphologically the intracellular compartment it represents. The TGN would appear to be a prime candidate. In addition to playing a pivotal role in the secretory pathway, sorting newly synthesized proteins to their correct final location (Griffiths and Simons, 1986), this organelle is also known to be involved in the recycling of certain proteins from the plasma membrane (Breitfeld et al., 1985; Duncan and Kornfeld, 1986; Stoorvogel et al., 1989; Ladinsky and Howell, 1992; Reaves et al., 1993; Meresse and Hoflack, 1993). TGN38 is an example of an integral membrane protein which recycles between the TGN and the plasma membrane; however, at steady state, the vast majority of TGN38 is in the TGN (Ladinsky and Howell, 1992; Reaves et al., 1993). The availability of epitope-mapped polyclonal and monoclonal antibodies to TGN38, as well as antibodies to GLUT4, enabled us to address whether the intracellular pool of GLUT4 is in the TGN.

Analysis of highly purified plasma membrane and LDM membranes isolated from basal or insulin-treated cells demonstrated that GLUT4 is translocated to the plasma membrane in response to insulin, with a concomitant loss of GLUT4 from the LDM fraction (Figure 1). However, TGN38 distribution was unaffected by insulin treatment (Figure 1), the protein remaining essentially completely localized to the LDM fraction. Previous workers have demonstrated that the GLUT4-containing vesicles can be specifically removed from the LDM by immunoadsorption with antibodies against the C-terminus of GLUT4. We have isolated the GLUT4-containing vesicles from resting cells, and analysed these vesicles and the post-adsorption LDM for the presence of TGN38. The results (Figure 2) demonstrated that GLUT4 vesicles were effectively removed by this procedure, and that (i) no immunoreactive TGN38 was present in these vesicles, and (ii) the levels of TGN38 in the LDM were not depleted after removal of the GLUT4-containing vesicles. These experiments indicate that TGN38 is not a major component of the GLUT4-containing vesicles, and does not translocate to the plasma membrane in response to insulin.

By way of further analysing the co-localization of these two proteins, we used a complimentary immunoadsorption approach to isolate TGN38-containing vesicles from basal and insulin-treated cells. The result (Figure 3) shows that a small amount of GLUT4 could be detected in the TGN38-containing vesicles. Analysis of these vesicles from several independent preparations indicated that between 5 and 10% of the total LDM GLUT4 was present in these vesicles. Depletion of TGN38-containing vesicles from the LDM fraction had no significant effect on the GLUT4 immunoblot signal obtained with these samples. These results indicate that TGN38 is not a major component of the GLUT4-containing vesicles from 3T3-L1 adipocytes, since between 90 and 95% of the total LDM GLUT4 does not co-localize with TGN38. The fraction of GLUT4 which co-localizes with TGN38 may well represent molecules present in a common part of the respective recycling pathways. Hence we suggest that the TGN is not the major location of GLUT4 protein in resting adipocytes.

No GLUT4 was detectable in vesicles adsorbed with either random IgG (Figure 3, lanes 2 and 5) or anti-GLUT4 (Figure 3, lanes 3 and 6); the GLUT4 protein remains tightly associated with the anti-GLUT4 antibodies throughout the Triton procedure used to release other vesicle proteins. Immunoblot analysis of the *Staph. a.* pellets in SDS/PAGE buffer containing 8 M urea

and 20 mM dithiothreitol demonstrated the presence of high levels of GLUT4 protein in the vesicles coated with anti-GLUT4 antibodies, but not in vesicles coated with either anti-TGN38 or irrelevant IgG (results not shown). The absence of any significant amount of co-localization of GLUT4 and TGN38 in insulin-stimulated cells further suggests that the re-cycling pathway of GLUT4 which occurs even in the continuous presence of insulin (Jhun et al., 1992; Yang and Holman, 1993) does not involve significant accumulation of GLUT4 in the TGN. The cells used in Figure 3 were exposed to insulin for 30 min, thus allowing ample time for several rounds of GLUT4 re-cycling, on the basis of the half-times reported by Jhun et al. (1992) and Yang and Holman (1993). The inability to detect TGN38 in the GLUT4-containing vesicles may be a reflection of the relatively weak immunoblot signal obtained with this antibody (compare the blots in Figure 1).

Immunofluorescence analysis of methanol-fixed 3T3-L1 adipocytes provides further evidence that TGN38 and GLUT4 do not co-localize in these cells. The patterns of staining observed with antibodies to the different proteins are clearly dissimilar (Figures 4a and 4b). This set of experiments also confirmed that the intracellular pool of GLUT4 fails to co-localize with either lysosomes or the Golgi stack, but does show some co-localization with the M6PR/IGFIR (Figures 4c and 5). This latter observation is of interest, since others have shown that incubation of rat adipocytes and H-35 hepatoma cells in the presence of insulin leads to an increase in cell-surface IGFII receptors (Oppenheimer et al., 1983; Wardzala et al., 1984; Oka et al., 1984; Appell et al., 1988). One might therefore expect at least a portion of the intracellular GLUT4 and M6PR/IGFIR to reside in the same intracellular compartment.

Washing insulin-treated adipocytes with low-pH buffer has been shown to reverse efficiently the stimulation of transport (Gibbs et al., 1986), and moreover to result in the re-sequestration of GLUT4 to its intracellular compartment (Yang et al., 1992; Yang and Holman, 1993). We have evaluated the role of the TGN as a sorting point for the re-sequestration of GLUT4 molecules after insulin reversal. We have performed immunoadsorption experiments with anti-TGN38 antibodies from cells which have been maximally stimulated with insulin, then reversed for 5 or 30 min. Table 1 shows that deoxyglucose transport was effectively reversed by this treatment. Analysis of the TGN38-containing vesicles further shows that, even under conditions of re-accumulation of GLUT4 into the intracellular compartment, the extent of the co-localization of GLUT4 and TGN38 does not increase (Figure 6). Thus we suggest that significant accumulation of GLUT4 in the TGN does not occur.

In summary, the data presented herein demonstrate that the insulin-responsive glucose transporter GLUT4 and the TGN marker protein TGN38 do not co-localize within the intracellular vesicles of 3T3-L1 adipocytes. Therefore, we suggest that the intracellular pool to which GLUT4 is localized in resting 3T3-L1 adipocytes is not the TGN. Upon reversal of insulin-stimulated glucose transporter translocation, we seen no evidence of GLUT4 re-sequestration involving the TGN.

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