Abstract. Antibodies specific for the insulin-regulatable glucose transporter (GLUT 4) were used to immunolocalize this protein in brown adipose tissue from basal- and insulin-treated rats. Cryosections of fixed tissue were incubated with antibodies, which were subsequently labeled with Protein A/gold and examined by EM. Antibodies against albumin and cathepsin D were also used with gold particles of different sizes to identify early and late endosomes, respectively. Under basal conditions 99% of the GLUT 4 labeling was located within the cell. Labeling was predominantly in the trans-Golgi reticulum and tubulo-vesicular structures elsewhere in the cytoplasm. In insulin-stimulated cells ~40% of the GLUT 4 labeling was at the cell surface, where it was randomly distributed, except for occasional clustering in coated pits. Moreover, after insulin treatment, GLUT 4 was also enriched in early endosomes. We conclude that translocation of GLUT 4 to the cell surface is the major mechanism by which insulin increases glucose transport. In addition, these results suggest that in the presence of insulin GLUT 4 recycles from the cell surface, probably via the coated pit-endosome pathway that has been characterized for cell surface receptors, and also that insulin causes the redistribution of GLUT 4 by stimulating exocytosis from GLUT 4-containing tubulo-vesicular structures, rather than by slowing endocytosis of GLUT 4.

Glucose is a fundamental energy-yielding substrate for most mammalian cells. Specific transporters facilitate the entry of glucose into cells in an energy-independent fashion. Both chronic and acute regulation of glucose transport have been described. Hormonal and nutritional factors regulate the expression of glucose transporters in various cell types (Garvey et al., 1989; Kahn et al., 1989; Sivitz et al., 1989; Tordjman et al., 1989; Bourre et al., 1990). Alternatively, in muscle and fat cells glucose transport can be modified on a minute by minute basis in a process that involves the movement of transporter proteins from an intracellular domain to the cell surface (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). This regulation is mediated by various extracellular stimuli, notably insulin, and does not require protein synthesis.

Several mammalian glucose transporter species have been identified by cloning and sequencing (reviewed in Gould and Bell, 1990). There is considerable amino acid sequence homology between these proteins, and structurally they are very similar. Aside from sequence differences, the tissue distribution of each transporter is one of the most distinguishing features. The five members of this family that have been described are: the HepG2/erythrocyte type transporter (GLUT 1), which is expressed in many tissues including brain and muscle; the insulin-regulatable glucose transporter (GLUT 4), which is expressed in muscle and fat (Birnbaum, 1989; James et al., 1989; Charron et al., 1989; Kaestner et al., 1989); and GLUT 5, which is expressed primarily in the jejunum (Kayano et al., 1990).

GLUT 4 is of particular interest because its expression is confined to fat and muscle, the tissues in which insulin-stimulated glucose transport occurs (James et al., 1989; James et al., 1986a,b). Moreover, it appears to be the major glucose transporter isoform expressed in these cells (Zorzano et al., 1989; Calderhead et al., 1990a,b). Insulin causes the rapid translocation of GLUT 4 from intracellular sites to the plasma membrane in fat and muscle cells (James et al., 1988; James et al., 1989; Birnbaum, 1989; Hirshman et al., 1990; Douen et al., 1990). The increase in GLUT 4 in the plasma membrane of rat white adipocytes, as assessed by immunoblots of GLUT 4 in plasma membranes isolated from basal and insulin-treated cells, accounts for a significant portion of the increase in the rate of glucose transport in response to insulin (James et al., 1989; Birnbaum, 1989; Zorzano et al., 1989).

An understanding of the insulin-stimulated translocation of GLUT 4 requires knowledge of the cellular organelles involved in the storage and trafficking of GLUT 4. In the pres-
ent study we have used immunoelectron microscopy to determine the cellular distribution of GLUT 4 in brown adipose tissue obtained from rats in the basal and insulin-treated states. Brown adipose tissue was chosen for these studies for several reasons. Insulin stimulates glucose used in treated states. Brown adipose tissue was chosen for these studies for several reasons. Insulin stimulates glucose transport in brown adipocytes, and they provide evidence that this multi-trafficked, such that the amount of GLUT 4 at the plasma membrane, such that the amount of GLUT 4 at the

tissue surface increases by at least 40-fold; moreover, GLUT 4 is seen to enter the coated pit/coated vesicle/early endosome pathway typical for receptor-mediated endocytosis. These findings indicate that translocation of GLUT 4 is the major mechanism by which insulin stimulates glucose transport in brown adipocytes, and they provide evidence that this multi-spanning membrane protein can recycle by the same route as receptors. The data support the hypothesis that insulin increases GLUT 4 levels at the cell surface by triggering its exocytic movement, rather than by inhibiting endocytosis of GLUT 4.

Materials and Methods

Animals

Male Wistar rats (wt ~100 g) were fasted overnight with free access to water. The next morning the animals were maintained in lattice-bottomed cages at 4°C for varying times up to 8 h. For insulin treatment, animals were injected intraperitoneally 20–50 min before fixation with PBS containing glucose (1 g/kg) and insulin (8 U/kg). For fixation, animals were anesthetized with Nembutal (90 mg/kg i.p.) and perfused, within 4 min after the injection, through the left ventricle over a 30-s period with PBS containing 10 U/ml heparin to flush blood out of the tissues. This flush was followed by a perfusion of fixative (2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for 5 min. Brown adipose tissue (BAT) was collected at the hilus side of the kidneys. It is noteworthy that we also sampled interscapular BAT and found the distribution of GLUT 4 to be identical to that observed in renal BAT (data not shown).

Tissue Processing and Cryosectioning

Fixation was prolonged by immersing the tissue samples for 1–2 h in the perfusion fixative. Tissues were stored in 2% paraformaldehyde in PBS at 4°C. Ultrarichotomy was performed using a slightly modified Tokuyasu procedure (Tokuyasu, 1980), as described previously (Slot et al., 1988). Tissue fragments were washed in PBS for a few minutes and immersed in 2.3 M sucrose in PBS for at least 1 h. Tissue blocks were mounted on specimen holders and frozen in liquid nitrogen. Thin (100 nm) sections for EM and semi-thin sections (200 nm) for light microscopy (LM) were cut at ~100°C using a Reichert/LKB Nova-cryo Nova system with a diamond knife prepared for ultrarichotomy (Diatome, Ltd., Bienne, Switzerland). Sections were picked up, thawed on the surface of a 2.3 M sucrose drop, and transferrable to formvar–carbon–coated copper grids for EM, or to gelatin-coated glass slides for LM. These sections were subsequently immunolabeled (see below). EM sections were then washed four times by floating on drops of distilled water for 1 min. Sections were stained for 5 min on a drop of uranyl acetate–oxalate, pH 7, washed on distilled water again, and finally flooded for 10 min on drops of 1.8% methyl cellulose (25% centipoise; Fluka AG, Buchs, Switzerland) containing 0.3% uranyl acetate at 4°C. The sections were taken up in a wire loop (3-mm diam), excess methyl cellulose was sucked into filter paper, and grids were dried before taking them from the loop. The sections were studied and photographed in an electron microscope (either Model 1200 EX; JEOL, USA, Electron Optics Div., Peabody, MA; or model 301; Philips Electronic Instruments, Inc., Mahwah, NJ). LM sections, after immunolabeling, were embedded under cover glasses in polymethyl methacrylate (Polysciences, Warrington, PA) and photographed under a polychromatic microscope (Reichert Jung S.A., Paris) in bright field and Nomarski interference contrast.

Immunolabeling

Three antibody preparations were used to localize GLUT 4: the mAb 1F8, which was initially used to detect GLUT 4 in fat and muscle tissue (James et al., 1988) and for further characterization of GLUT 4 (James et al., 1989); the IgG fraction of a rabbit antisera raised against the carboxy-terminal peptide encompassing the last 12 amino acids of GLUT 4 (anti-GLUT 4b) (James et al., 1989); affinity purified antibodies isolated from a rabbit antisem against the carboxy-terminal peptide consisting of the last 119 amino acids of GLUT 4 (anti-GLUT 4a) (Calderhead et al., 1990a). The IgG fraction of a rabbit antisem against rat albumin was prepared as described previously (Brands et al., 1983). The IgG fractions from rabbit antisera against cathepsin D and mouse IgG were gifts from Drs. Andresj Hasilik, Medical School, University Münster, Germany and Jannie Borst, Netherlands Cancer Institute, Amsterdam, respectively. Antibodies were used at a concentration of ~5 µg/ml in PBS containing 1% BSA. Gold markers of various sizes were prepared by tannic acid-citrate reduction (Slot and Geuze, 1985) and coupled to protein A (Rothe et al., 1978; Slot et al., 1988). Markers with diameters of 6, 9, or 14 nm were diluted in PBS/1% BSA to OD values at 520 nm of 0.1, 0.2 or 0.3, respectively, shortly before use.

For EM immunolabeling, grids were floated at room temperature on drops of the following solutions successively: (a) 2% gelatin in PBS (10 min); (b) PBS/0.02% glycine (10 min); (c) PBS/1% BSA (5 min); (d) antibody against GLUT 4 (30–60 min); (e) PBS/1% BSA (5 × 1 min); (f) only after the use of mAb 1F8 in step d, rabbit anti-mouse IgG (30 min) and 5 washes in PBS/1% BSA again; (g) protein A-gold (30 min); (h) PBS/1% BSA (4 × 5 min). In our initial double-labeling experiments this procedure was followed by a 5-min incubation in excess protein A (50 µg/ml) and a repetition of steps (d), (e), (g), and (h), except that anti-albumin was used this time in step (d) and a gold marker of different size was used in step (g). In contrast to the good results we had with this double-labeling procedure in previous studies (Geuze et al., 1981, 1983, 1988), we found a serious reduction, and to some extent, a redistribution of GLUT 4 labeling (see Fig. 5 D). This problem was solved by a simple modification of the procedure. Instead of the incubation with excess protein A the singly labeled sections were put successively through PBS, 1% glutaraldehyde in PBS (10 min), PBS, PBS/0.02% glycine, and PBS/1% BSA before starting the anti-albumin incubation. We usually combined a 9-nm gold marker for GLUT 4 with a 6-nm one for albumin. Triple labeling was performed by inserting the labeling for cathepsin D, marked by 14-nm protein A/gold, between the GLUT 4 and albumin (6 nm) labeling. In this case glutaraldehyde stabilization was done at two stages, after the gold marking of anti-GLUT 4 and after the marking of anti-cathepsin D. The glutaraldehyde treatment prevented any interference between the different antibody–gold complexes on the section (Fig. 6 B).

LM immunolabeling was similar to the above single labeling procedure for EM. After the labeling the glass slides were washed with distilled water, and silver enhancement was performed as described (Holgate et al., 1983; Slot et al., 1988).

Tissue Structure

For structural studies the tissue was perfusion fixed in 2% paraformaldehyde/2.5% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.4 after a 30-s flush with 10 mM Hepes/150 mM NaCl/2 mM CaCl2, pH 7.4. The fixation was continued by immersion of the tissue in the same fixative (1 h) and in 1% OsO4 (1 h). Then tissue fragments were dehydrated in ethanol and embedded in Epon. For LM observations, semi-thin sections were stained with methylene blue, and for EM, ultrathin sections were briefly stained with uranyl acetate and lead citrate.

Results

Morphology of Brown Adipose Tissue

The ultrastructure of brown adipose tissue (BAT) has been characterised. Animals were perfused, either in vivo or in vitro, with fixatives such as glutaraldehyde or formaldehyde, followed by dehydration and embedding in plastic or Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope. The ultrastructure of BAT is characterized by the presence of numerous lipid-filled vacuoles, which are the hallmark of brown adipocytes. The cytoplasm of brown adipocytes contains numerous mitochondria, which are the site of fatty acid oxidation. The peroxisomes, which are involved in beta-oxidation, are also abundant. The Golgi apparatus is well developed, and the endoplasmic reticulum is extensively developed. The plasma membrane is rich in GLUT4, which is responsible for glucose uptake in brown adipose tissue.
Figure 1. LM (A and B) and EM (C and D) micrographs of Epon-embedded BAT. (A) Tissue from an overnight fasted rat. The adipocytes contain many lipid droplets (li) with nuclei (n) squeezed in between them. (C) Blood capillaries. (B) Tissue from an overnight fasted rat subsequently maintained at 4°C for 5 h. As shown, this treatment results in a decrease in lipid levels. The numerous small dots in the cytoplasm are mitochondria. (C and D) EM views of BAT from a fasted and cold-treated rat. (C) Adipocyte at low magnification. Lipid stores are reduced to small droplets (arrows) in the cytoplasm, which is studded with mitochondria, except for some mitochondrion-free areas. These areas are often found in the periphery of the cell (arrowheads). (D) Mitochondrion-free area with Golgi elements (g), dense lysosomes (l), and vesicles or vacuoles of various sizes. Bars, (A and B) 10 μm; (C) 5 μm; (D) 0.5 μm.
Figure 2. Light micrographs of semi-thin cryosections of BAT labeled with mAb 1F8. Bright-field (A and C) and interference-contrast images (B and D) are shown of the same fields. Under basal conditions (A) apart from a diffuse labeling, pronounced positive areas occur in the adipocytes, often situated close to the cell border (arrows), but overall labeling of the cell surface is absent. These GLUT 4-positive areas appear less abundant in insulin-stimulated tissue (C), which may reflect a decrease in labeling of these regions. In addition, because of their location with respect to the cell surface it is now much harder to distinguish these regions from the general labeling which is now clearly present at the surface of adipocytes, where they border each other (small arrowheads) as well as where they face endothelial cells (double arrowheads). Endothelial cells are not labeled (large arrowheads). c, Blood capillaries. Bar, 10 μm.

described previously and is reviewed by Afzelius (1970). Two aspects that dominate the cytoplasm are the extensive multilocular fat storage (Fig. 1 A) and the numerous mitochondria (Fig. 1, B and C) with well-defined cristae (Fig. 1 D). These properties clearly distinguish brown fat cells from white fat cells, in which mitochondria are relatively scarce and there is one large lipid droplet which occupies about 90% of the cytoplasm. BAT is so densely vascularized (Fig. 1, A–C) that each adipocyte is in contact with at least two capillaries and one third of their surface faces the endothelium (Aherne and Hull, 1966). The fat deposits that were present in tissue from a fed animal made it very difficult to deal with BAT in ultracryotomy. Thus, to improve the quality of tissue sections, animals were both fasted and briefly cold treated to reduce the mass of intracellular lipid. Each of these treatments independently did not induce a significant decrease in lipid mass, whereas if rats were fasted overnight and then incubated at 4°C for 4–5 h, there was a dramatic reduction in lipid mass (Fig. 1 B). These conditions did not affect the morphology of the tissue other than the reduced lipid storage.
nor did they induce apparent changes in the distribution of GLUT 4 (data not shown). Thus, all studies shown here were conducted using these experimental conditions.

Brown adipocytes had a central nucleus and were studded with mitochondria (Fig. 1 C). Some areas just beneath the cell surface were devoid of mitochondria. These areas (Fig. 1 D) contained elements of the Golgi complex and dense structures which were identified as lysosomes using cathepsin D as a marker (see Fig. 6, A-C). In addition, a heterogeneous population of tubules and vesicles were present in these areas. These will be discussed below in relation to GLUT 4 distribution. Most of the EM data reported here focus on these mitochondrial-free areas because GLUT 4 is concentrated within these regions. Small invaginations (~50 nm), apparently representing caveolae, were often observed at the cell surface. Occasionally larger invaginations (>100 nm) occurred, sometimes with typical furcated appearance (see Fig. 6 A). Coated pits and vesicles were also present. No obvious ultrastructural changes were observed in cells after insulin administration.

**Antibodies against GLUT 4**

The GLUT 4 specific antibodies that are used in these experiments have been described in detail previously (James et al., 1988, 1989; Calderhead et al., 1990a). These antibodies are specific for GLUT 4 with no detectable cross reactivity with other transporter isoforms, each of which has a different carboxy-terminal sequence (Kayano et al., 1990). We obtained a similar immunolabeling pattern at the LM and EM level using each of these antibodies (Slot et al., 1990). Significant labeling of the transporter in thin sections with mAb 1F8 (Fig. 2) was only obtained after amplification with the rabbit anti-mouse IgG (see Materials and Methods). However, this procedure renders this antibody less useful for EM studies (Slot et al., 1988). In addition, mAb 1F8 gave some spurious side reactions (Slot et al., 1990). Thus, to avoid these problems, most of the experiments described here, except for those shown in Fig. 2, used the anti-peptide antibodies. In general, the anti-GLUT 4\textsubscript{i} antibody gave stronger labeling than the anti-GLUT 4\textsubscript{a} antibody. Therefore, we have used the anti-GLUT\textsubscript{i} antibody for each of the EM illustrations shown. In quantitative experiments we used the anti-GLUT 4\textsubscript{i} antibody (Table I) and anti-GLUT 4\textsubscript{a} (see Fig. 7) antibodies. In control thin sections, which were incubated with preimmune serum, or with the antipeptide antibodies saturated with preimmune serum, or with the antipeptide antibodies saturated with the appropriate peptide before use, no significant labeling was observed in the adipocyte (data not shown).

**LM Immunolabeling of GLUT 4**

At the LM level, the immunosilver reaction in basal tissue (Fig. 2, A and B) indicated an accumulation of GLUT 4 in certain areas of the adipocytes which, according to subsequent EM observations (see below), corresponds to the mitochondrial-free cytoplasmic areas. Outside these areas a diffuse punctate reaction was observed in the cells but there was no indication of cell surface labeling. After insulin stimulation (Fig. 2, C and D) the cellular accumulations were less pronounced and the most striking feature was the labeling of the cell surface. GLUT 4 appeared to be present at similar density all along the cell border of the adipocytes, at sites where the cells face other adipocytes as well as where they surround blood capillaries. In agreement with our previous studies (Slot et al., 1990), endothelial cells were not significantly labeled with any one of the three GLUT 4 antibodies used in these studies.

**EM Immunolabeling of GLUT 4**

In one set of experiments, sections of basal and insulin-stimulated BAT were labeled only with antibodies against GLUT 4, and the relative distribution of GLUT 4 within the cell was quantified by counting gold particles (Table I). In adipocytes of basal tissue most of the GLUT 4 labeling was associated with small vesicles and/or tubules (Fig. 3). Most notably, under these conditions the plasma membrane, including coated pits, was virtually devoid of immunoreactive GLUT 4 (~1% of total cell label). The GLUT 4 positive tubulo-vesicular (TV) structures were most abundant in the mitochondrial-free areas, which correspond roughly with the Golgi area as defined in Table I. Approximately 44% of the label was counted in this area. A similar proportion (49%) of the label was counted over TV structures that were scattered throughout the cytoplasm, both close to the cell surface and deep in the cell between mitochondria. Clusters of the small GLUT 4 positive structures were often observed in the mitochondrial-free areas, frequently in the immediate vicinity of vacuoles with diameters up to 1 μm, which were later recognized as endosomes (see below). Sometimes the vacuoles appeared empty, apart from material at the margins (Fig. 3 B), whereas others were filled with flocculent material or membranes giving a multivesicular appearance (Fig. 3 C). Some of the TV elements were associated with the stacks of Golgi cisternae. The stack itself was usually sparsely labeled except for one cisterna at the same side where associated TV structures were found (Fig. 3 A). This region of the cell is analogous to that previously referred to as the trans-Golgi reticulum (TGR) (Geuze et al., 1985, 1987, 1988; Griffiths and Simons, 1986; Blok et al., 1988). The Golgi stacks were often in a position parallel to the cell surface with the TGR directed towards the cell center (Fig. 3).
Figure 4. Electron micrographs of insulin-stimulated BAT. (A) Strong labeling of the adipocyte cell membrane between contiguous adipocytes as well as those facing an endothelial cell (e). (B) A mitochondrion-free area in an adipocyte. Many labeled T-V elements (asterisks) are in close association with early endosomal vacuoles (ee). The plasma membrane is also labeled and labeling of the TGR is still evident. Bar, 200 nm.

A), but other orientations were also observed (Fig. 4 B). Coated vesicles were observed primarily in the mitochondrion-free areas. These were sometimes labeled for GLUT 4.

The most impressive change in the distribution of GLUT 4 after insulin administration was the substantial increase in cell surface labeling. Approximately 40% of total cell labeling was associated with the cell membrane in stimulated adipocytes (Table I). Although there was some variation in

Figure 3. Electron micrographs of cryosections of nonstimulated BAT immunolabeled for GLUT 4. EM Figs. 3–6 are marked identically for cellular structures and are from experiments where anti-GLUT 4 was used for GLUT 4 labeling. (A) Mitochondrion-free areas in two adjacent cells. Labeling of the TGR (large arrows) and disperse (small arrowheads) or clustered (asterisks) T-V elements is shown. Labeling of other structures such as the plasma membrane (large arrowheads), Golgi cisternae (g), lysosomes (l), mitochondria (m), and nucleus (n) is not significant. Occasionally some gold was associated with coated vesicles (double arrowheads). (B) Clusters of GLUT 4-enriched T-V elements near an early endosomal vacuole (ee). (C) Labeling of the TGR and a cluster of T-V elements close to a late endosomal vacuole (le). Bar, 200 nm.
the density of GLUT 4 labeling along the cell surface there was no consistency to this with respect to a particular domain of the membrane. In general, the labeling intensity was similar at the cell surface facing the endothelial cells compared with that next to other adipocytes (Fig. 4 A), and in regions apposing mitochondria (Fig. 4 A) compared with those lining the mitochondrion-free areas (Fig. 4 B). In insulin-stimulated cells GLUT 4 was enriched in some coated pits and coated vesicles (Fig. 5, A and B), suggesting that GLUT 4 participates in the coated vesicle-mediated endocytic pathway in the presence of insulin. However, GLUT 4 was not observed in every coated pit or vesicle. Occasionally a gold particle was associated with caveolae and larger noncoated invaginations of the plasma membrane, but these images never gave the impression that GLUT 4 was enriched in these structures. Nevertheless, we can not exclude the possibility that GLUT 4 is also internalized via these noncoated invaginations. The intracellular distribution of GLUT 4 in cells from insulin-treated rats was essentially the same as that observed in the nonstimulated state. However, quantitation indicated that insulin resulted in a 60 and 32% decrease in labeling of membranous structures in the Golgi area and at other locations, respectively (Table I). One difference in the intracellular distribution of GLUT 4 with insulin was the labeling of endosomal vacuoles that were often associated with GLUT 4 positive vesicles and tubules (Fig. 4 B, see below).

In a second set of experiments sections of BAT were labeled for both GLUT 4 and rat albumin (Fig. 5) or for GLUT 4, rat albumin, and cathepsin D (Fig. 6), and the distribution of GLUT 4 was quantified (Fig. 7). The label for albumin provided clear identification of structures in the endocytic pathway, because albumin is not synthesized in brown fat, as indicated by the absence of Golgi labeling (Fig. 6 B); and the label for cathepsin D permitted identification of prelysosomes and lysosomes. Based upon morphology and immunolabeling for GLUT 4 and/or albumin, two separate classes of endosomes were recognized. The empty vacuoles, that we refer to as early endosomes, were the most common. They exhibited perimetric albumin labeling with a density often less than that observed at the cell surface (Fig. 5, B and C). In insulin-stimulated cells, but not basal cells, they were positive for GLUT 4 and the density of GLUT 4 labeling was often greater than was observed at the cell surface. Continuities were observed between some early endosomes and the small GLUT 4 positive TV structures. Elongated tubular extensions connected to endosomes were more prominent in stimulated (Figs. 4 B, 5, D and E) than in basal cells (Figs. 4 B, C and A). In these complex structures separation seemed to occur between albumin, which was mainly restricted to the vacuolar part, and GLUT 4, which seemed enriched in the tubular part and surrounding vesicular profiles. The second class of endosomes, referred to as late endosomes, contained internal vesicles and flocculant material. The late endosomes seemed to contain more albumin than the early endosomes, which was apparent from a more dense albumin reaction (Fig. 5 C) that was often present in the central part of these vacuoles (Fig. 6 C). There was no significant labeling of the late endosome with the GLUT 4 antibody in either stimulated or nonstimulated cells. Further justification for classifying these two vacuoles as early and late endosomes is based on cathepsin D-labeling studies. This lysosomal protein was never detected in early endosomes; there was a weak reaction in some (Fig. 6 C), but not all (Fig. 6 B) late endosomes, and a strong labeling of lysosomes with dense (Fig. 6, A–C) or mixed (Fig. 6 D) contents.

Relative quantitation of GLUT 4 labeling in the above-mentioned structures (Fig. 7) substantiates our qualitative observations. The data demonstrate that ~80% of the label was associated with the typical small TV structures in the basal state, and this was reduced by 50% in response to insulin. GLUT 4 is translocated from these TV elements at all locations in the cytoplasm (structures 1–5 in Fig. 7), including the TGR.

**Discussion**

In basal adipocytes GLUT 4 was located almost exclusively within the cell (Fig. 7); this finding is consistent with the low glucose utilization rate in BAT under these conditions (James et al., 1986a,b). After insulin administration there was a marked redistribution of GLUT 4, such that the cell surface labeling was increased from 1% in the basal state to ~40% of the total GLUT 4 immunoreactivity in stimulated cells (Table I). Because surface labeling in the basal state was close to background levels, GLUT 4 is probably effectively excluded from the plasma membrane in the absence of stimulation. These data are consistent with quantitation of GLUT 4 in different membrane fractions obtained from white adipocytes incubated in the absence or presence of insulin (James et al., 1989; Birnbaum, 1989). The amount of GLUT 4 in plasma membrane isolated from white adipocytes is increased by about 10-fold with insulin, commensurate with a 50% decrease in the amount recovered in the intracellular fraction. In the present studies, the magnitude of the insulin-dependent increase in GLUT 4 at the membrane is quite striking.

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**Figure 5.** Distribution of GLUT 4 and rat albumin in BAT from an insulin-stimulated rat. GLUT 4 is marked with 10-nm gold and albumin with 5-nm gold. (A) The cell border of an adipocyte facing an endothelial cell (e). Albumin is present in the intercellular space and in a small vesicle (arrow), which is presumably related to noncoated invaginations of the plasma membrane (see also Fig. 6 A). Labeling of GLUT 4 is observed at the cell surface, within a coated pit (see also Fig. 6 B) and a coated vesicle (double arrowhead). (B) Vacuoles of the type referred to as early endosomes, which are labeled for albumin and GLUT 4. (C) Examples of early (ee) and late (le) endosomes. The late endosome contains vesicular and amorphous material. In contrast to the early endosome, there is little if any GLUT 4 labeling of the late endosome whereas albumin labeling is more dense than in the early endosomal vacuoles. Labeling of GLUT 4 but not albumin was observed in TV elements clustered near the vacuoles. The double arrows indicate a putative connection between an early endosomal vacuole and TV elements. Such connections are more clearly depicted in (D) and (E). GLUT 4 seems enriched in tubular extensions and surrounding TV elements compared to the moderately labeled vacuole, which in contrast contains most of the albumin. Labeling of GLUT 4 is low in (D) and seems to be partly replaced by the albumin marker (small arrows). This is because of omission of the glutaraldehyde stabilization during the double-labeling procedure in this case (see Materials and Methods). Bar, 200 nm
Figure 6. Distribution of albumin (5-nm gold), GLUT 4 (10-nm gold), and cathepsin D (15 nm) in nonstimulated BAT. (A) An early endosome (ee) contains albumin but not cathepsin D and is not significantly labeled for GLUT 4. Caveolae (c) and a larger noncoated invagination (arrow) are evident on the cell surface, which is devoid of GLUT 4 labeling. The albumin labeled tubular structure (t) possibly originates from, or is continuous with such invaginations. GLUT 4 positive TV elements are spread underneath the cell surface. Cathepsin D is present in small dense lysosomes. (B) There is no GLUT 4 in the Golgi cisternae (g) but labeling of the TGR, which is not very extended in this case, is obvious. GLUT 4 labeling of disperse TV elements is shown while the plasma membrane and the endosomal vacuole, which in this case is considered to be a late endosome because of its amorphous and vesicular interior, are not labeled. Cathepsin D is not detected in this vacuole. However, it appears abundant in dense, sometimes elongated, lysosomes (l). Another late endosome (le) in C has a small amount of label for cathepsin D. (D) A pre-lysosomal vacuole with mixed contents. The presence of albumin indicates the endocytic origin, whereas its heterogeneous contents together with cathepsin D labeling suggest that it has undergone fusion with an active lysosomal vacuole. A cluster of GLUT 4-positive TV elements can be seen in close vicinity of the vacuole. The quality of the triple labeling is clearly shown in B where the 5-, 10-, and 15-nm markers occur well separated from each other in late endosomes, TGR, and lysosomes, respectively. This low level of contamination between the three reactions could only be achieved using glutaraldehyde stabilization during the labeling procedure (see Materials and Methods). Bar, 200 nm.
Figure 7. Relative quantification of GLUT 4 distribution throughout organelles of BAT from nonstimulated and insulin-stimulated rats. Quantitation was performed as described in Table 1, except that double-labeling experiments were used to obtain more detail and anti-GLUT 4 was used for immunolabeling. The structures denoted on the left are: (1) Golgi region (mainly TGR); (2) T-V elements distributed underneath (within 0.5 μm) plasma membrane; (3) clusters of T-V elements; (4) T-V elements distributed through the cytoplasm; (5) T-V elements connected or close to late endosomal vacuoles (6); (7) T-V elements connected or close to early endosomal vacuoles (8); (9) noncoated invaginations of the plasma membrane; (10) coated pits and vesicles; (11) plasma membrane; (12) cytoplasm. The percent gold associated with each structure is shown. Each value is the mean of three countings of at least 1,600 gold particles in different sections. Shown in parentheses is the standard error.

An effect of this magnitude (40-fold higher than basal) accounts entirely for the 15–30 fold increases in glucose transport that are typically observed in brown and white adipocytes following insulin stimulation (James et al., 1986a, b; Toyoda et al., 1987). This finding is of importance because it has been suggested that insulin may also stimulate glucose transport via a mechanism other than translocation. The data that supports this hypothesis is the observation that following incubation of white adipocytes with insulin the increase in transporters in the isolated plasma membrane fraction is less than the increase in glucose transport (Joost et al., 1988). However, this argument is limited by the possibility that the plasma membrane preparation is contaminated with some intracellular membranes containing GLUT 4. While our present observations do not rigorously rule out alternate mechanisms for insulin stimulation of glucose transport, they certainly do not support this contention.

In the only previous study of the type described here, we examined the distribution of glucose transporters in basal and insulin-treated 3T3-L1 adipocytes (Blok et al., 1988). This cell type contains both GLUT 1 and GLUT 4, in the ratio of 3.4 to 1 (GLUT 1/GLUT 4) (Calderhead et al., 1990a). The affinity-purified antibodies used in this earlier study were raised against GLUT 1 purified from human erythrocytes. We have since established that these antibodies react 10 times more strongly with GLUT 1 than GLUT 4 on immunoblots (Kitagawa, K., and G. E. Lienhard, unpublished results). Because of this specificity and the preponderance of GLUT 1 in 3T3-L1 adipocytes, the GLUT isotype examined in this earlier study was almost certainly largely GLUT 1. Consistent with this conclusion is the finding that insulin causes a much less marked translocation of GLUT 1; the proportion in the plasma membrane rises from 15% in basal 3T3-L1 adipocytes to 43% in insulin-treated cells.

The virtual exclusion of GLUT 4 from the membrane in the absence of insulin is critical to the regulation of this protein. This distribution is in marked contrast to the distribution reported for other transporter isoforms under basal conditions. Besides the 15% of GLUT 1 on the surface of basal 3T3-L1 adipocytes noted above, in nonstimulated Hep G2 cells (unpublished data) and in brain endothelial cells (Gerhart et al., 1989) GLUT 1 is primarily on the cell surface. The same is true for GLUT 2 in hepatocytes (Ciaraldi et al., 1986) and in pancreatic β cells (Orci et al., 1989). Establishing the basis for this difference in distribution should provide important insight into the mechanism of insulin action.

In stimulated cells we observed increased labeling of GLUT 4 in coated pits, coated vesicles and small vacuoles that we have classified as early endosomes because of the colocalization with endocytosed albumin and the absence of internal vesicles and cathepsin D labeling. These early endosomes were often continuous with tubular extensions in which the GLUT 4 to albumin ratio appeared to be sig-
The localization of GLUT 4 in coated pits and early endosomes is of particular interest for the following reasons. First, to our knowledge, this is the first indication that a number of cell surface receptors, an NPXY domain within a putative membrane-spanning domains (Mueckler et al., 1985; James et al., 1989). Recent evidence indicates that for a number of cell surface receptors, an NPXY domain within the juxta membrane region is involved in coated vesicle targeting (reviewed in Chen et al., 1990). It will be of interest to determine whether there is a similar targeting sequence for internalization of polytopic membrane proteins. Third, this evidence that GLUT 4 is continuously endocytosed in insulin-treated adipocytes points to a tentative conclusion concerning the locus of insulin action. GLUT 4 could be effectively excluded from the membrane in the basal state by intracellular sequestration or by an efficient internalization process. Thus, the redistribution of GLUT 4 to the cell surface in response to insulin could be due to an increased rate of exocytosis of the transporter or to a decreased rate of its endocytosis, respectively. The increased presence of GLUT 4 within elements of the endocytic pathway in the presence of insulin, suggests that the major effect of insulin is to increase the rate of movement of GLUT 4 to the cell surface.

In the basal state GLUT 4 is localized almost entirely to small TV structures in the Golgi area and elsewhere, often in clusters. For the reason described above, we propose that the effect of insulin is to allow GLUT 4 to relocate from TV structures to the plasma membrane and to participate in the recycling pathway (Fig. 8). Moreover, we suggest that upon insulin withdrawal the positive signal driving movement of GLUT 4 to the cell surface is removed such that GLUT 4 accumulates again in TV structures which appear to be withdrawn from the early endosomes. This provides a mechanism to explain the observation that after insulin removal GLUT 4 rapidly disappears from the cell surface of adipocytes and reappears in the same subcellular fraction where it was found in basal cells (Karnieli et al., 1981). Establishing the identity and the location of the sorting factors that determine the intracellular targeting of GLUT 4, as described above, may provide important information about insulin action. The early endosome or the TGR are the most likely locations for specific sorting. It remains to be determined whether the GLUT 4-containing TV structures that appear to bud from the early endosome in the presence of insulin are targeted directly to the plasma membrane or if they must first return to the TGR.

In addition to its specific effect on GLUT 4, a more general effect of insulin is also indicated by the fact that it increases the cell surface concentration of other proteins in adipocytes. The levels of the mannose-6-phosphate receptors, transferrin and α2-macroglobulin receptors and of GLUT 1 increase 1.5- to 3.5-fold (reviewed in Lienhard, 1989). There is considerable evidence that the basis of this effect is largely a general stimulation of membrane trafficking from the TGR and early endosomes to the cell surface by a factor of about twofold (Lienhard, 1989). Although this effect on membrane trafficking will contribute to the increase in surface GLUT 4, the much larger magnitude of the effect with GLUT 4 indicates that there is an additional aspect of regulation unique to GLUT 4, probably involving its specific exclusion from the recycling pathway in the basal state.
References

Afzdius, B. A. 1970. Brown adipose tissue: its gross anatomy, histology, and cytology. In Brown Adipose Tissue. Linberg, O., editor. Elsevier Science Publishing Co., Inc., New York. 1-31.

Aherne, W., and D. Hull. 1966. Brown adipose tissue and heat production in the newborn infant. J. Pathol. Bacteriol. 91:223-234.

Blok, J., E. M. Gibbs, G. E. Lienhard, J. W. Slot, and H. J. Geuze. 1988. Insulin-induced translocation of glucose transporters from post-Golgi compartments to the plasma membrane of 3T3 L1 adipocytes. J. Cell Biol. 106:69-76.

Bourey, R. E., L. Koranyi, D. E. James, M. Mueckler, and M. A. Permutt. 1990. Effects of altered glucose homeostasis on glucose transporter expression in skeletal muscle of the rat. J. Clin. Invest. 86:542-547.

Brands, R., J. W. Slot, and H. J. Geuze. 1983. Albumin localization in rat liver parenchymal cells. Eur. J. Cell Biol. 32:99-107.

Calderhead, D. M., K. Kitagawa, L. I. Tanner, G. D. Holman, and G. E. Lienhard. 1990a. Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. J. Biol. Chem. 265:13800-13808.

Ciaraldi, T. P., R. Horuk, and S. Matthaei. 1986. Biochemical and functional characterization in skeletal muscle of the rat. J. Clin. Invest. 86:404-411.

Douen, A. G., T. Ramlal, S. Rastogi, P. J. Bilan, G. D. Cartee, M. Vranic, J. Thorpe, and A. L. Schwartz. 1987. Membranes of sorting or-parachymal cells. Biochem. J. 249:155-161.

Gerhart, D. Z., R. J. LeVasseur, M. A. Broderius, and L. R. Drewes. 1989. A glucose transporter-like accounts for insulin stimulation of glucose transport in B16-H11 myocytes. Biochem. J. 269:597-601.

Charron, M. J., F. C. Brosius, L. S. Alper, and H. F. Lodish. 1989a. A glucose transport protein expressed predominantly in insulin-responsive tissues. Proc. Natl. Acad. Sci. USA. 86:2535-2539.

Chen, W. J., J. L. Goldstein, and M. S. Brown. 1990. NFXP1, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. 265:3116-3123.

Crawford, P. R., Y. Liu, and S. Mathias. 1989. Biosynthetic characterization of the rat liver glucose transport system. Biochem. J. 240:115-123.

Cushard, S. W., and L. J. Wardzala. 1980. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. J. Biol. Chem. 255:4758-4762.

Doun, A. G., T. Ramlal, S. Rastogi, P. J. Bilan, G. D. Cartee, M. Vranic, J. Thorpe, and A. L. Schwartz. 1990. Intracellular site of asialoglycoprotein receptor ligand uncoupling: double labeling immunoelectron microscopy on ultrathin frozen sections. J. Cell Biol. 101:2253-2262.

Geuze, H. J., J. W. Slot, P. A. van der Ley, and R. C. T. Scheffer. 1981. Use of colloid gold particles in double-labeling immunoelectron microscopy on ultrathin frozen sections. J. Cell Biol. 89:563-565.

Geuze, H. J., J. W. Slot, G. J. Strous, H. F. Lodish, and A. L. Schwartz. 1983. Intranuclear site of asialoglycoprotein receptor ligand uncoupling: double labeling immunoelectron microscopy during receptor-mediated endocytosis. Cell. 32:277-287.

Geuze, H. J., J. W. Slot, G. J. Strous, A. Hasilik, and K. von Figura. 1985. Possible pathways for lysosomal enzyme delivery. J. Cell Biol. 101:2223-2225.

Geuze, H. J., J. W. Slot, and A. L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. J. Cell Biol. 104:1715-1723.

Hiscox, H. W., J. W. Slot, G. J. Strous, J. W. Slot, J. E. Bleekemolen, and I. Mellman. 1988. Sorting of Mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. J. Cell Biol. 107:2491-2501.

Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. 1:1-39.

Gould, G. W., and G. I. Bell. 1990. Facilitative glucose transporters: an expanding family. TIBS (Trends Biochem. Sci). 15:18-23.

Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. Science ( Wash. DC). 234:438-443.

Hirshman, M. F., L. J. Goodyear, L. J. Wardzala, E. D. Horton, and E. S. Horton. 1990. Identification of an intracellular pool of glucose transporters from basal and insulin-stimulated rat skeletal muscle. J. Biol. Chem. 265:987-991.

Holgate, C. S., P. Jackson, Ph. N. Cowen, and C. C. Bird. 1983. Immunogold-silver staining: new method of immunostaining with enhanced sensitivity. J. Histochem. Cytochem. 31:938-944.

James, D. E., K. M. Burleigh, and E. W. Knaeghe. 1986a. In vivo glucose metabolism in individual tissues of the rat. J. Biol. Chem. 261:6356-6374.