Multiple endosomal recycling pathways in rat adipose cells

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Adipose and skeletal-muscle cells can translocate several membrane proteins from intracellular compartment(s) to the cell surface in an insulin-dependent fashion. Among these proteins is Glut4, a physiologically important glucose transporter which mediates insulin's effect on blood glucose clearance. Under basal conditions, Glut4 is localized in uniform, intracellular membrane vesicles with an average diameter of 50–70 nm and a sedimentation coefficient of 100–120 S. The nature of this compartment and its trafficking pathway to the plasma membrane is still unresolved. We show here that, in addition to Glut4, the aminopeptidase gp160 or insulin-responsive aminopeptidase ('IRAP'), sortilin, and an acutely recycling population of the insulin-like growth factor-II/mannose 6-phosphate receptor, this compartment includes 60 % of the intracellular population of the transferrin receptor. We used subcellular fractionation, cell-surface biotinylation, and radioactive-ligand (^{125}I-transferrin) uptake to demonstrate that the transferrin receptor recycles between this compartment and the plasma membrane in response to insulin along with Glut4 and other protein components of these vesicles. The co-localization of Glut4 and several endosomal markers in the terminally differentiated fat-cells during several stages of their cycling pathways suggests that the 'Glut4 pathway' may derive from the hormone-insensitive endosomes of undifferentiated preadipocytes. The insulin receptor is excluded from Glut4-containing vesicles in both insulin-stimulated and unstimulated adipocytes, and thus it is likely to traffic independently from Glut4 through different intracellular compartments. Our data show that, in adipose cells, the ligand-dependent recycling pathway of the insulin receptor is structurally separated from the ligand-independent pathway of the transferrin receptor, and that Glut4 is specifically targetted to the latter.

INTRODUCTION

An important physiological function of adipose cells is to recruit glucose transporters, especially Glut4, from an intracellular pool to the cell surface in response to insulin. Glut4 translocation to surface membranes in adipocytes and skeletal muscle and its function there accounts for most or all of insulin's action on blood glucose clearance in mammalian organisms [1–8]. Although intracellular Glut4 traffic has been intensively studied since its independent discovery by Cushman and Wardzala [9] and Suzuki and Kono [10], much remains unknown about its biochemical basis, regulation and relationship to other established cellular pathways of protein traffic and secretion. In particular, it is not clear whether insulin-sensitive Glut4 trafficking represents a specialized cellular trafficking route specific to fat and muscle or represents a variant of the normal endosome-to-cell-surface recycling pathway differing only in that insulin influences one or more steps in the kinetics of the process [11].

When Glut4 is expressed in NIH-3T3 and 3T3-L1 fibroblasts, it is compartmentalized intracellularly [12,13], where it co-localizes, partially, with the insulin-like growth factor II/mannose 6-phosphate (IGF-II/Man 6-*P*) receptor and also, with the transferrin receptor (TfR) [13]. Glut4 shows no co-localization with lysosomal or Golgi markers, but is present in the same compartment with internalized FITC-labelled wheatgerm agglutinin [13]. These studies provide good evidence that Glut4, after expression in non-differentiated cells, can be targeted to endosomes. However, as noted above, the nature of the Glut4-containing compartment in mature adipocytes remains unclear. Thus our interests have been concentrated on defining the identity and structural organization of this compartment in native adipose cells which express Glut4 *in vivo*. Since these cells are difficult to study by immunogold electron microscopy, we [14–16] and others [17,18] have established experimental approaches which allow us to isolate specifically Glut4-containing membrane vesicles by immunoadsorption in order to identify and to characterize their protein components. In the course of these studies, we and others have demonstrated that the Glut4-containing compartment includes a limited number of major component proteins. These are Glut4 itself, insulin-responsive aminopeptidase (IRAP) [19–21], the IGF-II/Man 6-*P* receptor [22], a recently identified [23] receptor-like protein gp110 or sortilin [24], and TfR (the present study). These proteins are translocated by or from this compartment to the cell surface in an insulin-dependent fashion ([16,22]; the present study). The Glut4-containing compartment is also enriched in secretory carrier-associated membrane proteins (SCAMPs) [15,25], members of the cellubrevin family [18,26–28], and low molecular weight GTP-binding proteins [29]. Along with this, the Glut4-compartment in adipocytes is segregated from Glut1 [14,30], caveolin [31], Golgi markers TGN38 ([32,33]; TGN is *trans*-Golgi network) and galactosyltransferase [34] and excludes lysosomal markers ([33]; K. V. Kandror and P. F. Pilch, unpublished work).

Therefore it appears that, in native adipose cells, as in transfected fibroblasts and preadipocytes, Glut4 co-localizes and traffics along with the well-established protein markers of the endosomal recycling pathway, namely the IGF-II/Man 6-*P* and TfR. Moreover, all the recycling population of the IGF-II/Man 6-*P* receptor [22] and at least half of the total TfR pool (the present study) share the same basal compartment and recycling pathway as does Glut4. This suggests that the 'Glut4-pathway' may be related to, or may directly evolve from, the ligand-independent receptor-mediated recycling pathway present in fibroblast-like preadipocytes in the course of their differentiation.

On the other hand, we found the major pool of the intracellular

Abbreviations used: Glut, glucose transporter; IGF, insulin-like growth factor; IRAP, insulin-responsive aminopeptidase; Man 6-*P*, mannose 6-phosphate; SCAMPs, secretory carrier-associated membrane proteins; TfR, transferrin receptor; LM, light microsomes; TGN, *trans*-Golgi network; NHS, *N*-hydroxysuccinimide.

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insulin receptor to be excluded from the Glut4-containing compartment. This suggests that the 'Glut4 pathway' in mature adipocytes is different from endosomes responsible for endocytosis of the insulin receptor. This finding supports the trafficking model according to which insulin stimulates exocytosis of the intracellular Glut4-containing vesicles simultaneously with (or immediately after) endocytosis of insulin receptors [35,36], so that the bulk of the two proteins is unlikely to share the same compartment.

MATERIALS AND METHODS

Antibodies

In the present study we used the monoclonal anti-Glut4 antibody 1F8 [37], polyclonal anti-(insulin receptor) antibody R1064 [38] and monoclonal anti-TfR antibody H68 (kindly given by Dr. I. Trowbridge, Salk Institute, La Jolla, CA, U.S.A.).

Labelling, biotinylation and fractionation of rat adipocytes

Adipocytes were isolated from the epididymal fat-pads of male Sprague–Dawley rats (200–250 g) by collagenase digestion [39] and transferred to KRP buffer (12.5 mM Hepes/120 mM NaCl/6 mM KCl/1.2 mM $MgSO_4$/1 mM $CaCl_2$/0.6 mM Na_2HPO_4/0.4 mM NaH_2PO_4/2.5 mM D-glucose/2% BSA, pH 7.4). In biotinylation experiments, before use with cells, the buffer was pre-incubated with 0.4–0.5 mg/ml of sulpho-N-hydroxysuccinimide acetate (sulfo-NHS-acetate; Pierce) for 3–4 h at 37 °C and overnight at 4 °C to block free amino groups of the BSA present in the buffer. Insulin was administered to cells (where indicated) to a final concentration 10 nM. Sulfo-NHS-biotin was added to cells 2 min after insulin to final concentration of 0.5 mg/ml. Biotinylation was usually performed for 16–17 min at 37 °C, then 1 M Tris, pH 7.4 and 0.2 M KCN was added, to final concentrations 50 mM and 2 mM respectively, for 5–15 min. After that, cells were washed three or four times with HES buffer (20 mM Hepes/250 mM sucrose/1 mM EDTA/5 mM benzamidine/1 mM PMSF/1 μM pepstatin/1 μM aprotinin/1 μM leupeptin, pH 7.4) cooled to 14–16 °C, homogenized with a Potter–Elvehjem Teflon pestle, and subcellular fractions were prepared as previously described [40]. Isolated fractions were resuspended in PBS, which contained all of the protease inhibitors listed above.

Immunoadsorption of Glut4 containing membranes (vesicles)

Protein A-purified 1F8 antibody, as well as non-specific mouse IgG (Sigma), were each coupled to acrylic beads (Reactigel GF2000; Pierce) at a concentration 0.4 and 0.6 mg of antibody/ml of resin respectively, according to the manufacturer's instructions. Before use the beads were saturated with 2% BSA in PBS for at least 1 h and washed with PBS. The light microsomes (LM) from rat adipocytes were incubated separately with each of the specific and non-specific antibody-coupled beads overnight at 4 °C. The beads were washed three times with PBS/10 mM Tris, pH 7.4, and the adsorbed material was eluted with either 1% Triton X-100 in PBS or Laemmli sample buffer without 2-mercaptoethanol.

^{125}I-insulin binding to subcellular membrane fractions

Membrane fractions (20 μl, 1–5 mg/ml) in PBS with 1% Triton X-100 were mixed with 180 ml of 1% BSA in PBS which contained 25–125 pM ^{125}I-insulin (NEN; 0.01–0.05 mCi/tube) and incubated for 45 min at room temperature. After that, 250 μl of 1.25 mg/ml γ-globulin and 250 ml of 25% poly(ethylene glycol) (M_r 8000) (both in ice-cold sodium phosphate buffer, pH 7.4) were added. Samples were vortex-mixed and, after 45 min incubation at 4 °C, microcentrifuged for 20 min at 4 °C. The supernatant was aspirated and the pellets were counted for radioactivity in an LKB γ-radiation counter for 10 min. In parallel experiments, binding was measured in the presence of unlabelled insulin (3 μM) to determine the non-specific insulin binding, which was then subtracted from the total to obtain receptor-specific binding.

Subfractionation of intracellular microsomes on sucrose gradient

The LM fraction from rat adipocytes was suspended in PBS and was loaded on to a 4.6 ml continuous 10–30%-(w/v)-sucrose gradient and centrifuged for 50 min in a SW-50.1 rotor at 48000 rev./min [30]. Each gradient was collected into 24 fractions starting from the bottom of the tube.

Internalization and cell-surface binding of ^{125}I-transferrin

For ^{125}I-transferrin binding, iodinated transferrin (Amersham) was added to the suspension of rat adipocytes at 0.22 mCi/ml (0.3 nM) alone or together with 75 nM of non-radioactive transferrin (Sigma). As in the biotinylation experiments, insulin was added to final concentration 10 nM for 18–20 min. After that, cells were treated with 2 mM KCN for 5 min at room temperature, washed and homogenized as described above.

Gel electrophoresis and immunoblotting

Proteins were separated in SDS/polyacrylamide gels as described by Laemmli [41], but without reducing agents and were transferred to Immobilon-P membrane (Millipore) in 25 mM Tris/192 mM glycine. Following transfer, the membrane was blocked with 10% non-fat dry milk in PBS for 2 h at 37 °C. Proteins were revealed with specific antibodies, horseradish-peroxidase-conjugated secondary antibodies (Sigma) and an enhanced chemiluminescence substrate kit (NEN). For quantification, autoradiograms were scanned in a computing densitometer (Molecular Dynamics). Biotinylated proteins were stained with 2000-fold diluted streptavidin/alkaline phosphatase conjugate obtained from Boehringer and used according to the manufacturer's instructions.

Protein content

Protein was determined with the BCA (bicinchoninic acid) kit (Pierce) according to manufacturer's instructions.

RESULTS

The TfR and the Glut4 intracellular trafficking pathway

Figure 1 demonstrates the subcellular distribution of the transferrin receptor in insulin-treated and untreated rat adipocytes. We show by Western blotting that the amount of this receptor is increased in the plasma membrane upon insulin stimulation and is concomitantly decreased in the fraction known as LM which contains intracellular pools or many, if not all, recycling proteins in adipocytes, including Glut4 (reviewed in [7,8]). Thus TfR demonstrates a clear insulin-dependent recruitment to the cell surface of adipose cells. This finding is in complete agreement with the results of the two earlier studies where TfR translocation was first described in adipocytes using ^{125}I-transferrin binding as an assay [42,43].

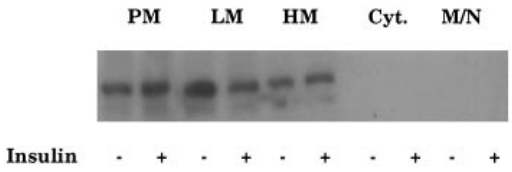

Figure 1 Distribution of TfR in subcellular fractions of rat adipocytes treated or not treated with insulin

Rat fat-cells were isolated and fractionated as described in the Materials and methods section. Membrane protein (50 μg/fraction) was electrophoresed, transferred to PVDF membrane, and blotted with anti-receptor antibodies. Abbreviations: PM, plasma membrane; HM, heavy microsomes; Cyt., cytosol; M/N, combined fraction of mitochondria and nuclei. A representative blot from three independent experiments is shown.

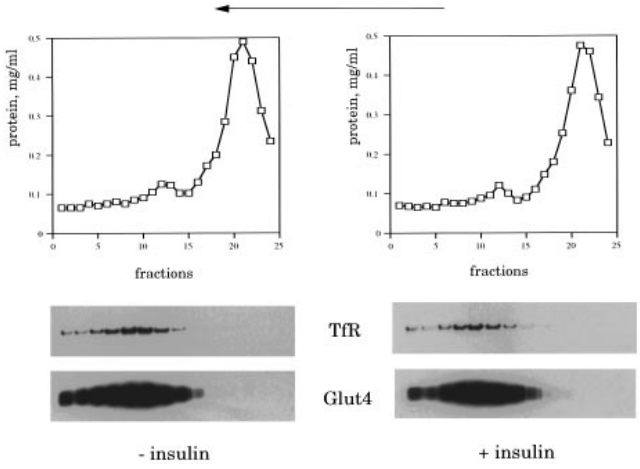

Figure 2 Sedimentation distribution of TfR and Glut4-containing intracellular microsomes in sucrose gradients

LMs (0.2 mg of protein) from insulin-treated and untreated adipocytes were fractionated in a 10–30%-sucrose gradient as described in the Materials and methods section. The horizontal arrow shows the direction of sedimentation. After centrifugation and fractionation, odd-numbered fractions were analysed for Glut4 and TfR by Western blotting.

Since TfR demonstrates a similar type of insulin-induced plasma-membrane recruitment as does Glut4, we further studied the potential co-localization of these two proteins by centrifugation in a sucrose gradient. Figure 2 shows that after adipocyte homogenization and subcellular fractionation, intracellular pools of the TfR and Glut4 overlap almost completely, with both proteins being present in homogeneous membrane vesicles with the sedimentation coefficient of 100–120 S (see also [30]). It is noteworthy that both the TfR receptor and Glut4-containing microsomes are very well separated from the bulk of microsomal proteins. Interestingly, all known recycling proteins in rat adipocytes are found in microsomal particles which have essentially the same sedimentational and buoyant-density characteristics. This is true even for those proteins which have been shown to be structurally segregated from Glut4, such as Glut1 [14,30] and caveolin [31]. Knowing this, we studied the potential co-localization of TfR and Glut4 by immunoadsorption.

Figure 3 demonstrates the result of a representative experiment in which we specifically immunoadsorbed 87% of Glut4-containing membranes with anti-transporter monoclonal antibody coupled to acrylic beads without any noticeable adsorption of Glut4 by non-specific IgG-coupled beads. About 97–98% of the

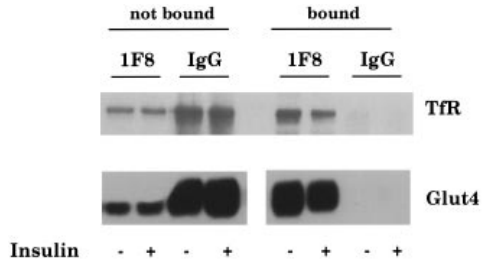

Figure 3 TfR is localized in Glut4-containing vesicles

LM (0.5 mg each) from insulin-treated and untreated cells were immunoadsorbed with 150 μl of 1F8- or IgG-coupled beads. The distribution of the TfR protein and Glut4 in supernatant and eluate from 1F8-beads and non-specific IgG was determined by Western blotting and quantified by computing densitometry of autoradiograms. This experiment was repeated three times with virtually similar results. Material immunoadsorbed with 1F8-beads from both basal and insulin-stimulated adipocytes contains 90 ± 5% of Glut4 and 58 ± 7% of the TfR present in LMs, with no detectable signals in the eluate from non-specific IgG. A representative blot from three independent experiments is shown.

total membrane protein was recovered in the supernatant of the immunoadsorption and the washing fractions (not shown; see also [14]), which is consistent with the results of the sucrose-gradient centrifugation (Figure 2) and suggests that Glut4 is present in a very limited membrane population (see also [30]). Under these conditions, we are able to bring down 61% of the total intracellular population of TfR. As expected, no TfR is immunoadsorbed with non-specific antibodies (lanes marked IgG). Moreover, we were able to identify a TfR band on silver-stained gels after electrophoretical separation of immunoadsorbed Glut4-containing membranes or vesicles (Figure 4). The overall polypeptide composition of Glut4-containing vesicles

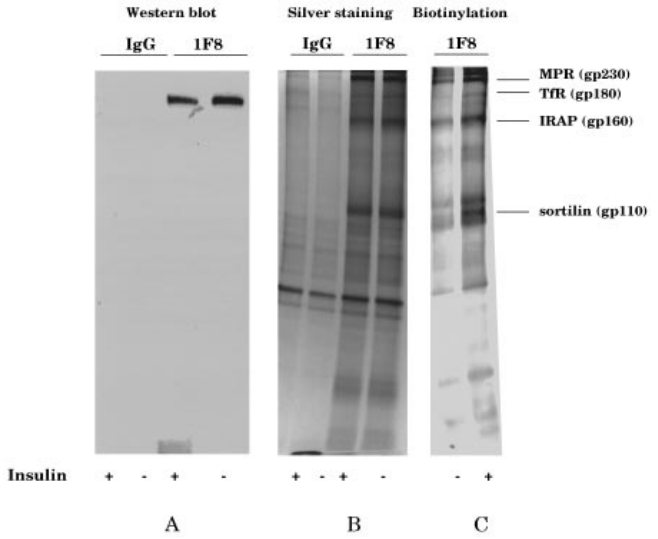

Figure 4 Identification of TfR among the component proteins of Glut4-containing vesicles

Rat adipocytes were biotinylated in the absence and in the presence of insulin as described in the Materials and methods section. LM (0.7 mg each) from these cells was immunoadsorbed with 150 μl of 1F8-beads or IgG-beads and eluted with 1% Triton X-100. The eluate was divided into three parts and electrophoresed. (**A**) Western blot with anti-TfR antibodies; (**B**) silver staining of the gel; (**C**) Western blot stained with streptavidin–alkaline phosphatase conjugate for biotinylated proteins.

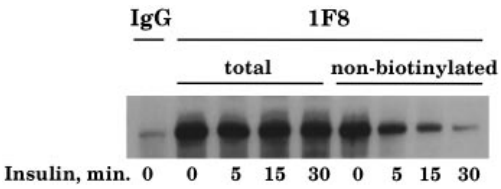

Figure 5 Separation of biotinylated and non-biotinylated pools of TfR present in Glut4-containing vesicles

Rat adipocytes were incubated with insulin and sulfo-NHS-biotin for 0, 5, 15 and 30 min, and LM (0.4 mg each) from these cells was immunoadsorbed with 100 μl of 1F8-beads or IgG-beads and eluted with 1% Triton X-100. The eluate was divided into two halves. The first half was left for control analysis; the second was passed over 20 μl of streptavidin–agarose (Pierce) overnight at 4 °C. Material not bound to streptavidin–agarose (non-biotinylated) was analysed along with control samples (total) by Western blotting with anti-TfR antibodies.

Table 1 Recovery of ^{125}I-transferrin in subcellular fractions of rat adipocytes

^{125}I-transferrin (0.3 nM) alone or together with 75 nM of non-radioactive transferrin was added to cells which were then exposed (+) or not (−) to insulin in immediate succession. After incubation, cells were washed and fractionated into plasma membrane, LM and cytosol, and radioactivity and protein content were measured in these fractions. The results of non-specific binding (not shown) were subtracted from the experimental values. The results are representative of two independent experiments.

Fraction	Insulin	Total ^{125}I-transferrin bound (fmol)	Specific binding of ^{125}I-transferrin (fmol/mg)
Plasma membrane	−	7.28	13.91
	+	15.91	29.04
LM	−	6.02	7.22
	+	9.62	18.39

Table 2 ^{125}I-transferrin is internalized via Glut4-containing vesicles

A portion of the LM fraction (0.12 mg each), obtained as in the experiment shown in Table 1, was immunoadsorbed with 100 μl of 1F8- or IgG-beads, and total radioactivity was counted in supernatants and eluates of 1F8-coupled (specific) and IgG-coupled (non specific) beads. The results are representative of two independent experiments.

Fraction	Insulin	Total ^{125}I-transferrin recovered (fmol)
LM	−	0.87
	+	2.21
1F8-bead supernatant	−	0.61
	+	1.37
1F8-bead eluate	−	0.14
	+	0.73
IgG-bead supernatant	−	0.83
	+	2.08
IgG-bead eluate	−	0.01
	+	0.04

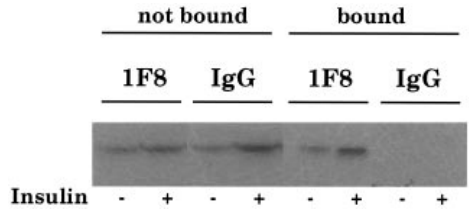

Figure 6 ^{125}I-transferrin is internalized into Glut4-containing vesicles

Cells were incubated with ^{125}I-transferrin as described in the Materials and methods section and then exposed to insulin (+) or not (−). After incubation, cells were washed and LM fractions (0.12 mg each) were immunoadsorbed with 100 μl of 1F8- or IgG-beads. The distribution of the ^{125}I-transferrin in supernatant and eluate from 1F8-beads and non-specific IgG was determined by electrophoresis and autoradiography.

appears to stay the same in both the absence and presence of insulin (Figure 4), although the amount of the material recovered in the latter case is decreased, owing to fusion of vesicles with the plasma membrane. TfR represents a relatively minor component of Glut4-containing vesicles in comparison with their major proteins, such as the IGF-II/Man 6-*P* receptor, IRAP and sortilin, which is most probably explained by the low level of TfR expression in fully differentiated adipocytes [42]. The number of glucose-transporter molecules as measured in [9] is calculated to be more than a 1000-fold greater than the number of TfRs as measured in [42].

In previous experiments we have labelled insulin-sensitive recycling proteins in adipocytes by cell-surface biotinylation [16]. As is seen in Figure 4(C), all specific high-M_r component proteins of Glut4-containing vesicles, including TfR, are biotinylated with a cell-impermeable reagent, sulfo-NHS-biotin, in an insulin-dependent fashion and, thus, recycle between this compartment and the cell surface in response to insulin. However, in agreement with the results of silver staining, biotin labelling of TfR, although detectable at its maximum time point, is still very low in comparison with biotinylation of the major vesicle component proteins, almost certainly because of the low abundancy of this receptor, as noted above. On the other hand, Western-blotting analysis of TfR is very sensitive (Figure 4A). Thus, in order to monitor transferrin receptor trafficking more closely, we undertook the following approach. Rat adipocytes were incubated with insulin and sulfo-NHS-biotin for different times, and then Glut4-containing vesicles were immunoadsorbed and solubilized in 1% Triton as described in the Materials and methods section. This material was divided into two and the first half was subjected to SDS/PAGE without further treatment. The second half was passed over streptavidin–agarose in order to separate biotinylated and non-biotinylated proteins present in Glut4 vesicles. Material not bound to streptavidin–agarose was analysed together with the first (control) half of the sample by Western blotting with specific anti-TfR monoclonal antibody and a chemiluminescence detection system (Figure 5). Note, however, that although all biotinylated proteins quantitatively bind to streptavidin–agarose (results not shown), it is not possible to elute them from this resin for quantitative analysis owing to the SDS-stable nature of biotin–streptavidin binding.

As seen in Figure 5, the amount of non-biotinylated TfR in Glut4-containing vesicles recovered in the streptavidin–agarose supernatant linearly decreases with time in such a way that, in 30 min, practically every molecule of TfR in Glut4-vesicles completes at least one round of turnover to the cell surface, and only 4% of the receptor remains non-biotinylated. These results support the data shown in Figure 4(C) and suggest that Glut4 vesicles deliver TfR to the surface of adipocytes along with their other 'cargo' proteins (Glut4, IRAP, and IGF-II/Man 6-*P* receptor, and, probably, sortilin).

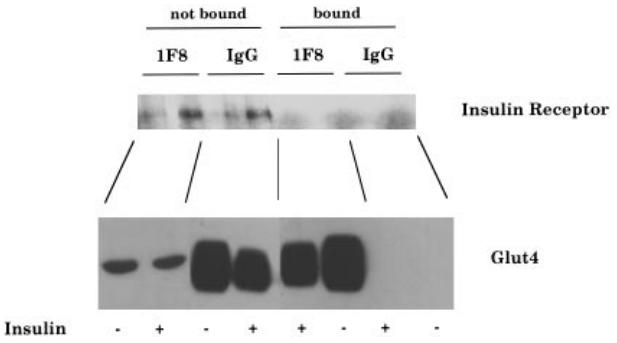

Figure 7 Insulin receptor protein is excluded from Glut4-containing vesicles

LM (0.5 mg each) from insulin-treated and untreated cells was immunoadsorbed with 150 μl of 1F8-beads. The distribution of the insulin-receptor protein and Glut4 in supernatant and eluate from 1F8- or IgG-coupled beads was determined by Western blotting. Equal volumes of supernatant and eluate were loaded on the gel. A representative blot from three independent experiments is shown.

Table 3 ^{125}I-insulin-binding activity is excluded from Glut4-containing vesicles

Fractions of LM (0.2 mg each, 0.44 mg/ml) from insulin-treated and untreated cells were immunoadsorbed with 140 μl of 1F8- or IgG-beads. ^{125}I-insulin-binding activity was determined in the original LM, supernatant, and eluate from 1F8-beads and non-specific IgG-beads (0.45 ml each fraction) as described in the Materials and methods section. The results of non-specific binding (not shown) were subtracted from the experimental values.

Fraction	Insulin	^{125}I-insulin bound (fmol/20 μl)
LM	−	0.18 ± 0.08
	+	0.34 ± 0.06
1F8-bead supernatant	−	0.10 ± 0.02
	+	0.36 ± 0.09
1F8-bead eluate	−	−0.03 ± 0.04
	+	0.07 ± 0.08
IgG-bead eluate	−	−0.05 ± 0.08
	+	0.09 ± 0.07

Next, we exposed rat adipocytes to radioactive transferrin in the absence and in the presence of insulin and prepared subcellular fractions of these cells. A 15 min exposure to insulin increases the amount of ^{125}I-transferrin both in the plasma-membrane fraction and in intracellular LM due to stimulation of the receptor recycling (Table 1). This result is consistent with the previously published data [42,43] as well as with our present findings (Figures 1, 4 and 5). When we immunoadsorbed Glut4-containing vesicles from the LM fraction of insulin-treated and untreated cells, we found that a large portion of the endocytosed radioactive transferrin (35% in the presence of insulin) is recovered in Glut4-containing vesicles (Table 2 and Figure 6). Considering that the latter comprise not more than 2–3% of LM, the specific ^{125}I-transferrin content in Glut4-containing vesicles is much higher than in the overall LM fraction.

Insulin receptor and the Glut4 intracellular trafficking pathway

The dynamics of insulin-receptor endocytosis and signalling in rat adipocytes have recently been studied in detail [36]. It has been shown, in particular, that 8–16 min after insulin administration, a maximal amount of the receptor ($\sim$ 50% of the total) is internalized from the plasma membrane into LM, the heterogeneous membrane fraction that also contains half the Glut4 at this time [36]. However, immunoadsorption experiments (Figure 7) show that the insulin receptor is largely excluded from the Glut4-containing compartment. This result has been confirmed by an independent method of ^{125}I-insulin binding, which also allows us to determine quantitatively the amount of the receptor in fractions (Table 3). Thus, by two independent approaches, no co-localization has been detected between intracellular insulin receptors and Glut4 in the LM fraction. This result is consistent with some early observations about the different sedimentation characteristics of intracellular membrane vesicles which contain internalized insulin and glucose-transporting activity [44,45].

DISCUSSION

Most or all of the abundant proteins present in Glut4-containing intracellular membrane vesicles from rat adipose cells, i.e., those proteins most prominent by the criteria of silver staining, have been identified in the last 2–3 years. It has been shown that these include all the acutely recycling population of the IGF-II/Man 6-*P* receptor [22], a significant fraction of the newly identified [23] protein sorting receptor sortilin [24], and more than half of the intracellular TfR pool (the present study). Thus Glut4 naturally expressed in mature rat adipose cells (the present study), and Glut4 expressed upon cDNA transfection in pre-adipocytes and fibroblasts [12,13], is targetted to an intracellular compartment enriched with well-known endosomal markers. This suggests that a developmental process may be the way by which the insulin-dependent 'Glut4 pathway' is formed in adipocytes. These cells differentiate from fibroblast-like pre-adipocytes *in vivo* and *in vitro* (for a recent review, see [46]). Preadipocytes do not express Glut4, but they express the receptors for IGF-II/Man 6-*P* and transferrin which constitutively recycle via a normal endocytic pathway. It appears that Glut4, which is induced relatively late in the differentiation program, is targeted to the same membrane compartment as is the majority of the cell-surface recycling population of these two receptors.

It has been shown, however, that incorporation of Glut4, by itself, does not confer insulin-responsiveness to these endosomes [12,13]. Thus it is still an open question as to how constitutively recycling endosomes acquire insulin-sensitivity. We hypothesize that there must be tissue-specific molecules, other than Glut4, which are induced during differentiation and confer insulin-sensitivity to the compartment. Yang et al. [47] have shown that, early in the fat-cell-differentiation program before Glut4 is expressed, formation of the insulin-sensitive pool of Glut1 glucose transporters requires their intracellular sequestration. This may be achieved by the anchoring of Glut4 (and Glut1)-containing vesicles inside the cell under basal conditions. Indirect support for this anchoring hypothesis derives from recent data showing that the introduction of the cytoplasmic portion of IRAP [48] and Glut4 [49] causes Glut4 translocation to the plasma membrane, presumably as a result of competing with the endogenous proteins for the putative 'anchor'. Under normal circumstances, insulin administration may cause vesicle translocation by phosphorylation of the 'anchor', for example, thus disassociating it from IRAP and/or Glut4 and allowing default fusion of the vesicle with the plasma membrane.

Our data complement previously published studies showing

that insulin stimulates transferrin binding and uptake in fat-cells [42,43], and extend them in several important ways. Thus, we show directly by Western blotting that TfR is redistributed between intracellular membranes and the plasma membrane in response to insulin (Figure 1). Moreover, we show that, in the absence of insulin, $> 50\%$ of the intracellular pool of this receptor co-localizes with Glut4 (Figures 2 and 3). Finally, we show, by cell-surface biotinylation followed by immunoadsorption of Glut4-containing vesicles, that the biotinylation of TfR is stimulated by insulin in a time-dependent fashion (Figures 4 and 5) (see also [16]). The simplest interpretation of these data is that Glut4 and TfRs are co-localized in the basal state and recycle together to and from the same compartment. However, we cannot completely rule out that there exists some very fast sorting step that separates Glut4 into a specialized, insulin-responsive vesicular compartment distinct from that containing these other proteins.

The existence of such a specialized compartment has been suggested by recent studies employing a compartment ablation technique using TfR conjugates which results in the ablation of only 40% of the Glut4, but all of the TfR-containing vesicles [27,33]. The authors of these studies concluded that a part of Glut4 pool (60% of total) may reside in a specialized 'unique' compartment segregated from the endosomal system and, therefore, traffics separately from the receptors for transferrin and IGF-II/Man 6-*P*. We offer an alternative explanation for these experiments. Because the level of the TfR expression in adipocytes is low in comparison with the IGF-II/Man 6-*P* receptor, IRAP and Glut4 (Figure 4), some Glut4 vesicles may simply lack this receptor. In fact, it has been previously shown that, in 3T3-L1 adipocytes, only one glucose-transporter-containing vesicle in three contains a TfR [50]. Although anti-Glut1 antibody was used in the present study in order to immunoisolate glucose-transporter-containing vesicles, it is known that in 3T3-L1 adipocytes, Glut1 and Glut4 are co-localized in the same vesicles [51]. Thus, the result that only 40% of intracellular Glut4 is ablated with a transferrin–horseradish peroxidase conjugate [27,33] is in very good agreement with the predicted 33% [50]. In any case, we employ rat fat-cells, whereas the above-cited studies [27,33] used cultured murine fat-cells, and the latter are generally thought to be less phenotypically mature than the former, in part because they express more Glut1 and Glut4 [51]. Thus, the different experimental systems may also explain the differing results.

A second important point our data raises is the apparent existence of separate endosomal compartments for the insulin receptor, on the one hand, and for the transferrin and IGF-II/Man 6-*P* receptors, the 'Glut4 pathway', on the other hand. Previous studies employing sucrose gradients had suggested that there were some differences between insulin-receptor-containing and glucose-transporter-containing compartments [44,45], but they could not rule out a complete separation of vesicles containing these two proteins, as we do here (Figure 7 and Table 3). This hypothesis is also consistent with the recent results of Warren et al. [52], who demonstrated that saturation of the endocytic pathway for TfR in HeLa cells does not affect the endocytosis of the epidermal-growth-factor receptor which may utilize a ligand-dependent endocytic pathway similar to, or identical with, that of the insulin receptor. Several studies using microscopy techniques have suggested, however, the existence of a single endosomal sorting compartment in fibroblasts [53] and hepatoma cell lines [54], although these cells lack the Glut4 compartment. Thus, our current efforts are focused on characterization of insulin-receptor-containing endosomes in order to determine how they may differ from these containing Glut4.

This work was supported by grants from the National Institutes of Health (R01DK52057 to K.V.K. and R01DK30425 and R01DK36424 to P.F.P.), by research grants 195054 and 197029 from the Juvenile Diabetes Foundation to K.V.K., and by a research grant from the American Diabetes Association to K.V.K.

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Received 19 September 1997/9 January 1998; accepted 12 February 1998