

# Immuno-localization of the Insulin Regulatable Glucose Transporter in Brown Adipose Tissue of the Rat

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**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; Bourey 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),<sup>1</sup> which is expressed in many tissues including brain and

fibroblasts (Mueckler et al., 1985); the liver type (GLUT 2), which is found in liver, kidney, and in  $\beta$  cells of the pancreas (Thorens et al., 1988); GLUT 3, which is expressed in many tissues including brain and placenta (Kayano et al., 1988); 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 immunoblotting 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-

1. *Abbreviations used in this paper:* ASGP-r, asialoglycoprotein receptor; BAT, brown adipose tissue; CURL, compartment of uncoupling receptors and ligands; GLUT 1-5, glucose transporter isoforms; LM, light microscopy; TGR, *trans*-Golgi reticulum; T-V, tubulo-vesicular.

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 rat brown adipose tissue *in vivo* by a factor of 15-fold (James et al., 1986a,b), and the level of expression of GLUT 4 in this tissue is high (James et al., 1989). Moreover, because the fat content of the tissue can be reduced, it is more suited for our immunocytochemical approach than white adipocytes, which have been used for many biochemical studies.

We find that in the basal state GLUT 4 is concentrated in tubulo-vesicular structures in the *trans*-Golgi region and elsewhere throughout the cytoplasm. Upon insulin treatment there is a marked shift in GLUT 4 from these sites to the plasma membrane, such that the amount of GLUT 4 at the cell 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. Ultracryotomy 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-cryoNova system with a diamond knife prepared for ultracryotomy (Diatome, Ltd., Bienne, Switzerland). Sections were picked up, thawed on the surface of a 2.3 M sucrose drop, and transferred 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 5 min. Sections were stained for 5 min on a drop of uranyl acetate-oxalate, pH 7, washed on distilled water again, and finally floated 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 polyvinyl alcohol (Moviol, Hoechst, Germany) and photographed under a polarizing 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 antiserum raised against the carboxy-terminal peptide encompassing the last 12 amino acids of GLUT 4 (anti-GLUT 4<sub>12</sub>) (James et al., 1989); affinity purified antibodies isolated from a rabbit antiserum against the carboxy-terminal peptide consisting of the last 19 amino acids of GLUT 4 (anti-GLUT 4<sub>19</sub>) (Calderhead et al., 1990a). The IgG fraction of a rabbit antiserum 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. Andrej 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 repeat 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 (9 nm) 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).

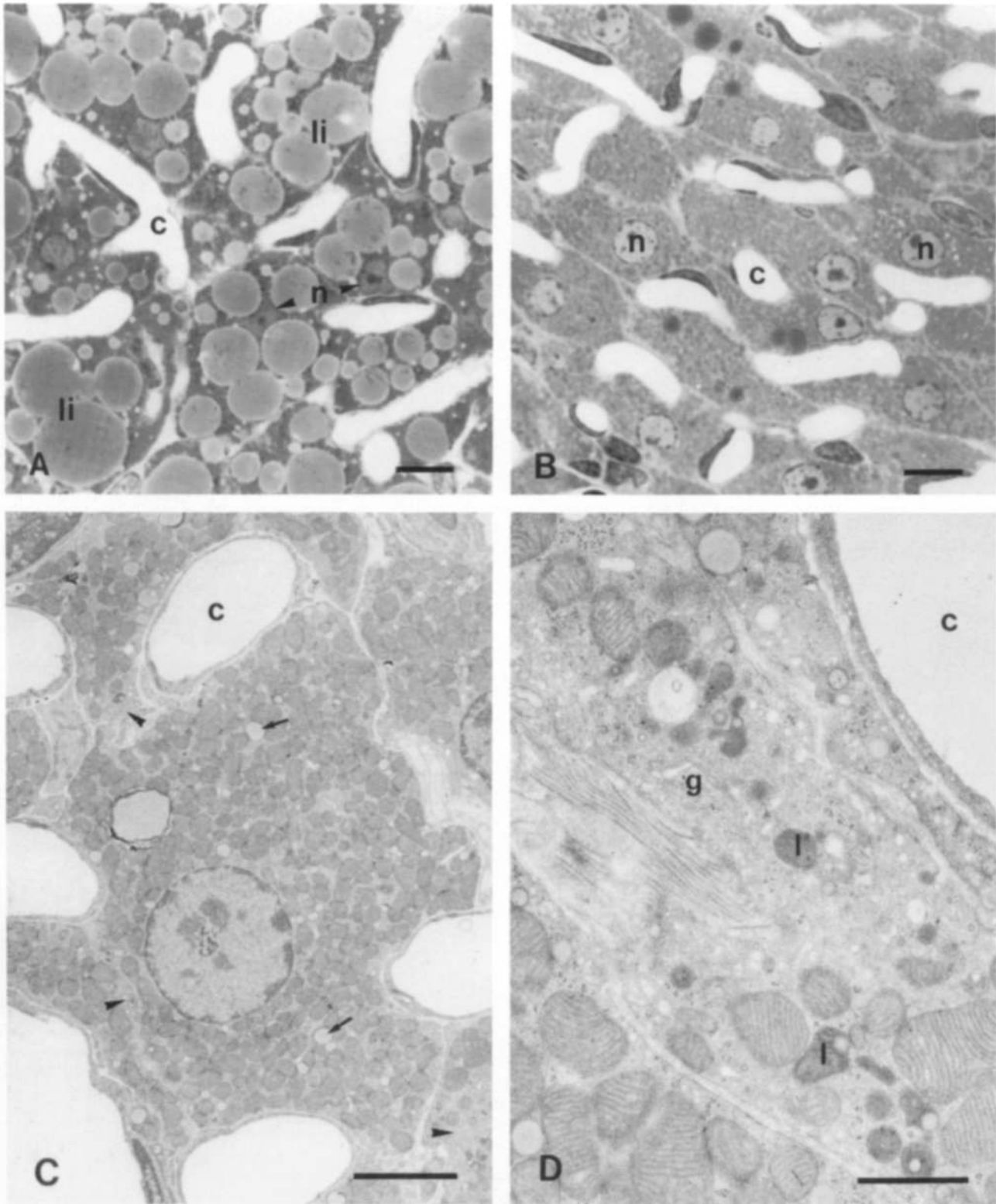
### Tissue Structure

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 CaCl<sub>2</sub>, pH 7.4. The fixation was continued by immersion of the tissue in the same fixative (1 h) and in 1% OsO<sub>4</sub> (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.

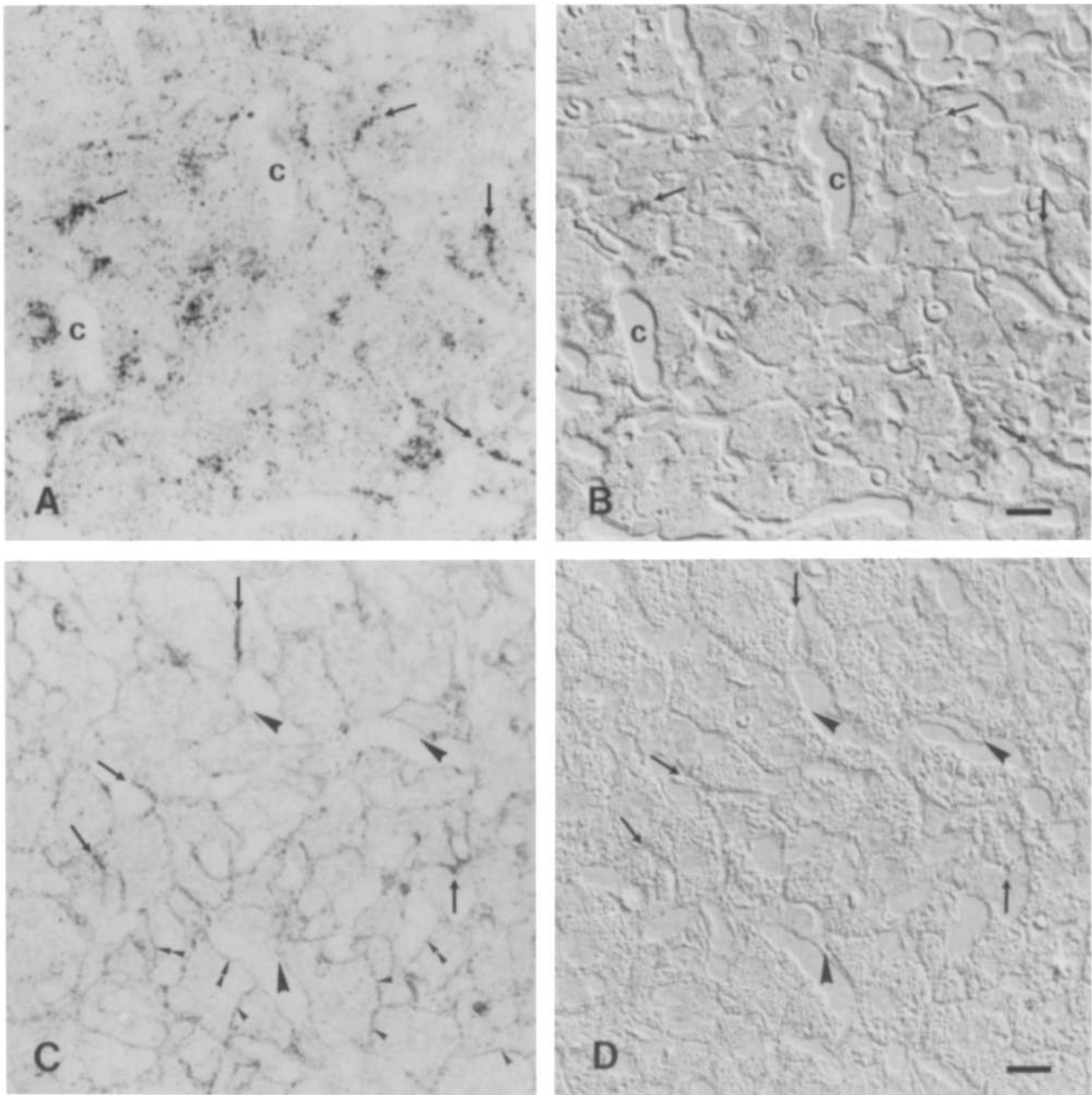
## Results

### Morphology of Brown Adipose Tissue

The ultrastructure of brown adipose tissue (BAT) has been



**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  $\mu\text{m}$ ; (C) 5  $\mu\text{m}$ ; (D) 0.5  $\mu\text{m}$ .



**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  $\mu$ m.

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 ultracytometry. 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 mitochondrion-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<sub>19</sub> antibody gave stronger labeling than the anti-GLUT 4<sub>12</sub> antibody. Therefore, we have used the anti-GLUT<sub>19</sub> antibody for each of the EM illustrations shown. In quantitative experiments we used the anti-GLUT 4<sub>12</sub> (Table I) and anti-GLUT 4<sub>19</sub> (see Fig. 7) antibodies. In control thin sections, which were incubated with preimmune serum, or with the anti-peptide 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 mitochondrion-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

Table I. Proportional Distribution of GLUT 4 Label in Cellular Membranes of Brown Adipocytes

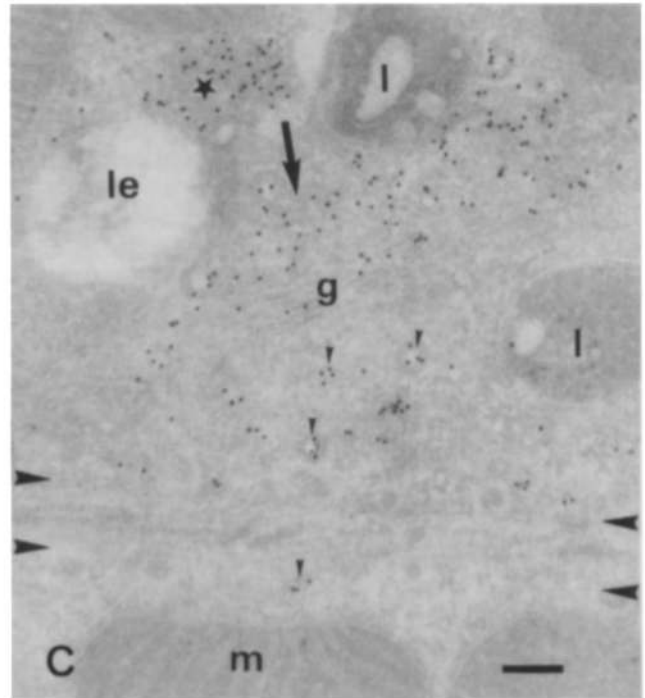
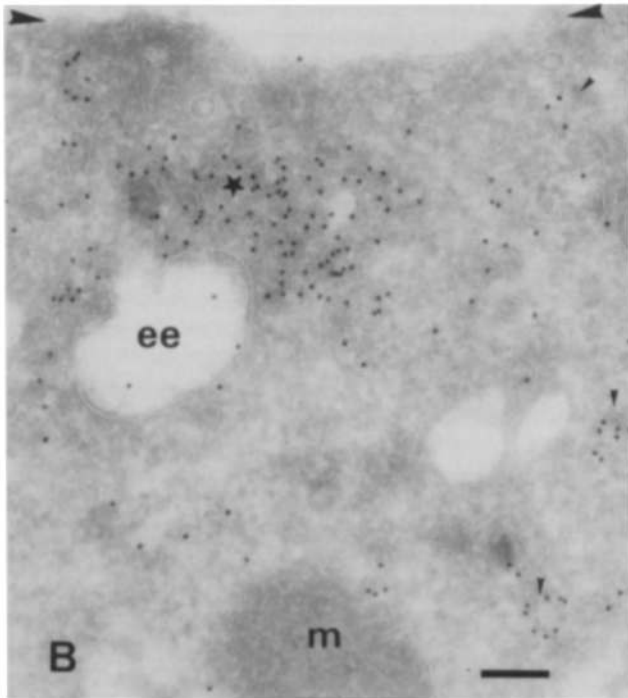
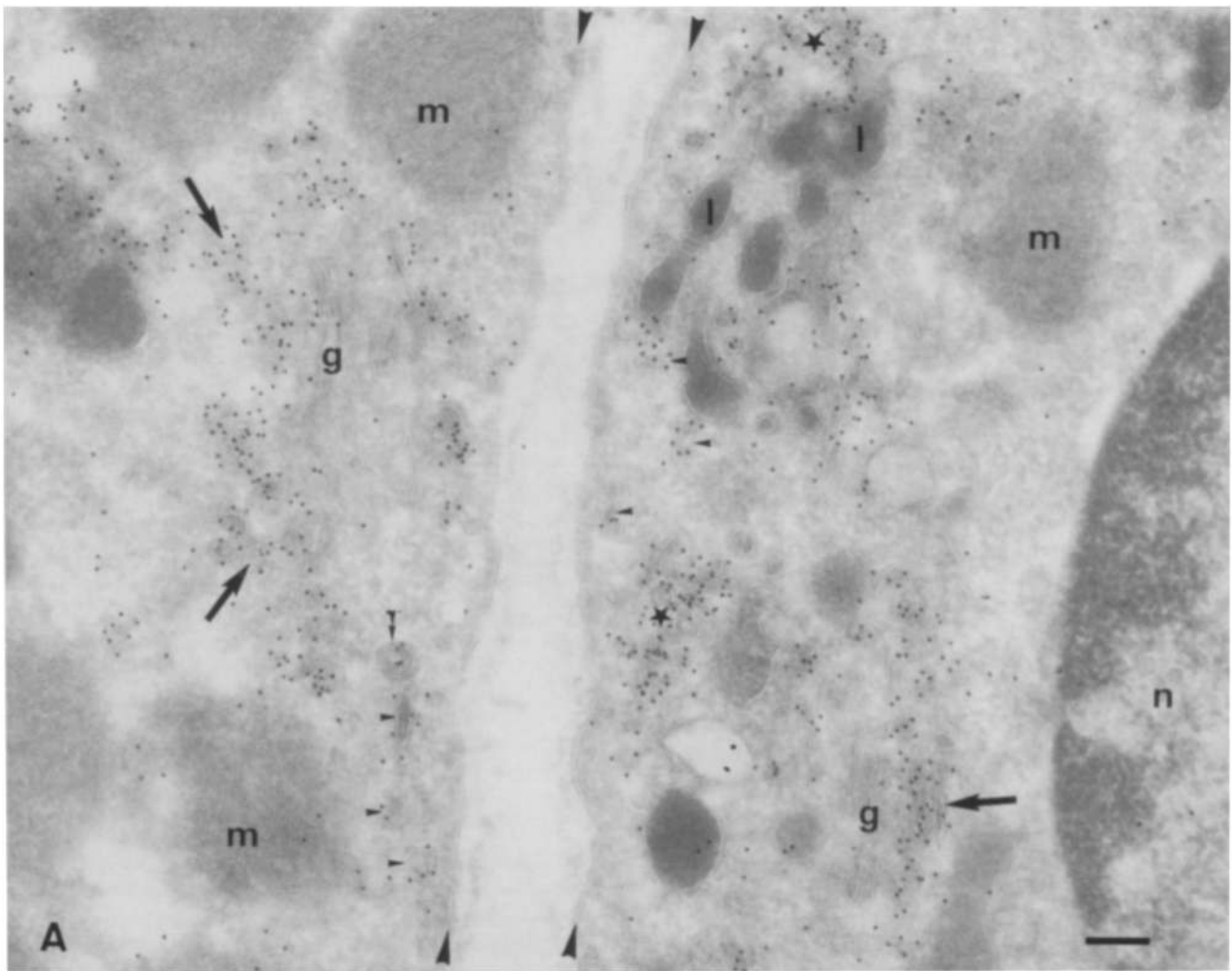
Animal number	Plasma membrane	Membranous structures		
		Golgi area	Other sites	Cytoplasm
- Insulin				
1	0.4	49.4	42.5	7.7
2	1.6	38.0	54.9	5.5
3	1.2	43.9	49.2	5.7
Mean	1.1	43.7	48.9	6.3
+ Insulin				
4	40.7	21.9	24.9	12.5
5	41.2	16.8	34.2	7.8
6	40.6	13.7	40.6	5.1
Mean	40.8	17.5	33.2	8.5

Cryosections of BAT from three basal and three stimulated rats were immunolabeled with anti-GLUT 4<sub>12</sub>. Gold particles located no more than 20 nm from a visible membrane structure were counted and assigned to one of the following organelles: plasma membrane, membranous structures (T-V elements, vacuoles) in the Golgi area, and elsewhere in the cell. The Golgi areas were taken fairly broadly to include the TGR and most of the structures accumulated in the mitochondrion-free parts of the cell. Gold particles not within 20 nm of visible membrane structures were assigned to the cytoplasm. These may represent background labeling and/or labeling of poorly defined structures. Labeling of nuclei and mitochondria was considered as nonspecific and therefore excluded from the countings. For each animal ~1,200 gold particles in two different sections were counted.

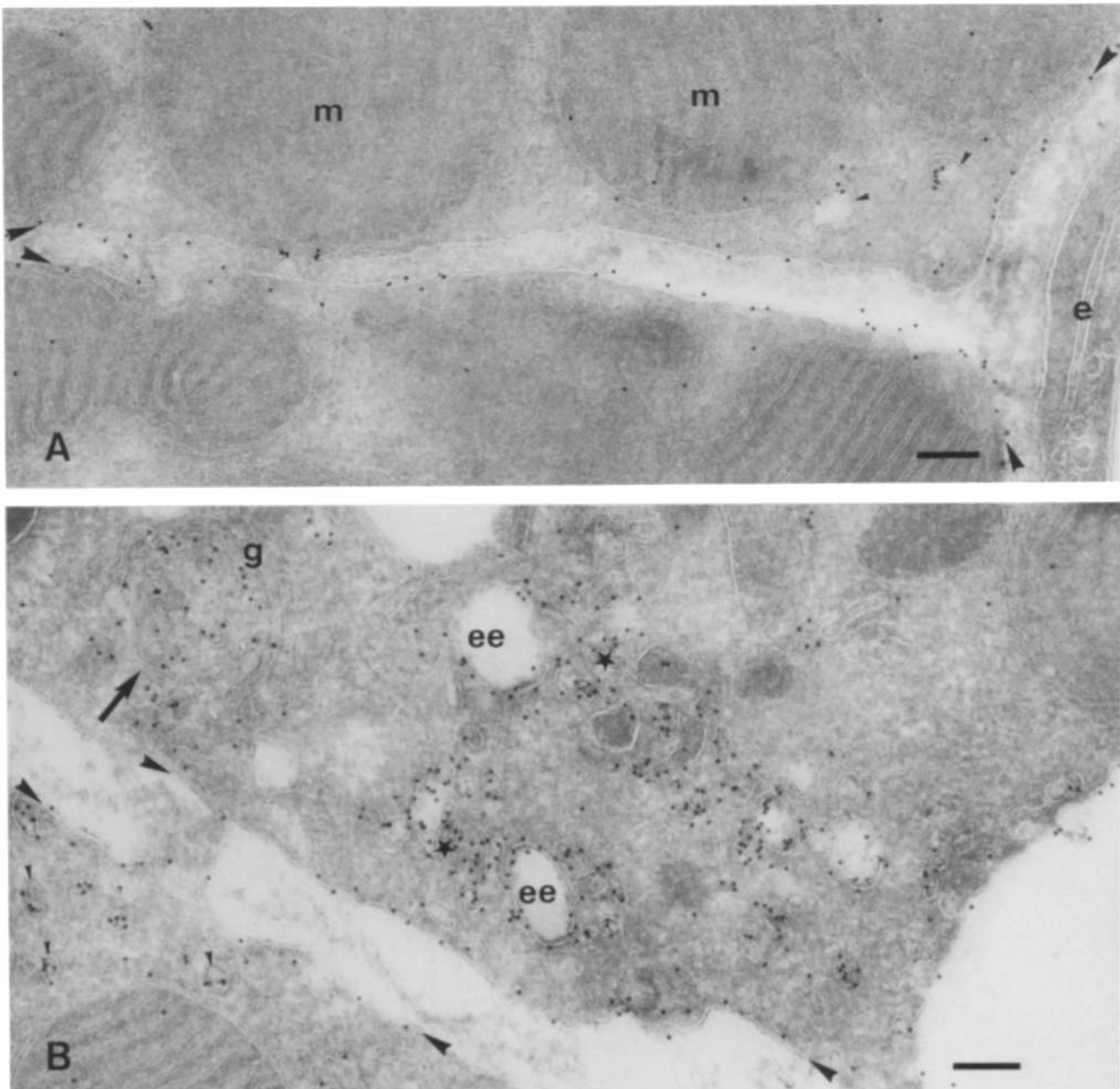
studies (Slot et al., 1990), endothelial cells were not significantly labeled with any one of the three GLUT 4 antibodies used in these studies.

### 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 (T-V) structures were most abundant in the mitochondrion-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 T-V 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 mitochondrion-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 T-V 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 T-V 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







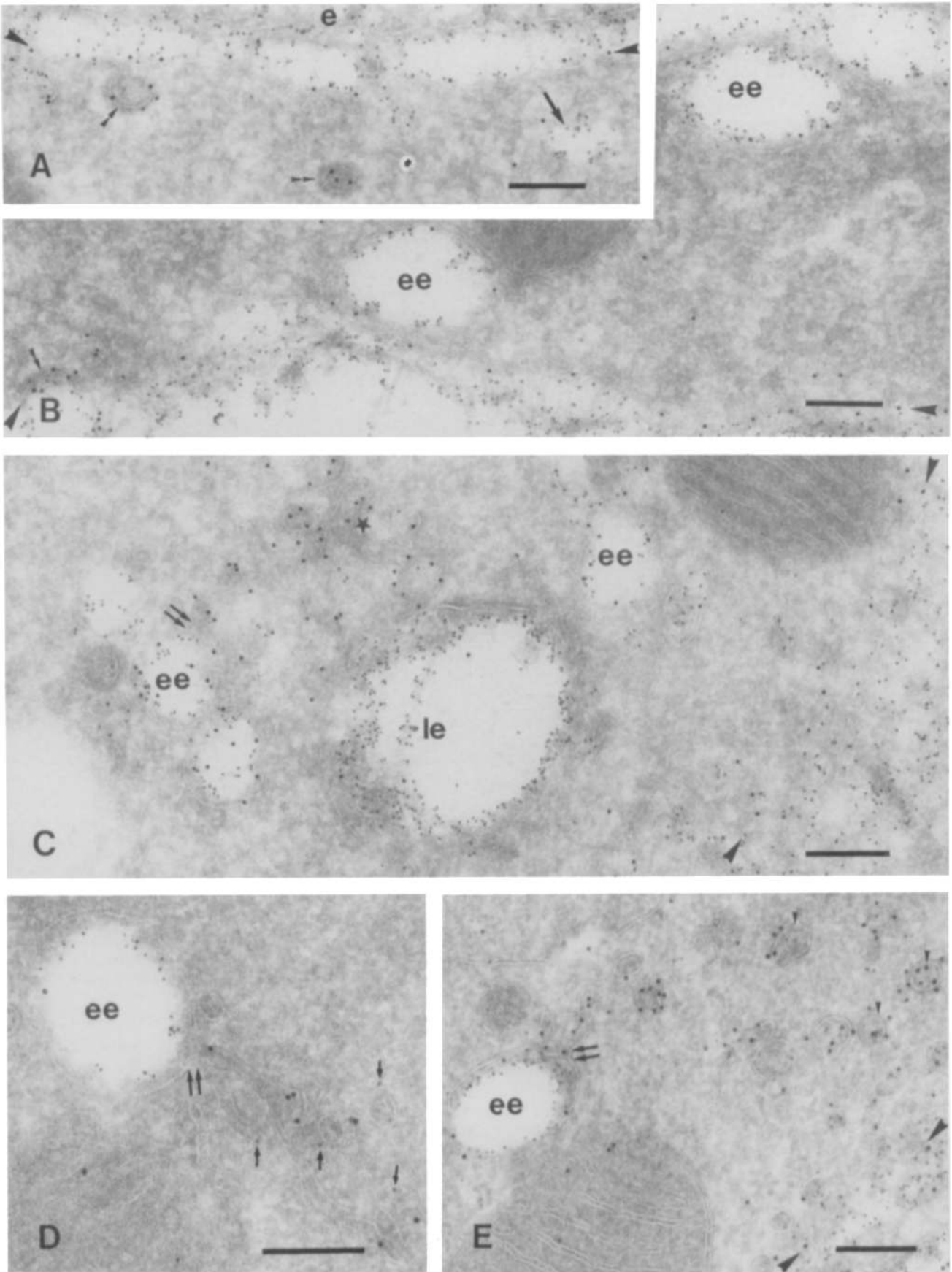
**Figure 4.** Electron micrographs of insulin-stimulated BAT. (A) Strong labeling of the adipocyte cell membrane between contiguous adipocytes as well as those facing an endothelial cell (*e*). (B) A mitochondrion-free area in an adipocyte. Many labeled T-V elements (*asterisks*) are in close association 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<sub>19</sub> 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 non-coated 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 T-V structures. Elongated tubular extensions connected to endosomes were more prominent in stimulated (Figs. 4 B, 5, D and E) than in basal cells (Figs.

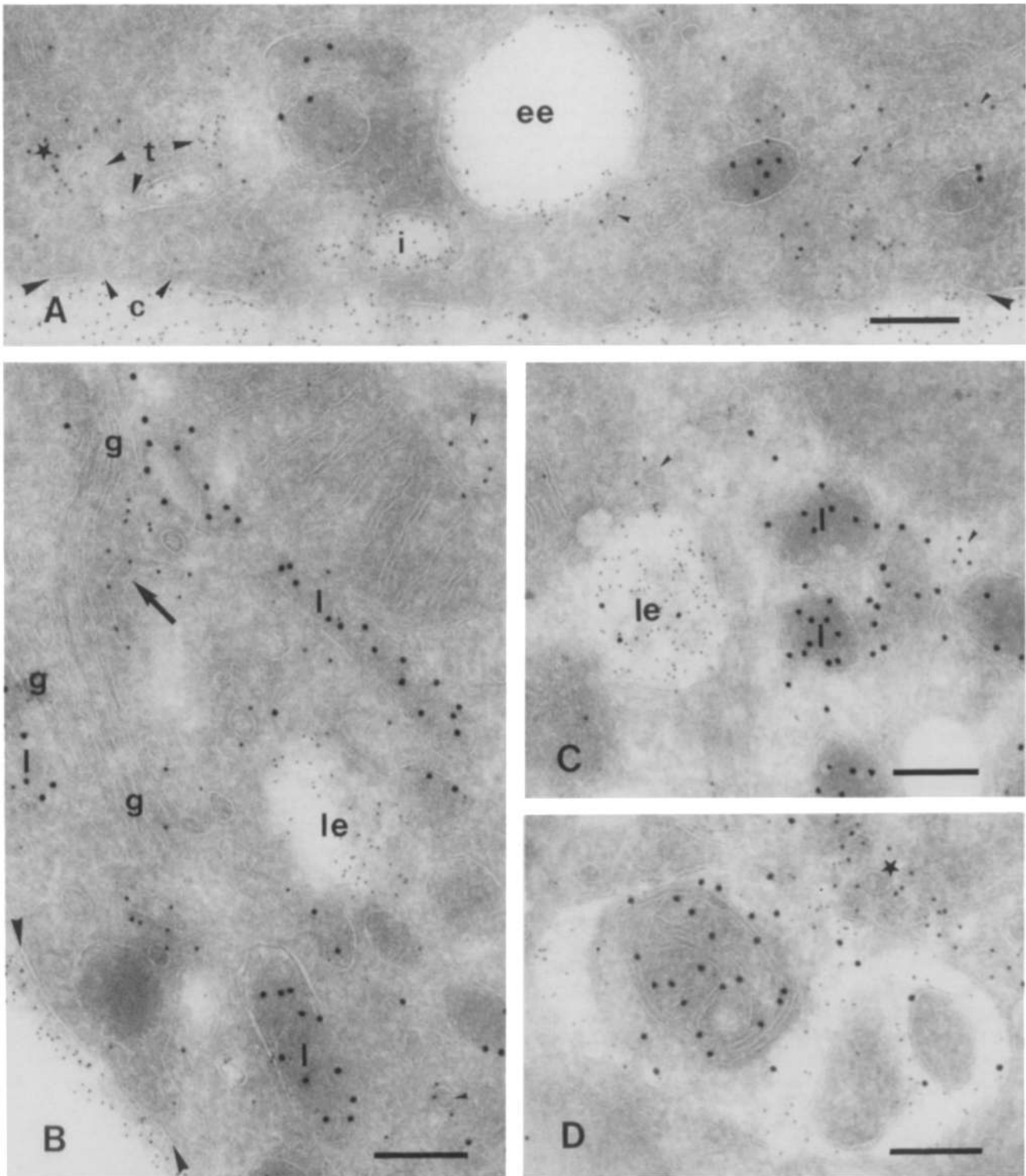
3 B, C and A). In these complex structures separation seemed to occur between albumin, which was mainly restricted to the vacuolar part, and GLUT 4, which seemed enriched in the tubular part and surrounding vesicular profiles. The second class of endosomes, referred to as late endosomes, contained internal 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 T-V structures in the basal state, and this was reduced by 50% in response to insulin. GLUT 4 is translocated from these T-V elements at all locations in the cytoplasm (structures 1-5 in Fig. 7), including the TGR.

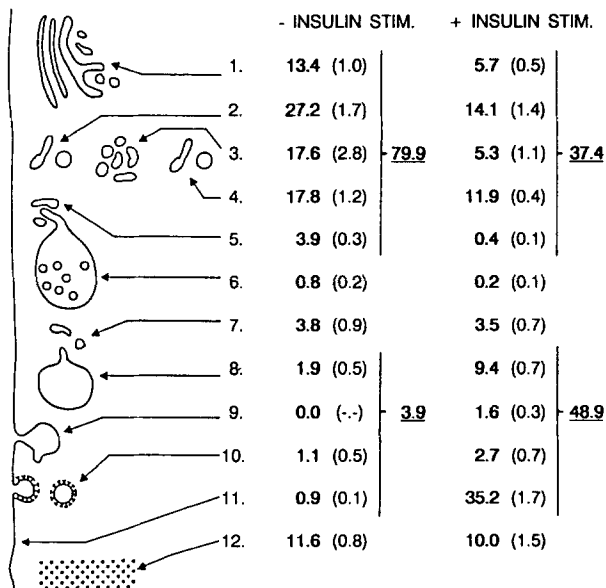
## Discussion

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.

**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 arrowheads*). (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 T-V elements clustered near the vacuoles. The double arrows indicate a putative connection between an early endosomal vacuole and T-V elements. Such connections are more clearly depicted in (D) and (E). GLUT 4 seems enriched in tubular extensions and surrounding T-V 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 T-V 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 T-V 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 T-V 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 I, except that double-labeling experiments were used to obtain more detail and anti-GLUT 4<sub>19</sub> 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  $\mu$ m) the 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.

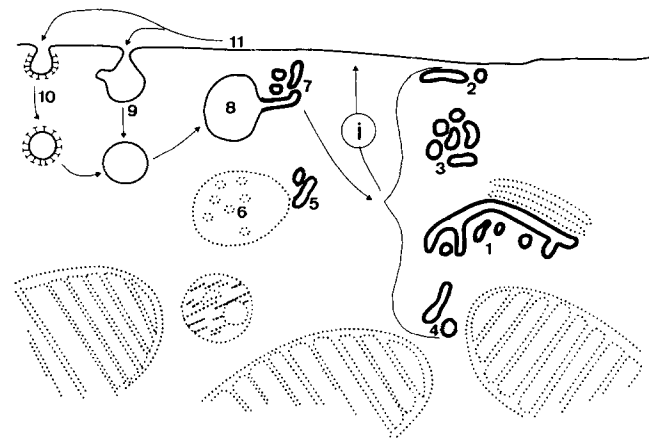
An effect of this magnitude ( $\geq 40$ -fold higher than basal) accounts entirely for the 15–30 fold increases in glucose transport that are typically observed in brown and white adipocytes following insulin stimulation (James et al., 1986a,b; Toyoda et al., 1987). This finding is of importance because 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  $\beta$  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-



**Figure 8.** Schematic summary of GLUT 4 distribution in BAT from nonstimulated (bold structures) and insulin-stimulated (structures drawn in bold and medium solid lines) rats. Quantitative aspects of the distribution and the identity of various structures are given in Fig. 7. The observations are consistent with a model where in the presence of insulin GLUT 4 molecules recycle between the cell surface and the endosomal system. GLUT 4 is primarily endocytosed via coated vesicles, which fuse with early endosomal vacuoles. GLUT 4 is extruded from these vacuoles at an early step via tubular extensions, reminiscent of the way receptors and ligands dissociate in CURL in liver cells (Geuze et al., 1983). In the continued presence of insulin GLUT 4 positive T-V elements may originate from the tubular extensions and mediate the return of GLUT 4 to the cell surface, either directly or via the TGR/secretory route. (i) Indicates the proposed level at which the regulatory effect of insulin interferes with this cycling pathway of GLUT 4. Without insulin GLUT 4 accumulates in T-V structures at locations 1–5.

nificantly higher than in the corresponding endosome. The distribution of GLUT 4 in insulin-stimulated cells is in many ways reminiscent of the distribution of the asialoglycoprotein receptor (ASGP-r) in rat liver cells (Geuze et al., 1983). Approximately 35% of the total cell complement of the ASGP-r is found at the cell surface. The receptor is also found in the compartment for uncoupling of receptors and ligands (CURL), which consisted of vacuoles and tubular extensions analogous to those containing GLUT 4 in brown adipocytes (Fig. 5, *D* and *E*). The dissociated ASGP ligand is retained in the fluid phase containing vacuoles, like albumin in adipocytes, while the receptor is enriched in the tubular extensions. Larger, often multivesicular vacuoles, though still in contact with receptor-positive tubules, do not contain a significant amount of the receptor (Geuze et al., 1987). The tubules presumably mediate the recycling of the ASGP-r to the cell surface. This recycling route via coated vesicles and early endosomes is well described for several receptor molecules at the cell surface, including those for low density lipoprotein (Goldstein et al., 1985) and transferrin (Stoorvogel et al., 1987). Kinetic studies have indicated that within a few minutes after endocytosis these receptor molecules are sorted from the endosomal pathway, after which they recycle to the plasma membrane. The present data suggest that GLUT 4 is actively involved in a similar recycling system through early endosomes in insulin-stimulated brown adipocytes (Fig. 8). The role that coated pits and vesicles may play in the recycling of GLUT 4 is not clear from our data. The quantitative nature of coated vesicle labeling in our studies may be affected by two factors. Firstly, we can not be certain that the GLUT 4-positive coated vesicles originate only from the plasma membrane and not for instance from the TGR, a structure often located near the cell surface. In fact, this may explain the presence of GLUT 4-positive coated vesicles in basal cells (Fig. 3 *A*). Secondly, depending on the position of the coated structures in the sections, the clathrin lattice may have hindered labeling of the cytosolic GLUT 4 epitope in many cases. Nevertheless, the qualitative observations indicate that in stimulated cells GLUT 4 is enriched in at least some surface connected coated pits (Fig. 5, *A* and *B*), which we take as strong evidence that at least part of the recycling of GLUT 4 is mediated by these structures. In addition, some internalization may occur via the noncoated invaginations of the cell membrane. However, there was no enrichment of GLUT 4 at these sites, suggesting that endocytosis of GLUT 4 via these structures presumably occurs by random interaction.

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 transporter undergoes recycling by the pathway that has been well established for cell surface receptors. Second, unlike these receptors, which contain a single transmembrane segment per polypeptide chain, the glucose transporter has 12 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 either 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 T-V 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 T-V 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 T-V 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 (Karnielli 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 small GLUT 4-containing T-V 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  $\alpha$ 2-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.

We would like to thank Tom van Rijn for preparing the photographs and Dr. Drago Loncar (Department of Biology, University of California, San Diego, CA) for advising us on treatment of the animals.

This work was supported by grants from the Juvenile Diabetes Foundation to J. W. Slot, the Muscular Dystrophy Association to D. E. James and J. W. Slot, the National Institutes of Health (DK25336) to G. E. Lienhard and National Institutes of Health (DK42503) to D. E. James.

Received for publication 11 October 1990 and in revised form 19 December 1990.

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