Translocation of the Glucose Transporter (GLUT4) to the Cell Surface in Permeabilized 3T3-L1 Adipocytes: Effects of ATP, Insulin, and GTPγS and Localization of GLUT4 to Clathrin Lattices

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Abstract. Insulin stimulates the movement of two glucose transporter isoforms (GLUT1 and GLUT4) to the plasma membrane (PM) in adipocytes. To study this process we have prepared highly purified PM fragments by gently sonicating 3T3-L1 adipocytes grown on glass coverslips. Using confocal laser immunofluorescence microscopy we observed increased PM labeling for GLUT1 (2.3-fold) and GLUT4 (eightfold) after insulin treatment in intact cells. EM immunolabeling of PM fragments indicated that in the nonstimulated state GLUT4 was mainly localized to flat clathrin lattices. Whereas GLUT4 labeling of clathrin lattices was only slightly increased after insulin treatment, labeling of uncoated PM regions was markedly increased with insulin. These data suggest that GLUT4 recycles from the cell surface both in the presence and absence of insulin. In streptolysin-*O* permeabilized adipocytes, insulin, and GTPγS increased PM levels of GLUT4 to a similar extent as observed with insulin in intact cells. In the absence of an exogenous ATP source the magnitude of these effects was considerably reduced. Removal of ATP per se caused a significant increase in cell surface levels of GLUT4 suggesting that ATP may be required for intracellular sequestration of these transporters. When insulin and GTPγS were added together, in the presence of ATP, PM GLUT4 levels were similar to levels observed when either insulin or GTPγS was added individually. Addition of GTPγS was able to overcome this ATP dependence of insulin-stimulated GLUT4 movement. GTPγS had no effect on constitutive secretion of adipsin in permeabilized cells. In addition, there was no effect of insulin or GTPγS on GLUT4 movement to the PM in noninsulin sensitive streptolysin-*O*-permeabilized 3T3-L1 fibroblasts overexpressing GLUT4. We conclude that the insulin-stimulated movement of GLUT4 to the cell surface in adipocytes may require ATP early in the insulin signaling pathway and a GTP-binding protein(s) at a later step(s). We propose that the association of GLUT4 with clathrin lattices may be important in maintaining the exclusive intracellular location of this transporter in the absence of insulin.

INSULIN stimulates glucose transport in muscle and fat cells by a process that in many ways resembles regulated exocytosis. Using immunogold labeling and EM, it has been shown that in the absence of insulin the major glucose transporter endogenous to adipocytes and myocytes (GLUT4) is almost completely sequestered in intracellular tubulo-vesicular structures that are clustered either in the *trans*-Golgi reticulum or in the cytoplasm (Slot et al., 1991a, b). Upon stimulation with insulin there is a marked increase in cell surface GLUT4 immunolabeling together with a decrease in labeling of the intracellular tubulo-vesicular elements (Slot et al., 1991a,b). In addition, the level of GLUT4 in endosomes and coated pits is increased with insulin suggesting that the transporter recycles from the plasma membrane (PM)[1] in the presence of insulin (Slot et al., 1991b).

These data indicate that insulin stimulates the cell surface levels of GLUT4 by facilitating exocytosis of GLUT4 from the intracellular tubulo-vesicular elements rather than by inhibiting endocytosis of GLUT4.

A major factor that has limited investigations of the mechanism of insulin-stimulated GLUT4 translocation in adipocytes and myocytes is that nearly all studies have been confined to intact cells. Considerable progress in elucidating mechanisms of regulated exocytosis in other cell types has been made using semiintact or permeabilized cells. Numerous methods, including the use of detergents, electropermeabilization, or mechanical disruption, have been used to puncture the PM of cells (for review see Tartakoff, 1989). A particular advantage of this approach is that the cytosolic constituents can be carefully manipulated allowing a more precise study of different effector systems than can be achieved using intact cells. In studies using permeabilized cell systems, a role for GTP binding proteins has been proposed for most forms of vesicle traffic (for review see Hall,

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1. *Abbreviations used in this paper:* IC, intracellular; KRP, Krebs-Ringer phosphate; LDH, lactate dehydrogenase; PM, plasma membrane; SLO, streptolysin-*O*; WGA, wheat germ agglutinin.

1990). Nonhydrolyzable analogs of GTP almost universally trigger regulated exocytosis (for review see Gomperts, 1990), while such analogs appear to inhibit the constitutive movement of proteins from the *trans*-Golgi reticulum (Gravotta et al., 1990; Miller and Moore, 1991). GTPγS has also been shown to inhibit constitutive protein traffic between the ER and the Golgi apparatus (Beckers and Balch, 1989), as well as between individual Golgi cisternae (Melançon et al., 1987).

These studies prompted us to develop a permeabilized cell system for investigating the mechanism of insulin-stimulated GLUT4 translocation, and to investigate the potential role of a GTP-binding protein in this process. We have used 3T3-L1 adipocytes for these studies because in contrast to rat adipocytes they can be studied while firmly attached to a substratum, which facilitates rapid manipulation of the incubation media. In addition, we have used this property to develop a new assay for the movement of glucose transporters to the cell surface. This assay involves the rapid removal of cells from the substratum by sonication, leaving highly purified PM fragments attached. These fragments are then analyzed by immunolabeling, to serve as a sensitive biochemical and morphological method for measuring glucose transporter translocation. Streptolysin-*O* (SLO), a bacterial endotoxin (Bhakdi et al., 1984), was used to permeabilize the cells. SLO has been used as a permeabilization reagent to examine the regulatory factors involved in exocytosis or constitutive secretion in a variety of cell types (Gravotta et al., 1990; Miller and Moore, 1991; Tartakoff, 1989). SLO has a number of advantages compared with other permeabilization techniques: (*a*) the size of the pores generated by SLO are sufficiently large to allow movement of both small and large molecules into or out of the cell; (*b*) SLO is quite selective for the PM so that intracellular organelles remain relatively unperturbed by the toxin; (*c*) SLO has been used to obtain quantitative permeabilization of cells; and (*d*) once the pores have been created, they remain viable for long periods of time. In SLO-permeabilized 3T3-L1 adipocytes we show that both insulin and GTPγS stimulate the translocation of GLUT4 to the PM with an efficiency similar to that observed with insulin in intact cells. We also show that neither insulin nor GTPγS is capable of stimulating GLUT4 translocation in noninsulin-sensitive cells that have been transfected with the GLUT4 cDNA. This may provide an ideal complementation scheme for identifying regulatory molecules.

Materials and Methods

Materials

SLO was obtained from Burroughs-Wellcome (Research Triangle Park, NC). SLO was prepared immediately before use as a 0.8-IU/ml stock in the intracellular (IC) buffer (see below). Creatine phosphate and creatine phosphokinase were obtained from Calbiochem Corp. (La Jolla, CA). ^{125}I-Goat anti-rabbit was obtained from ICN Radiochemicals Inc. (Irvine, CA). FITC-conjugated goat anti-rabbit IgG was obtained from Cappel Laboratories (West Chester, PA). Protein A conjugated with 10-nm gold was obtained from Janssen Life Science Products (Piscataway, NJ) through Amersham Corp. (Arlington Heights, IL). 3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). ATPγS was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Biotinylated wheat germ agglutinin (WGA) was obtained from Pierce Chem. Co. (Rockford, IL). All other chemicals were obtained from Sigma Chem. Co. (St. Louis, MO).

Antibodies

The glucose transporter antibodies that were used for these studies have been described previously (Piper et al., 1991). The rabbit anti-GLUT4 antiserum was raised against a synthetic peptide corresponding to the COOH-terminal 16 residues of the rat GLUT4 sequence (Haney et al., 1991); the rabbit anti-GLUT1 antiserum was raised against a synthetic peptide corresponding to the COOH-terminal 12 amino acids of the human GLUT1 sequence (Piper et al., 1991); the rabbit anti-Na/K-ATPase antiserum, kindly provided by Dr. Robert Mercer, was raised against the purified rat protein; the rabbit anti-adipsin antiserum, kindly provided by Dr. Bruce Spiegelman, was raised against purified baculovirus-expressed mouse adipsin (Kitagawa et al., 1989).

Cell Culture

Murine-derived 3T3-L1 fibroblasts were cultured in DME on either tissue culture dishes (10 cm) or ethanol-washed glass coverslips (No. 1) and differentiated as described previously (Piper et al., 1991). All experiments were performed on adipocytes 8–12 d after withdrawal from differentiation media. Adipocytes were rinsed three times with Krebs-Ringer phosphate buffer containing 2% BSA and 2.5 mM glucose (KRP) and incubated in KRP for 2 h at 37°C before experimentation. 3T3-L1 fibroblasts transfected with the rat GLUT4 cDNA (Haney et al., 1991) were also used in some experiments. These cells were treated identically to 3T3-L1 adipocytes (see above) except that they were not differentiated, and in some experiments GLUT4 expression was induced from the metallothionein promoter of the expression vector by incubating cells overnight in media containing 7 mM Na butyrate and 100 μM ZnCl₂. As shown previously (Haney et al., 1991) the level of expression of GLUT4 in noninduced transfectants was similar to that observed in 3T3-L1 adipocytes and induction resulted in a threefold increase in GLUT4 expression.

Permeabilization with SLO

Adipocytes were permeabilized using a procedure similar to that described previously (Miller and Moore, 1991). To determine the optimal concentration of SLO, 3T3-L1 cells were washed three times with IC buffer (140 mM potassium glutamate, 20 mM Hepes, 5 mM MgCl₂, 5 mM EGTA, 5 mM NaCl, pH 7.2) and incubated in IC buffer containing different SLO concentrations and 1 μg/ml propidium iodide for 5 min at 37°C. The degree of permeabilization was quantitated by counting fluorescent nuclei per cell number in several different fields. Greater than 95% of cells were permeabilized using 0.8 IU/ml of SLO for 5 min at 37°C. On the basis of these studies permeabilization was routinely performed in this manner. The cells were then washed three times in IC buffer, and incubated as described at 37°C. IC buffer containing 1 mg/ml BSA and 1 mM DTT was used as the incubation medium in all experiments.

Cell Incubations

Cells that were studied as intact were incubated in KRP containing either no additions (control), insulin (10⁻⁷ M), or GTPγS (100 μM) for 15 min at 37°C. When ATP was added, either MgATP (10 mM) or an ATP regeneration system (40 IU/ml creatine phosphokinase, 5 mM creatine phosphate, 1 mM ATP) was used. Under conditions without ATP, no exogenous ATP was added after permeabilization. In some experiments endogenous ATP was depleted by incubation of permeabilized cells with apyrase (5 U/ml) during the transport assay. Unless otherwise indicated, all incubations with experimental treatments were performed by incubating cells in IC buffer containing the various additions for 15 min at 37°C. In some experiments secretion of the serine protease adipsin from permeabilized adipocytes was studied. Cells were incubated as described above, with the exception that intact cells were incubated in KRP containing only 1 mg/ml BSA. 0.5-ml aliquots of the media were removed at different times after the initiation of the transport assay and incubated on ice. Adipsin levels in media samples were measured by immunoblotting (Kitagawa et al., 1989).

PM Lawn Technique

This method relied on a one-step procedure for obtaining PM lawns as described previously (Heuser, 1989; Lin et al., 1991; Moore et al., 1987). Cells cultured on glass coverslips were used for these studies. After experimental treatments, coverslips were washed in PBS, followed by a 10-s treatment with 0.5 mg/ml polylysine in PBS. The cells were swollen by three 5-s incubations in a hypotonic buffer (1/3× buffer A), transferred to buffer

A (70 mM KCl, 30 mM Hepes, 5 mM $MgCl_2$, 3 mM EGTA, pH 7.5), and immediately broken open by placing under an ultrasonic microprobe (Kontes Co., Vineland, NJ). For antibody labeling, sonicated cells were immediately transferred to 2% paraformaldehyde in buffer A. In some cases (see Fig. 7), sonicated cells were immediately transferred to 2% glutaraldehyde in buffer A. PM lawns were apparent by phase microscopy as small fragments attached to the coverslip. Conditions were established under which almost all of the cells on a coverslip could be removed by this method leaving behind a uniform lawn of PM fragments.

Assessment of Purification of PM Lawns

3T3-L1 adipocytes grown on glass coverlips were incubated at 0°C for 30 min in the presence or absence of biotinylated WGA at a concentration of 50 μg/ml in PBS. Cells were washed five times with PBS, PM lawns were prepared by sonication as indicated above, and coverslips were washed with HES buffer (20 mM Hepes, 1 mM EDTA, 250 mM sucrose, pH 7.4). The PM fragments were scraped from the coverslip into HES buffer using a rubber policeman. PM fragments were recovered by centrifugation at 200,000 g for 1 h at 4°C. To obtain total cell membranes, cells were scraped from the coverslip into HES buffer and homogenized by six passes through a 22-G needle. Homogenates were then centrifuged at 200,000 g for 1 h at 4°C to obtain a total membrane pellet. In some cases a crude plasma membrane fraction was obtained using conventional subcellular fractionation, as previously described (Piper et al., 1991). To quantitate biotinylated WGA bound to membranes, fractions were subjected to SDS-PAGE and transferred to nitrocellulose. Nitrocellulose blots were incubated with avidin-peroxidase for 30 min at 37°C, followed by three washes in PBS/1% Triton X-100. Blots were then incubated with substrate solution (20 mg 3-amino-9-ethyl-carbazole, 5% dimethylformamide, 0.018% hydrogen peroxide, 50 mM acetate, pH 5) for 15 min at room temperature.

Quantitative Immunofluorescence Microscopy

Fixed coverslips were prepared for immunofluorescence microscopy as described previously (Piper et al., 1991), with slight modifications. The primary antibody used was either 20 μg/ml protein A-purified anti-GLUT4 antiserum, 50-μg/ml afinity-purified anti-GLUT1 antiserum, or a 1:100 dilution of Na^+/K^+-ATPase antiserum. After mounting coverslips on slides as described (Piper et al., 1991), the PM lawns were visualized and imaged using a scanning laser confocal immunofluorescence microscope under a 40× objective for quantitation purposes and a 63× objective for the photographs. Images were enhanced, and relative intensities were scaled using analysis software (Bio-Rad Laboratories, Richmond, CA). All images were scaled identically for each antibody, regardless of the treatments used. Lawns from at least 24 cells were quantitated for each 40× image, and at least two images were quantitated on each coverslip. These values were then averaged to obtain a single data point for each experimental condition.

Electron Microscopy

For immunolabeling of PM lawns, paraformaldehyde-fixed coverslips were quenched, blocked, and labeled with primary antibody as described previously (Piper et al., 1991). Coverslips were then washed in PBS and transferred to PBS containing a 1:25 dilution of protein A-gold (10 nm, dialyzed against PBS) and 0.1% calf serum for 1 h at 22°C. After labeling, coverslips were washed three times in PBS and transferred to 2% glutaraldehyde in PBS. Both immunolabeled coverslips, and coverslips that were fixed in glutaraldehyde immediately after sonication were then frozen, freeze dried in a freeze-etch unit, and rotary replicas were made as described previously (Heuser, 1989). Replicas were examined on Formvar-coated grids for electron microscopy. The gold particles in PM fragments (12 μm²) from 17 (insulin) or 13 (basal) different fields from two different experiments were quantitated. Gold particles were identified as punctate white dots on the membrane surface, surrounded by white halos.

Other Assays

Protein assays were performed using the bicinchoninic acid reagent (Pierce Chem. Co.), or the fluorescamine kit (Sigma Chem. Co.) according to the manufacturer's instructions. ATP levels were measured using the firefly luciferase assay kit (Sigma Chem. Co.) according to the manufacturer's instructions. Lactate dehydrogenase activity was measured using a previously described method (Dunn and Holz, 1983).

Statistical Analysis

Data are presented as mean ± SEM. In most instances, permeabilized cell data was normalized to the insulin response observed in intact cells from the same experiment. This was accomplished by subtracting the average fluorescence intensity of the basal PM lawns from the average intensity of PM lawns for each experimental condition. The data were then expressed as a percentage of the average insulin effect observed in intact cells within each experiment. Data were analyzed by two- or three-way analyses of variance using an unweighted means model. In some instances, two-tailed t tests were performed.
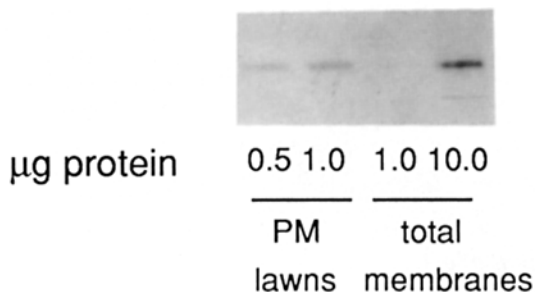
Results

Development of a Translocation Assay Using PM Lawns

In the present studies, we have used a previously described procedure for obtaining PM fragments or lawns (Heuser, 1989; Lin et al., 1991; Moore et al., 1987) to study GLUT4 translocation in 3T3-L1 adipocytes. This procedure is simple, rapid, and yields purified plasma membrane fragments with their cytoplasmic surfaces exposed. As shown in Fig. 1, the PM fragments generated by this procedure represent highly purified plasma membranes. As a measure of enrichment of the plasma membrane in this fraction, intact cells were labeled with biotinylated WGA at 0°C before isolation of the PM lawns. The PM lawns were sevenfold enriched in this cell surface marker when compared to total membranes (Fig. 1 a), representing an ∼25-fold purification over the cell homogenate. As a further index of the purity of this fraction, PM lawns were isolated from cells incubated in the presence or absence of insulin and immunoblotted with anti-GLUT4 antiserum (Fig. 1 b). Most notably, the PM fraction from basal cells contained negligible GLUT4. Hence, the insulin-dependent increase in GLUT4 levels in PM lawns was over ninefold. These data suggest that the higher levels of GLUT4 observed in basal PM isolated using conventional fractionation (Fig. 1 b) must arise from contamination of this fraction with intracellular membranes. By morphological criteria as well, these fragments are completely stripped of intracellular organelles yet retain structures characteristic of the PM such as coated pits, caveolae, and cytoskeletal elements (see Figs. 4 and 6). Furthermore, this PM lawn technique has been used to study assembly of clathrin-coated pits in vitro (Lin et al., 1991). Fig. 2 shows representative lawns that were isolated from basal or insulin-stimulated intact 3T3-L1 adipocytes and labeled with antibodies specific for GLUT1, GLUT4, and the Na^+/K^+-ATPase. The uniformity of Na^+/K^+-ATPase labeling among individual PM fragments indicates the reproducibility of this technique for isolating a purified plasma membrane fraction. In addition, large areas of PM fragments were obtained using this procedure, enabling a quantitative analysis of up to 80 individual fragments per coverslip using the confocal microscope.

There was no effect of insulin on the level of the Na^+/K^+-ATPase in PM lawns of intact cells, indicating that the overall composition of the PM was unchanged with insulin (Fig. 2, E and F). PM fragments from control cells exhibited high levels of GLUT1 labeling but very low levels of GLUT4 labeling. Insulin increased the levels of GLUT1 and GLUT4 in PM lawns by 2–3- and 8–10-fold, respectively (Fig. 2, A-D). Because of the difficulty in accurately quantifying the levels of GLUT4 in PM fragments from control cells, the

a) WGA-biotin

µg protein   0.5 1.0   1.0 10.0

            PM   total

       lawns   membranes

b) GLUT4

Insulin   −   +   −   +   −   +

       total   PM   crude
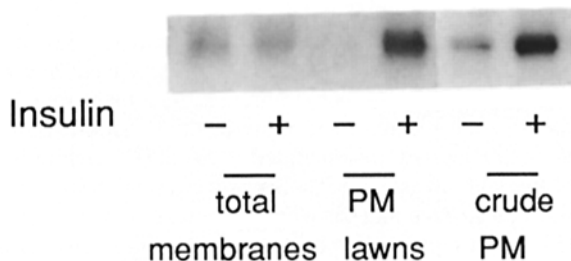
   membranes   lawns   PM

Figure 1. Assessment of the purification of PM lawns from 3T3-L1 adipocytes. (*a*) Cells attached to glass coverslips were incubated with biotinylated WGA (50 µg/ml) for 30 min at 0°C. Cells were washed in PBS, and PM lawn and total membrane fractions were obtained as described in the text. Protein was determined, and indicated amounts of PM lawns and total membranes were subjected to PAGE and transferred to nitrocellulose as previously described (Piper et al., 1991). Biotin was detected by a colorimetric assay as described in the text. (*b*) Cells attached to glass coverslips were incubated in the presence or absence of insulin (10^{-7} M) for 15 min at 37°C. PM lawns and total membrane fractions were prepared and protein was determined. 5 µg of PM lawns or total membranes and 10 µg of crude PM fractions were subjected to PAGE and Western blotting with an anti-GLUT4 antiserum as described previously (Piper et al., 1991).

magnitude of insulin-stimulated GLUT4 levels in the PM fraction may be underestimated using this technique. Nevertheless, the quantitative agreement between the immunofluorescence assay and direct immunoblotting of GLUT4 in PM lawns (see Fig. 1 *b*) provides additional validation for the use of the immunofluorescence assay. As shown in Fig. 2 *D*, variation in GLUT4 labeling between individual PM fragments was observed. This is not due to differential labeling efficiency or variability in the yield of the PM fraction because uniform labeling was observed using antibodies to GLUT1 and the Na⁺/K⁺-ATPase (Fig. 2, *B* and *F*). This variation most likely reflects the presence of cells at different stages of adipocyte differentiation because, unlike GLUT1 and the Na⁺/K⁺-ATPase, GLUT4 expression is switched on in a time-dependent manner during differentiation in 3T3-L1 cells (Tordjman et al., 1989). Despite this variation, all cells in any given field were used in our quantitative analysis of

PM labeling, which will also tend to underestimate the magnitude of GLUT4 translocation that occurs in fully differentiated adipocytes.

To further validate the PM lawn assay we compared the effects of insulin on GLUT1 and GLUT4 levels in the PM as determined by this method with the well-characterized differential centrifugation procedure (Piper et al., 1991; Simpson et al., 1983). There was good agreement between these methods in the relative amounts of GLUT1 in PM fractions from control and insulin-treated cells (Fig. 3). High levels of GLUT1 were evident in control PM fractions using both methods, and this labeling was increased by two- to threefold with insulin (Fig. 3). The magnitude of insulin-stimulated GLUT4 translocation was higher (8.1-fold ± 1.0, $n = 10$) using the PM lawn method compared with differential centrifugation (4.0-fold ± 1.3, $n = 5$). This is primarily due to the presence of significant amounts of GLUT4 in the PM fraction isolated by differential centrifugation from basal cells, which presumably derives from contamination of this fraction with intracellular membranes. It is noteworthy that using immunocytochemistry in brown adipose tissue (Slot et al., 1991*b*), cardiac muscle (Slot et al., 1991*a*), and 3T3-L1 adipocytes (Piper et al., 1991), we have observed very little GLUT4 labeling of cell surface membranes in control cells in agreement with the data obtained using the PM lawn method.

Distribution of GLUT4 in the Plasma Membrane As Determined by Immunogold Labeling

To study the distribution of GLUT4 in the PM from control and insulin-treated cells, PM lawns were fixed and labeled with the anti-GLUT4 antibody followed by protein A–gold (Fig. 4). Low levels of GLUT4 labeling were observed in the smooth uncoated regions of PM fragments from untreated control cells, while there was no detectable labeling of caveolae or cytoskeletal elements (Fig. 4 *a* and Table I). There was, however, significant GLUT4 labeling of clathrin lattices and clathrin-coated pits in PM fragments from nonstimulated cells. Treatment of cells with insulin caused a slight increase in GLUT4 labeling of clathrin lattices and pits (Table I; $t = 1.80$, df $= 28$, $0.10 > P > 0.05$), but a large (6.6-fold) increase in GLUT4 labeling of PM fragments outside of lattices and pits was observed (Table I; $t = 5.36$, df $= 28$, $P < 0.001$). As was the case in control cells, there was no labeling of either caveolae or cytoskeletal attachments after insulin treatment.

Use of the PM Lawn Assay to Demonstrate Insulin-Stimulated Translocation of GLUT4 in Permeabilized Cells

Several different methods were used to determine the extent of permeabilization caused by SLO. As shown in Fig. 5, we examined the staining of nuclei with propidium iodide. Intact cells incubated in IC buffer were not labeled with propidium iodide, whereas, over 95% of the nuclei from permeabilized cells were labeled with propidium iodide under the conditions used for the assay (Fig. 5). To examine whether both small and large molecules could diffuse out of the permeabilized cells, we measured release of ATP and lactate dehydrogenase (LDH) (150 kD) (Fig. 6). At 15 min
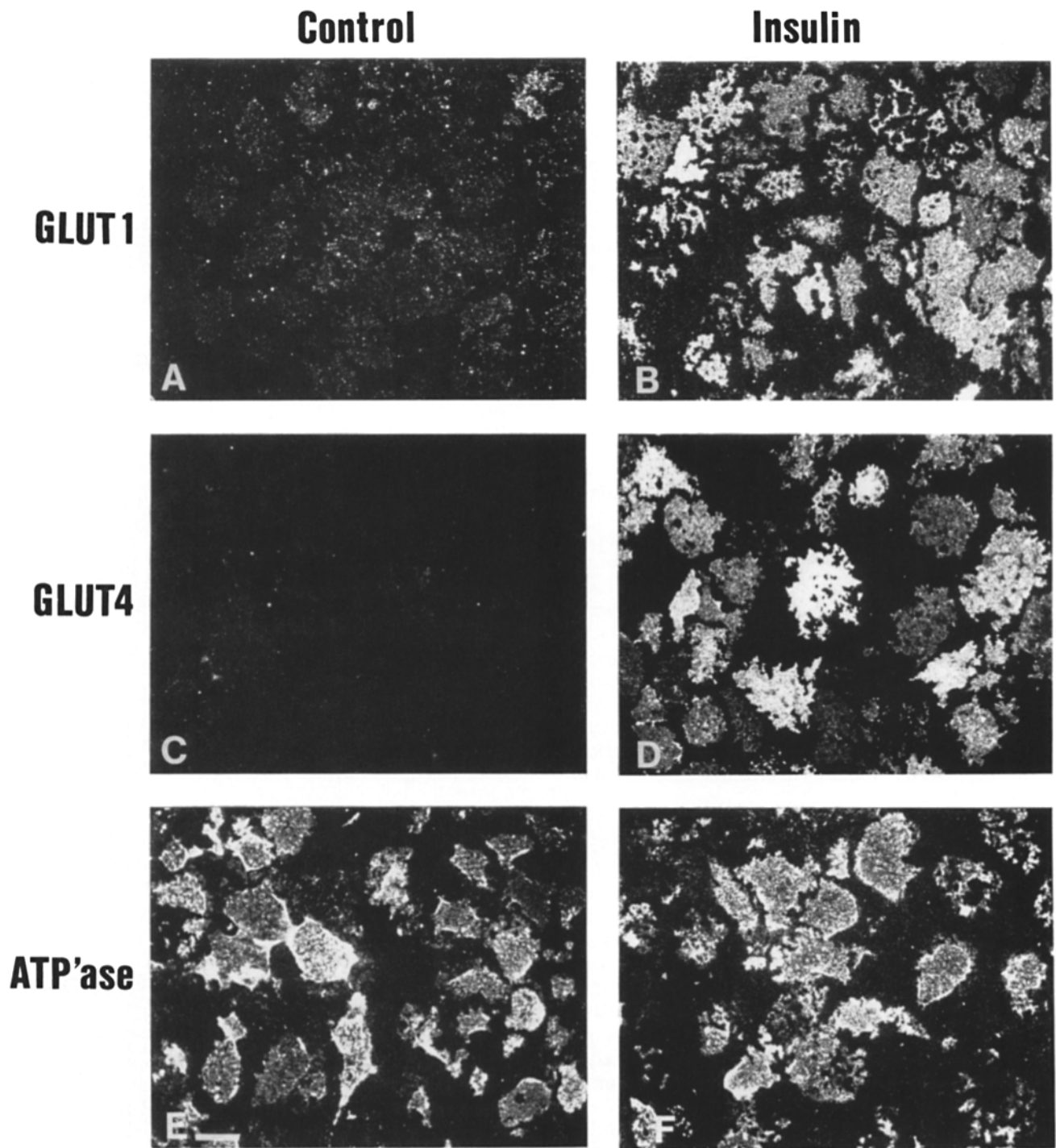
Figure 2. Immunofluorescence labeling of GLUT1, GLUT4, and the Na$^+$/K$^+$-ATPase in PM lawns isolated from intact 3T3-L1 adipocytes. Cells attached to glass coverslips were incubated in the absence (*A, C,* and *E*) or presence (*B, D,* and *F*) of insulin (10^{-7} M) for 15 min at 37°C. PM lawns were obtained as described in the text, fixed, and labeled with antibodies specific for GLUT1 (*A* and *B*), GLUT4 (*C* and *D*), or the Na$^+$/K$^+$-ATPase (*E* and *F*), followed by incubation with FITC-conjugated goat anti-rabbit IgG. PM lawns were examined using a scanning laser confocal immunofluorescence microscope. For each antibody, images were scaled identically regardless of the treatment. Bar, 15 μm.

after removal of the SLO, the termination point for most transport experiments, 95% of the intracellular ATP had been released. The release of LDH from SLO-permeabilized cells was linear for up to 30 min after removal of the SLO, such that only 33% of this protein was released from the cells

during the course of the assay (Fig. 6). As a final measure of permeabilization efficiency, we measured the amount of total cytosolic protein remaining in both intact and permeabilized cells at the end of the assay. Cytosol was prepared by homogenizing cells and removing membranes by centrifuga-
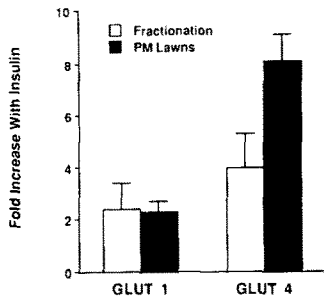
Figure 3. Quantitative comparison between subcellular fractionation and PM lawn techniques for measuring translocation of GLUT1 and GLUT4 in 3T3-L1 adipocytes. Intact 3T3-L1 adipocytes were incubated in the absence or presence of insulin (10^{-7} M) for 20 min at 37°C. Plasma membranes were isolated using either differential centrifugation (Piper et al., 1991) or the PM lawn method. GLUT1 and GLUT4 levels were quantified in these fractions by immunoblotting followed by quantitative densitometry of the autoradiograms, or by immunofluorescence microscopy, respectively. Data is expressed as the fold increase in levels of the glucose transporters with insulin above basal (mean ± SEM; $n = 5$ for fractionation, $n = 10$ for PM lawn method).

tion as described previously (Piper et al., 1991). There was a 25% decrease in the amount of cytosolic protein remaining in permeabilized cells as compared with intact cells at the conclusion of the transport assay (data not shown). To examine the integrity of the PM lawns after permeabilization, we compared PM fragments from intact and permeabilized cells by EM. The morphology of the PM lawns from permeabilized cells was well maintained and very similar to those of intact cells (Fig. 7). We did, however, observe significant changes in the morphology of the caveolae with permeabilization. The density of caveolae was increased with SLO treatment and the caveolae frequently formed large bunches consisting of up to 20 caveolae per bunch (Fig. 7 c). These were distinct from the rosettes that are often observed in intact cells.

To study the insulin-stimulated translocation of GLUT4 in permeabilized adipocytes, cells were treated with SLO, washed, and incubated with IC buffer containing an ATP regenerating system in the presence or absence of insulin (10^{-7} M) for 15 min (Fig. 8). GLUT4 labeling was measured by obtaining the average intensity of pixels within a PM fragment. PM fragments from at least 24 cells were quantitated for each 40× image, and at least two images were quantitated for each coverslip. These values were then averaged to obtain a single data point for each experimental condition. Permeabilization of basal adipocytes resulted in a significant increase in GLUT4 labeling over basal intact cells (permeabilized mean = 18.64 ± 2.86; control mean = 7.03 ± 0.78; t = 4.15; df = 28, P < 0.001). After incubation with insulin there was an increase in GLUT4 labeling of PM lawns (Fig. 8 B) to a level comparable to that observed in intact cells (see Fig. 2 D). The magnitude of the insulin effect in intact cells was variable between experiments. Because of this variability, we decided to normalize all permeabilized cell data with respect to the insulin effect obtained in intact cells for each experiment. To do this, the intensity of GLUT4

Table I. Quantitation of GLUT4-Immunogold Labeling in PM Fragments (Gold Particles/μm^2)

Region	Basal	Insulin
Coated lattices/pits	25.7 ± 5.8	34.8 ± 10.4
	(2.02 ± 0.21)	(2.78 ± 0.4)
Outside of clathrin areas	1.14 ± 0.33	7.51 ± 1.29*

Gold particles were quantitated, and results are expressed as the mean density of gold particles per μm^2 membrane ± SEM for 17 (basal) or 13 (insulin) different areas of plasma membrane (12 μm^2) from two different experiments. Numbers in parentheses refer to the mean number of gold particles per coated lattice/pit ± SEM.
* Significantly different from basal, P < 0.001.

labeling in the control intact cells was subtracted from each data point. Data were then expressed as a percentage of the insulin effect obtained in intact cells.

The ability of insulin to stimulate movement of GLUT4 in permeabilized cells required the addition of ATP because we did not observe a significant change with insulin when ATP was excluded from the transport assay (see below). The $t_{1/2}$ for insulin-stimulated movement in permeabilized cells was 7 min (Fig. 9) as compared with 6 min in intact cells (Piper et al., 1991). These data indicate that most of the essential factors required for insulin-stimulated movement of GLUT4 to the cell surface, with the possible exception of an energy source, are retained in SLO-permeabilized cells.

To show that we were reconstituting not only movement of GLUT4 to the cell surface, but also fusion of GLUT4 vesicles with the PM, the distribution of GLUT4 in the PM lawns of permeabilized cells was examined using immunocytochemistry. The pattern of GLUT4 labeling that we observed in permeabilized cells (data not shown) was essentially the same as that observed in intact cells (Fig. 4). These data provide direct evidence that in permeabilized 3T3-L1 adipocytes insulin stimulates both translocation and fusion of GLUT4 vesicles with the cell surface in a similar fashion to that observed in the intact cell.

Nucleotide Dependence of GLUT4 Translocation in Permeabilized Cells, as Measured by the PM Lawn Assay

While insulin-stimulated movement of GLUT4 to the PM was enhanced by the addition of ATP, evidence was also obtained to suggest that the removal of ATP resulted in translocation of GLUT4 in the absence of insulin (Fig. 10). A two-way analysis of variance revealed a main effect of insulin (F = 22.49; dfs = 1/40; P < 0.0005) and a significant interaction between insulin and ATP (F = 10.39; dfs = 1/40; P < 0.005). In the absence of an exogenous ATP source, or after apyrase or KCN treatment of cells, the levels of GLUT4 in the PM lawns of basal cells was significantly higher than those observed after ATP addition. The ATP analog ATPγS was as effective as ATP in preventing the

Figure 4. Electron microscopic localization of anti-GLUT4 IgG-gold (10 nm) in PM fragments isolated from basal (a) and insulin-treated (b) 3T3-L1 adipocytes. The circles indicate the location of GLUT4-gold particles on the membrane. Note the labeling of clathrin-coated lattices and pits (a and b), and some labeling outside of lattices and pits in (b). Bars, 0.25 μm.
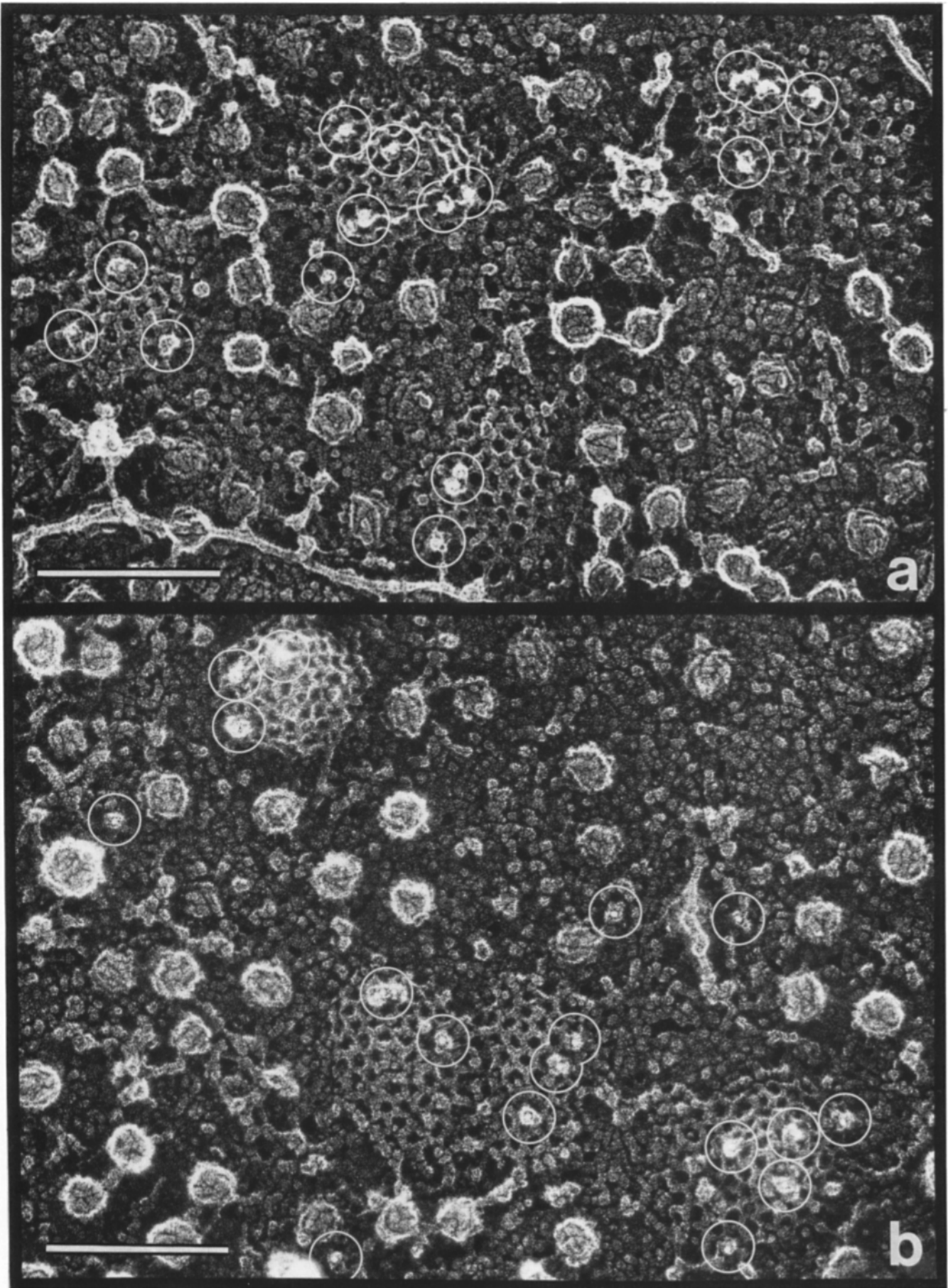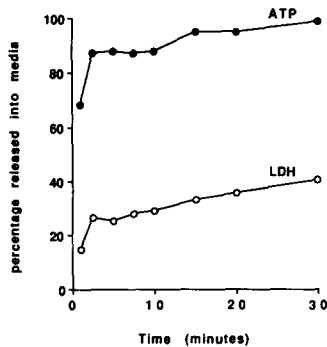
Permeabilized

Intact

1188
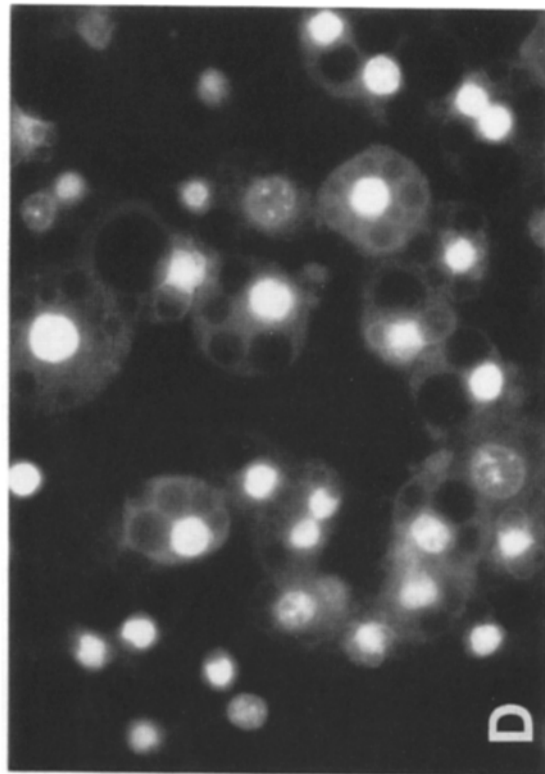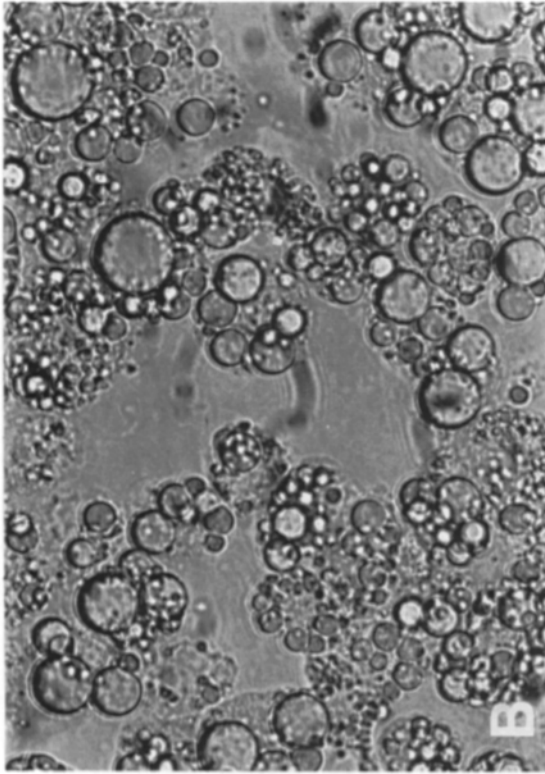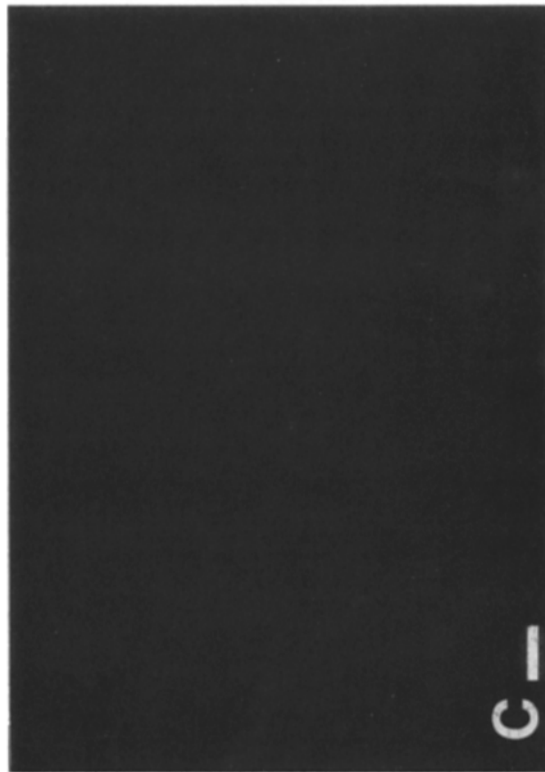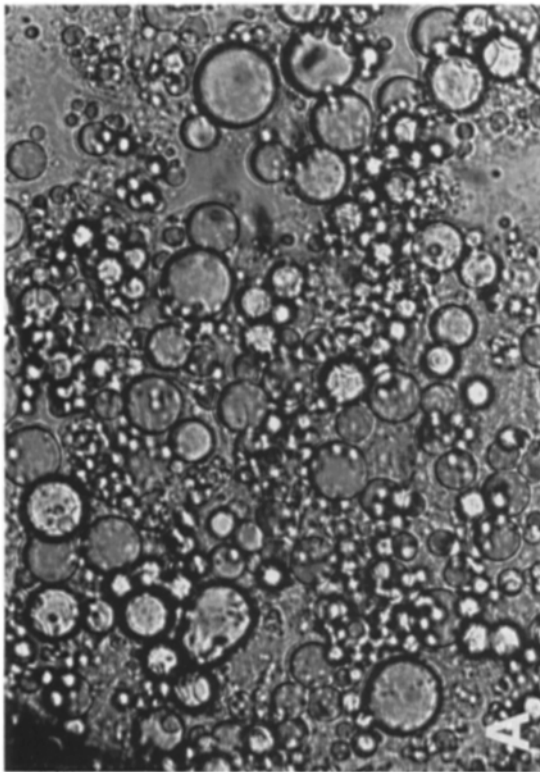
Figure 6. Release of ATP and LDH from permeabilized 3T3-L1 adipocytes. Adipocytes were permeabilized with SLO, washed with IC buffer, and incubated with IC buffer for 30 min at 37°C. Media samples were collected at the indicated times. Intact cells were studied under similar conditions except that they were incubated with KRP buffer throughout. At the end of the incubation period, cells were washed in IC buffer, scraped from the dish, and lysed by boiling for 3 min. Cells were pelleted, and the supernatant, as well as the media samples, were assayed for ATP activity (●) and LDH activity (○) as described in the text. Results are expressed as a percentage of the total ATP or LDH activity in nonpermeabilized cells.

elevated GLUT4 levels in basal plasma membrane fragments, suggesting a role for protein phosphorylation. The magnitude of insulin-stimulated GLUT4 translocation to the PM was markedly increased in the presence of an exogenous ATP supply (Fig. 10).

Use of the PM Lawn Assay to Demonstrate GTPγS-stimulated Translocation of GLUT4 in Permeabilized Cells

GTPγS, which has been shown to stimulate regulated exocytosis in many different cell types (Gomperts, 1990), stimulated the translocation of GLUT4 to the PM in permeabilized, but not intact, 3T3-L1 cells (Fig. 11). The single and combined effects of ATP, GTPγS, and insulin were examined in a series of experiments (Fig. 12). Addition of 100 μM GTPγS in the presence of ATP increased GLUT4 levels in PM lawns to the same extent as observed with insulin (compare Figs. 10 and 12). Another nonhydrolyzable analog of GTP, $G_{pp}NH_p$, also stimulated GLUT4 movement to the PM in the presence of ATP, whereas GDPβS, ATPγS, or $A_{pp}NH_p$ were without effect (data not shown). A three-way analysis of variance using ATP, insulin, and GTPγS as the variables, showed main effects of insulin (F = 10.86; dfs = 1/63; $P < 0.005$) and GTPγS (F = 10.53; dfs = 1/63; $P < 0.005$). A significant three-way interaction between the three factors (F = 4.07; dfs = 1/63; $P < 0.05$) suggested that the combined effect of insulin and GTPγS was ATP dependent. Two-way analyses of variance revealed a significant interaction between insulin and GTPγS in the presence (F = 5.20; dfs = 1/34; $P < 0.05$), but not in the absence (F = 0.31; dfs = 1/29; NS) of ATP. This was due to the additivity between insulin and GTPγS in the absence, but not the presence, of ATP. In an effort to further characterize this observation, we

examined the effects of insulin and GTPγS addition to permeabilized cells after an extensive 15-min washout to remove almost all of the intracellular ATP (see Fig. 6). However, in these experiments we were unable to observe significant movement to the PM with either agonist even in the presence of ATP. These studies suggest that other factors that regulate GLUT4 translocation may have been removed from permeabilized cells during this preincubation.

Reconstitution of Constitutive Secretion in Permeabilized 3T3-L1 Adipocytes—No Effect of GTPγS

As a critical control for any nonspecific effects of GTPγS on vesicle trafficking in our system, we investigated the effect of GTPγS on constitutive secretion in permeabilized 3T3-L1 cells. Previous studies in other cell types have shown that GTPγS either inhibits or has no effect on constitutive vesicle traffic (Beckers and Balch, 1989; Gravotta et al., 1990; Melançon et al., 1987; Miller and Moore, 1991). To study the constitutive secretory pathway in 3T3-L1 cells, we examined the release of adipsin, a serine protease that is secreted by adipocytes into the media (Table II). Adipsin was secreted at a high rate from intact 3T3-L1 adipocytes. In agreement with previous studies, insulin caused a modest stimulation of adipsin secretion in intact cells. In permeabilized cells, adipsin secretion was almost completely blocked by incubating cells at 0°C, or by incubating cells at 37°C in the absence of ATP. In the presence of ATP, however, the rate of adipsin secretion returned to levels comparable to those observed in intact cells. This is in agreement with numerous other studies showing an ATP dependence for the constitutive secretory pathway. In contrast to its effects on GLUT4, GTPγS had no significant effect on the rate of adipsin secretion in permeabilized 3T3-L1 cells (Table II). These data suggest that the GTPγS-mediated stimulation of GLUT4 translocation does not reflect a nonspecific effect of this analog on vesicle traffic to the cell surface.

Effect of Insulin and GTPγS on GLUT4 Movement in Noninsulin-sensitive Cells

3T3-L1 fibroblasts do not express GLUT4, nor do they exhibit insulin-dependent glucose transport (James et al., 1989). GLUT4 has been expressed in 3T3-L1 fibroblasts using DNA transfection and, morphologically, the targeting of this transporter in these cells is similar to that observed in adipocytes (Haney et al., 1991). Incubation of nonpermeabilized 3T3-L1 fibroblast transfectants with insulin or IGF I did not cause a significant change in GLUT4 labeling of PM lawns (data not shown), consistent with previous findings (Haney et al., 1991). These studies were performed using cells in which GLUT4 expression was induced via the metallothionein promoter yielding levels of GLUT4 at least two-

Figure 5. Quantitative permeabilization of 3T3-L1 adipocytes with SLO. 3T3-L1 adipocytes were washed three times with IC buffer and incubated for 5 min in IC buffer containing 0.8 IU/ml of SLO at 37°C (B and D). Intact cells (A and C) were maintained in IC buffer throughout. Cells were washed and incubated with IC buffer containing the membrane-impermeant fluorescent dye propidium iodide (1 μg/ml) for an additional 3 min, followed by three washes in PBS. Cells were examined by either phase (A and B) or fluorescence (C and D) microscopy. Bar, 15 μm.

Permeabilized
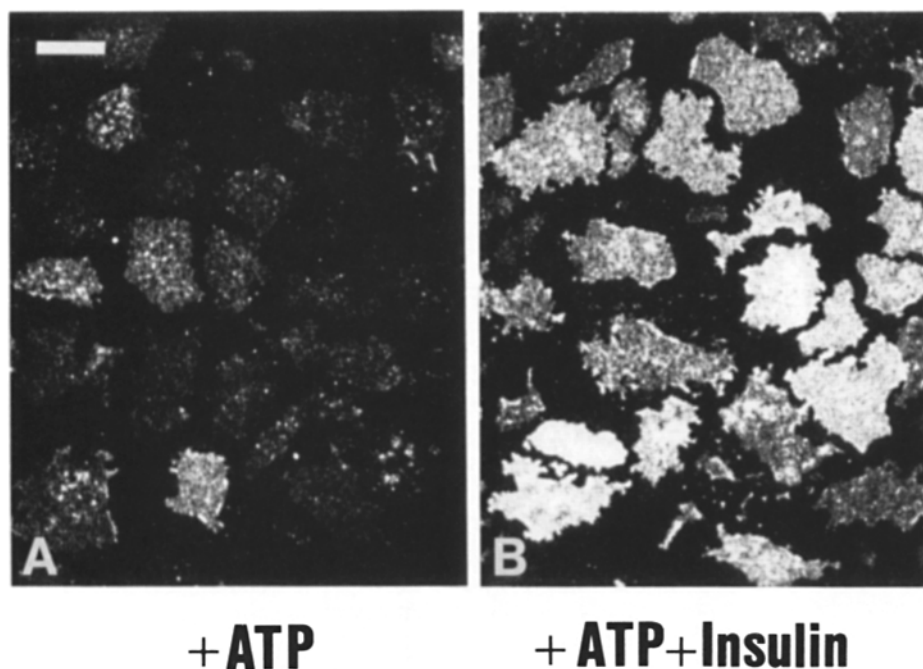

+ATP   +ATP+Insulin
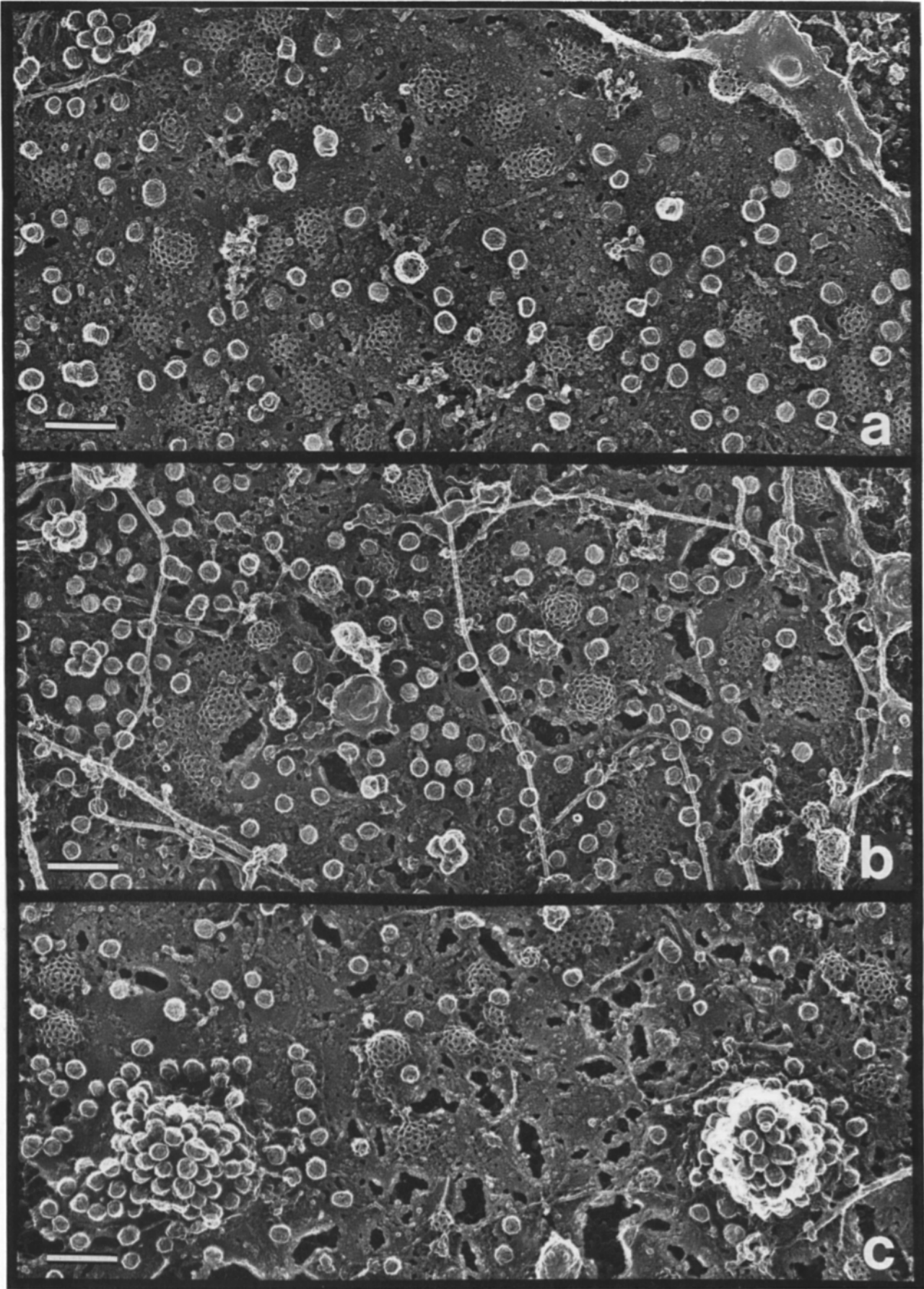
Figure 8. Effect of insulin on GLUT4 translocation in permeabilized 3T3-L1 adipocytes. Adipocytes attached to glass coverslips were permeabilized with SLO and incubated in IC buffer containing an ATP regenerating system for 15 min with no additions (*A*) or with 10^{-7} M insulin (*B*). PM lawns were obtained as described in the text, fixed, and labeled with antibodies specific for GLUT4 followed by FITC-conjugated goat anti–rabbit IgG. Bar, 15 μm.

to threefold higher than observed in adipocytes. As shown in Fig. 13 *F*, the 3T3-L1 fibroblast transfectants used in these studies contained very high levels of GLUT4 labeling in the perinuclear region. Hence, it seems likely that if GLUT4 had moved under these conditions we would have been able to detect it using our assay. These data indicate that critical elements of the insulin signal transduction machinery required for GLUT4 translocation may be absent from 3T3-L1 fibroblasts.

We next wanted to test the effects of GTPγS in permeabilized fibroblasts because we reasoned that this nucleotide may operate beyond the point at which the insulin/IGF I effect is compromised in these cells. Neither insulin, IGF I, nor GTPγS stimulated movement of GLUT4 to the PM in permeabilized fibroblasts (Fig. 13, *A–C*). To visualize the PM fragments from fibroblasts, the contrast settings in the analysis software on the confocal microscope were increased compared with the settings used for adipocyte experiments. Under these conditions we only observed nonspecific labeling due to entrapment of the fluorescent secondary antibody within the folded edges of individual PM fragments. Inclusion of cytosol isolated from 3T3-L1 adipocytes, bovine brain, rat brain, or rat skeletal muscle during the transport assay did not provide any significant movement of GLUT4 in the transfectants (data not shown).

Discussion

A critical determinant for studying the movement of a mem-

brane protein between two cellular compartments is that the assay must be able to assess that the reporter protein has undergone movement from the donor compartment and also that it has fused with the acceptor membrane. In the case of the glucose transporter, the development of such an assay has been difficult because there is no evidence that the transporter undergoes any covalent modification during its transit from the intracellular tubulovesicular elements to the cell surface. Most studies of glucose transporter movement in the cell have used differential centrifugation procedures to isolate a PM fraction (Simpson et al., 1983; Suzuki and Kono, 1980). However, this fraction is somewhat crude and may contain other intracellular membranes. Thus, this assay does not provide direct proof for incorporation of the transporter into the PM. A method for isolating PM fragments that are free of contaminating intracellular organelles has been described (Heuser, 1989; Lin et al., 1991; Moore et al., 1987). We have used three criteria to show that plasma membranes isolated from 3T3-L1 adipocytes using this technique are highly purified. Morphologically, these fragments appear to contain only elements derived from the PM. The level of GLUT4 in the PM lawns isolated from basal cells is very low, in agreement with previous localization studies (Slot et al., 1991*b*). Most importantly, we observed an enrichment in cell surface–bound biotinylated WGA in the PM lawns by ~25-fold as compared with a total cell homogenate. We have used this method to study factors that are involved in controlling the movement of the glucose transporter to the cell surface in 3T3-L1 adipocytes. GLUT1 and GLUT4 immunolabeling

Figure 7. Deep-etch freeze-fracture electron micrographs of PM lawns isolated from intact (*a*) and permeabilized (*b* and *c*) 3T3-L1 adipocytes. Note the clusters of caveolae in *c*. Bars, 0.25 μm.
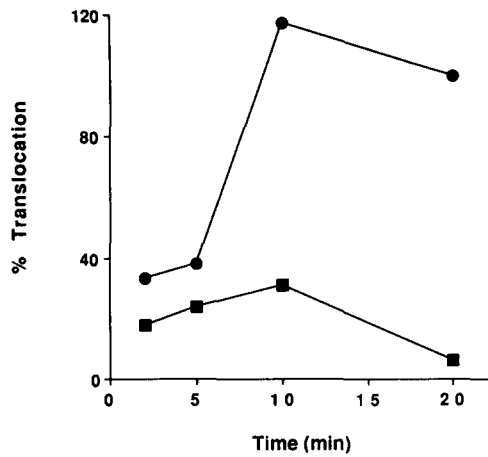
Figure 9. Time course of insulin-stimulated translocation of GLUT4 in permeabilized 3T3-L1 adipocytes. Cells were permeabilized with SLO, washed with IC buffer, and incubated for different times as indicated in the absence (■) or presence (●) of 10^{-7} M insulin in IC buffer containing an ATP regenerating system. GLUT4 levels were quantified in PM lawns using immunofluorescence microscopy. Percent translocation refers to the relative intensity of GLUT4 labeling in PM lawns under the conditions indicated expressed as a percentage of GLUT4 labeling in PM lawns from intact cells treated with 10^{-7} M insulin for 15 min at 37°C. The intensity of GLUT4 labeling in control intact cells was subtracted from each data point.

of these fragments was readily detectable using immunofluorescence microscopy. The relative labeling of these antigens in PM fragments from control and insulin-treated cells was quantitatively comparable to that observed using alternate methods. This method also provides the more rigorous re-
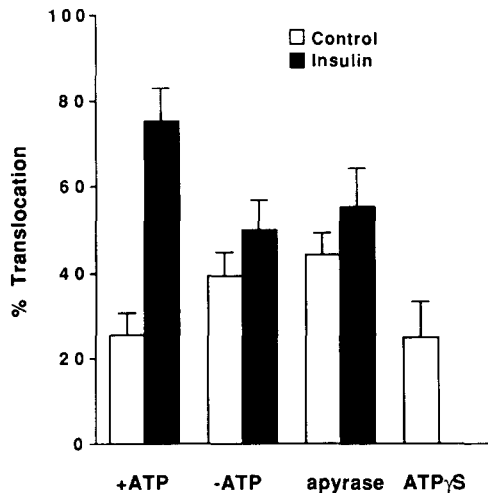

Figure 10. Effects of ATP on insulin-induced translocation of GLUT4 in permeabilized 3T3-L1 adipocytes. Adipocytes attached to glass coverslips were permeabilized with SLO followed by an incubation in IC buffer containing an ATP regenerating system (+ATP), no additions (−ATP), 5 U/ml apyrase (apyrase), or 100 μM ATPγS (ATPγS), in the presence or absence of 10^{-7} M insulin. PM lawns were quantitated as described in the text. Percent translocation was calculated as described in Fig. 9 (mean ± SEM from 4 to 13 different experiments).

quirements that vesicles must fuse with the plasma membrane, and that the transporters must be incorporated into the PM in order to be detected.

A major advantage of the PM lawn method is that when coupled with EM it can be used to verify the morphological integrity of the fragments. In addition, using gold-labeled secondary antibody it is possible to study the distribution of the antigen within the PM at the EM level (Fig. 4 and Table I). In nonstimulated adipocytes GLUT4 labeling of the PM was low, consistent with other studies (Piper et al., 1991; Slot et al., 1991b). However, significant GLUT4 labeling of clathrin lattices in the plasma membrane of nonstimulated cells was observed. These lattices contained the highest density of GLUT4 labeling of any PM structure in basal cells (Table I). In addition, GLUT4 labeling of the flat clathrin lattices was slightly increased with insulin treatment. In support of these findings we have previously observed an insulin-dependent increase in GLUT4 labeling of clathrin-coated pits and vesicles in rat brown adipocytes and cardiac myocytes using ultra-thin section EM (Slot et al., 1991a,b). The implication from these data is that GLUT4, like many cell surface receptors, constantly recycles from the PM both in the absence and presence of insulin. The presence of GLUT4 in clathrin lattices of nonstimulated cells suggests that under these conditions at least a proportion of the intracellular GLUT4 makes its way into the recycling pathway. Hence, the predominant intracellular location of this transporter in nonstimulated cells is probably a function both of its sequestration within the tubulo-vesicular elements as well as its efficient internalization from the cell surface. These properties suggest that, like cell surface receptors that recycle between the *trans*-Golgi reticulum and the PM, (for review see Pearse and Robinson, 1990), GLUT4 probably has a specific domain that interacts with adaptor proteins. Given the difference in secondary structure between GLUT4 and the other proteins that bind to adaptins (Pearse and Robinson, 1990), it will be of interest to see if there is any conservation in the targeting domain between these proteins. As to how insulin influences the subcellular distribution of GLUT4 is not presently clear. This may occur either by stimulation of GLUT4 exocytosis, slowing of internalization, or a combination of both. The insulin-dependent increase in GLUT4 labeling of early endosomes previously observed in brown adipose tissue suggests that stimulation of GLUT4 exocytosis may be the major step regulated by insulin (Slot et al., 1991b).

Permeabilized and semiintact cells have been invaluable for studying the regulation of vesicle and protein targeting. A major goal of the present studies was to establish a permeabilized cell system for investigating insulin-stimulated movement of the glucose transporter to the cell surface in 3T3-L1 adipocytes. A number of criteria indicated that 3T3-L1 adipocytes were quantitatively permeabilized with SLO. Propidium iodide was excluded from intact cells whereas it was efficiently taken up by >95% of cells after the addition of SLO (Fig. 5). ATP levels in permeabilized cells declined to 5% of those observed in intact cells within 15 min after addition of SLO. Also, in contrast to its effects in permeabilized cells, GTPγS had no effect on GLUT4 translocation to the PM in intact cells (Fig. 11 A). In terms of vesicle-mediated trafficking, the integrity of the permeabilized cells appeared to be preserved. Insulin stimulated the transloca-
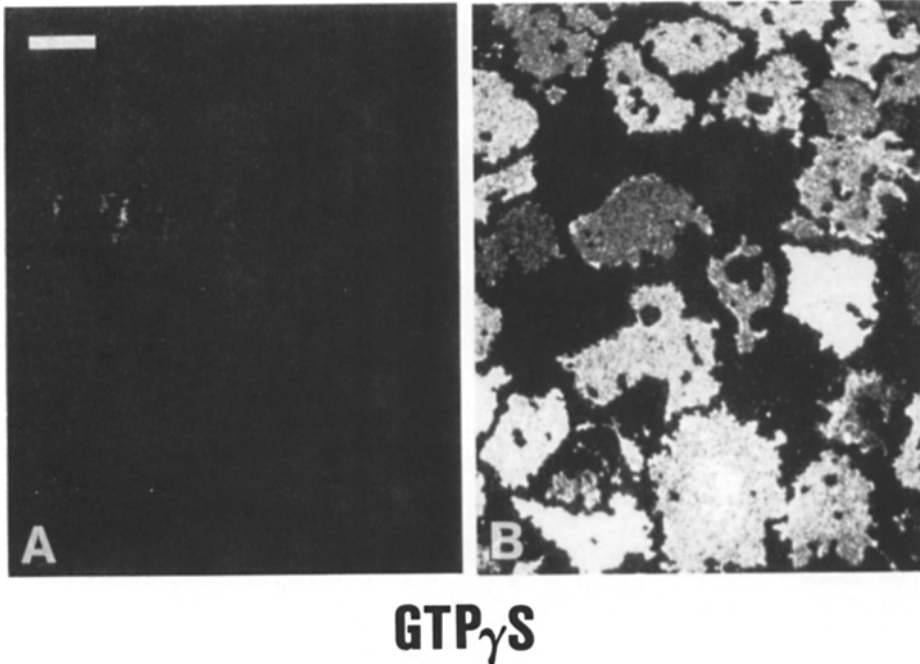
Intact

Permeabilized

Figure 11. Effect of GTPγS on translocation of GLUT4 in intact (*A*) and permeabilized (*B*) 3T3-L1 adipocytes. Intact or permeabilized adipocytes were incubated with 100 μM GTPγS for 15 min at 37°C, and PM lawns were obtained as described in the text. PM lawns were then labeled with an antibody to GLUT4 followed by immunofluorescence microscopy. Bar, 15 μm.

tion of GLUT4 to the PM in permeabilized 3T3-L1 adipocytes with similar efficiency and kinetics to that observed in intact cells (Fig. 9). In intact 3T3-L1 adipocytes, maximal insulin stimulation causes a change in the steady-state distribution of GLUT4 (Piper et al., 1991). However, even under these conditions, only a finite proportion of the intracellular GLUT4 is redistributed to the cell surface, suggesting that either GLUT4 is rapidly internalized, or that there is a large
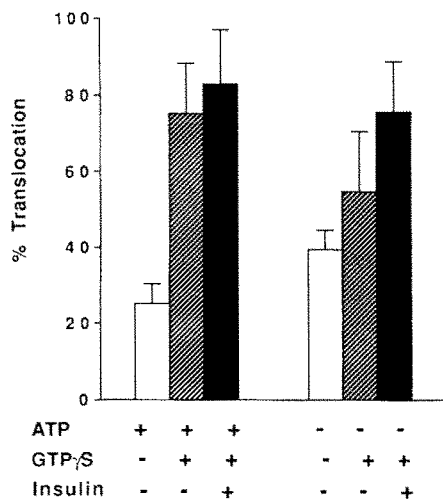
subcompartment of GLUT4 that does not recycle after insulin stimulation. The fact that we observed a similar change in the steady-state distribution of GLUT4 in insulin-treated permeabilized cells to that observed in intact cells suggests that all elements of GLUT4 regulation (i.e., recycling and/or sequestration) are maintained after treatment with SLO. The morphology of the PM fragments and the distribution of GLUT4 among different regions of the plasma membrane appeared to be identical in intact and permeabilized cells (Fig. 7). Furthermore, other vesicle-mediated processes such as adipsin secretion continued to function efficiently in permeabilized cells (Table II). This was an important control in these studies indicating that multiple secretory pathways, aside from the GLUT4 pathway, were operating under these conditions.

Both insulin and GTPγS stimulated the movement of

Figure 12. Effects of ATP and insulin on GTPγS-induced translocation of GLUT4 in permeabilized 3T3-L1 adipocytes. Adipocytes attached to glass coverslips were permeabilized with SLO and incubated in IC buffer in the presence (*+ATP*) or absence (*−ATP*) of an ATP regenerating system containing insulin (10⁻⁷ M), GTPγS (100 μM), or both insulin and GTPγS as indicated. Percent translocation was calculated as described in Fig. 9 (mean ± SEM from 5 to 13 different experiments).

Table II. Reconstitution of Adipsin Secretion in Permeabilized 3T3-L1 Adipocytes

Condition	Adipsin Secretion
	(*U/min*)
Intact − insulin	1.36
Intact + insulin	3.11
SLO − ATP	0.40
SLO + ATP	2.61
SLO + ATP/+GTPγS	2.32
SLO + ATP/0°C	0.11

Adipocytes were permeabilized with SLO, washed with IC buffer, and incubated with IC buffer containing the indicated treatments for 30 min at 37°C. Media samples were collected at various intervals for measurement of adipsin by immunoblotting. Intact cells were studied under similar conditions except that they were incubated with KRP buffer throughout. The final concentration of the various additions was as follows: ATP, 10 mM; GTPγS, 100 μM; insulin, 10⁻⁷ M.
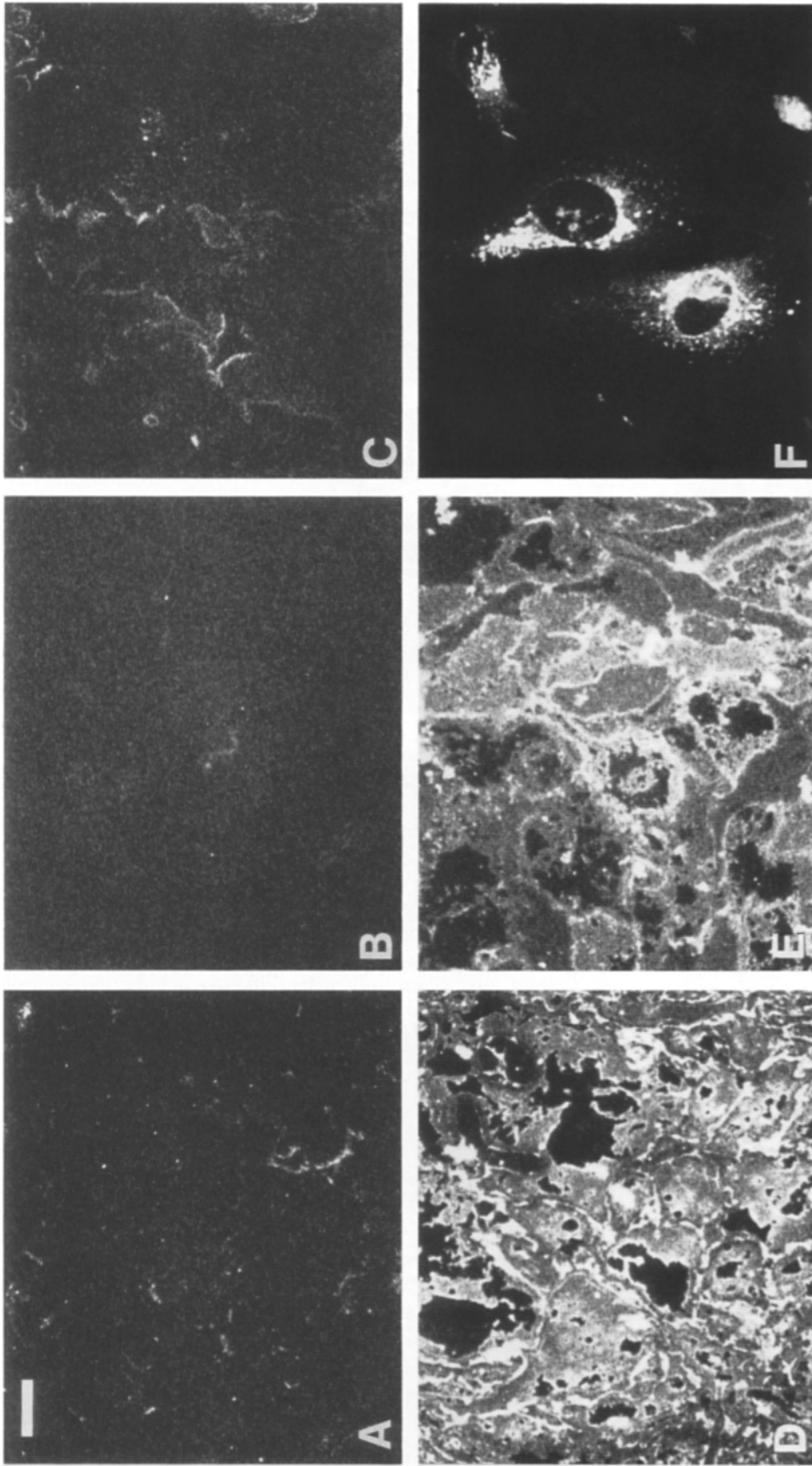
Figure 13. Effects of insulin+IGFl and GTPγS in permeabilized 3T3-L1 fibroblasts transfected with GLUT4. PM lawns from SLO-permeabilized 3T3-L1 fibroblasts expressing similar levels of GLUT4 to those observed in adipocytes (Haney et al., 1991) were analyzed using the same procedures as described for 3T3-L1 adipocytes in the presence of ATP. Shown are PM lawns from control (*A*), insulin- and IGFl-treated (*B*), or GTPγS-treated cells (*C*) labeled with anti-GLUT4 antisera (*A–C*). PM lawns from control cells were labeled with anti-Na⁺/K⁺-ATPase antisera (*D*) or anti-GLUTl antisera (*E*), and examined using the scanning laser confocal microscope. *F* shows whole cells labeled with anti-GLUT4 antisera. Bar, 30 μm.

GLUT4 to the PM in permeabilized 3T3-L1 adipocytes. These data are in agreement with a recent study (Baldini et al., 1991) where it was shown that insulin and GTPγS stimulated GLUT4 translocation in rat adipocytes that were permeabilized with α-toxin. In contrast to the studies by Baldini et al. (1991), we found that in order to obtain the same level of translocation with insulin or GTPγS in permeabilized cells that is observed with insulin in intact cells, it was necessary to provide a source of ATP during the transport assay. We observed that removal of ATP per se activated GLUT4 translocation, an effect that was prevented by the addition of exogenous ATP. Previous studies have suggested that ATP is involved in maintaining low glucose transport rates in the nonstimulated state. Metabolic poisons or hypoxia, for example, markedly activate glucose transport in skeletal muscle and heart (for review see Gould, 1984). One implication from these data is that the exclusion of GLUT4 from the plasma membrane is regulated by phosphorylation/dephosphorylation of a regulatory protein(s). In support of this hypothesis, it has recently been shown that the phosphatase inhibitor, okadaic acid, causes a marked increase in the levels of GLUT4 in the PM of intact rat adipocytes (Lawrence et al., 1990). Furthermore, removal of ATP has been shown to prime or stimulate regulated exocytosis in mast cells (Tatham and Gomperts, 1989) and in basophilic leukemia cells (De Matteis et al., 1991). The observation that ATPγS was able to substitute for ATP in preventing the increase in cell surface GLUT4 levels in the absence of insulin (Fig. 10) is also consistent with regulation by a phosphoprotein. In contrast to adipocytes, 3T3-L1 fibroblasts transfected with GLUT4 did not demonstrate increased PM GLUT4 levels after removal of cytosolic ATP (data not shown). Because these cells are also incapable of hosting the insulin-facilitated movement of GLUT4 to the PM, it is conceivable that insulin treatment and removal of ATP stimulate exocytosis of GLUT4 via a common pathway in adipocytes, whereas fibroblasts may be missing factor(s) common to this pathway.

GTPγS and G$_{pp}$NH$_p$ were as effective as insulin in stimulating movement of GLUT4 to the cell surface in permeabilized cells. This effect appeared to be specific to nonhydrolyzable analogues of GTP because other analogues (GDPβS, ATPγS, and A$_{pp}$NH$_p$) were without effect on GLUT4 movement. The fact that GTPγS was not additive with insulin in stimulating GLUT4 translocation in the presence of ATP suggests that these two effectors may act at different loci along the same pathway. We can not exclude the possibility that the amount of transporter translocated with either insulin or GTPγS alone represents the maximal level of translocation obtainable under these conditions. However, this seems unlikely because we have previously shown that at a maximal concentration of insulin only 10% of the intracellular GLUT4 compartment is translocated to the PM in intact cells (Piper et al., 1991). The ability of GTPγS plus insulin to cause recruitment of GLUT4 to the PM without ATP addition was a curious finding in view of the limited effectiveness of each agonist on its own. Based on the absence of any additivity between either effector in the presence of ATP we feel it is unlikely that these data reflect the operation of separate signal transduction pathways. On the contrary, we interpret these data to imply a direct interaction between insulin and GTPγS in the signal transduction pathway for GLUT4 recruitment to the PM. The ability of the two agonists to-

gether to overcome the ATP dependence indicates that insulin and GTPγS may operate in a cooperative fashion to trigger GLUT4 movement. For example, insulin may regulate the ability of a regulatory GTP binding protein to function. Further study will be required to elucidate the interaction between these agonists and to define the ATP dependence of distinct steps in the pathway.

Several reports have suggested that GTPγS inhibits constitutive secretion (Miller and Moore, 1991), whereas it stimulates the regulated secretory pathway (Gomperts, 1990). The GTPγS-stimulated movement of GLUT4 to the PM in adipocytes (Fig. 12) suggests that the regulated movement of GLUT4 in insulin-sensitive cells may be analogous to the regulated secretory pathway in so-called professional secretory cells. This stimulation did not occur in 3T3-L1 fibroblasts transfected with GLUT4 (Fig. 13). The level of GLUT4 expression observed in these cells was comparable to that observed in 3T3-L1 adipocytes (Haney et al., 1991) so that we should have been able to detect movement in our assay even if it had occurred at a slightly reduced efficiency compared with adipocytes. We were also unable to complement GLUT4 movement in these cells by adding cytosol from insulin-sensitive cells (data not shown). These data lend further support to the hypothesis that the "regulated" pathway of insulin-stimulated glucose transporter translocation is cell specific, and that the expression of GLUT4 per se is not sufficient for insulin-stimulated glucose transport. To directly compare the regulated movement of GLUT4 with the constitutive secretory pathway in adipocytes, we studied the effect of GTPγS on adipsin secretion. GTPγS neither stimulated nor inhibited adipsin secretion in permeabilized adipocytes. The fact that GTPγS did not inhibit adipsin secretion probably indicates, by analogy with other studies (Gravotta et al., 1990), that we are studying a late stage of the constitutive secretory pathway beyond the point of GTPγS inhibition.

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