Targeting of the "Insulin-responsive" Glucose Transporter (GLUT4) to the Regulated Secretory Pathway in PC12 Cells

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Abstract. Insulin-activated glucose transport depends on the efficient sorting of facilitated hexose transporter isoforms to distinct subcellular locales. GLUT4, the "insulin-responsive" glucose transporter, is sequestered intracellularly, redistributing to the cell surface only in the presence of hormone. To test the hypothesis that the biosynthesis of the insulin-responsive compartment is analogous to the targeting of proteins to the regulated secretory pathway, GLUT4 was expressed in the neuroendocrine cell line, PC12. Localization of the transporter in differentiated PC12 cells by indirect immunofluorescence revealed GLUT4 to be in the perinuclear region and in the distal processes. Although, by immunofluorescence microscopy, GLUT4 co-localized with the endosomal protein transferrin receptor and the small synaptic vesicle (SSV) marker protein synaptophysin, fractionation by velocity gradient centrifugation revealed that GLUT4 was excluded from SSV. Immunoelectron microscopic localization indicated that GLUT4 was indeed targeted to early and late endosomes, but in addition was concentrated in large dense core vesicles (LDCV). This latter observation was confirmed by the following experiments: (*a*) an antibody directed against GLUT4 immunoadsorbed the LDCV marker protein secretogranin, as assayed by Western blot; (*b*) ~85% of secretogranin metabolically labeled with ^{35}S-labeled sulfate and allowed to progress into secretory vesicles was coadsorbed by an antibody directed against GLUT4; and (*c*) GLUT4 was readily detected in LDCV purified by ultracentrifugation. These data suggest that GLUT4 is specifically sorted to a specialized secretory compartment in PC12 cells.

One of the most prominent and best-characterized effects of insulin is to promote the uptake of sugar into muscle and adipose tissue during the absorptive period. Abundant experimental evidence has indicated that this physiological response is in large part mediated by the redistribution of glucose transporters from an intracellular site to the plasma membrane, thereby augmenting the facilitated flux of glucose into the cell (4). Thus, a complete understanding of the molecular mechanisms underlying insulin-stimulated glucose uptake involves the characterization of the routes of intracellular trafficking of hexose carriers.

Non-active glucose transport in mammalian cells is catalyzed by a class of integral membrane glycoproteins, of which there are currently five recognized isoforms (2). One such facilitated transporter, GLUT4, displays a tissue distribution correlating with insulin responsiveness, and has been implicated as essential to the full, hormonal activation of transport (3, 13, 22). After stimulation of adipose cells with insulin, there is a substantial (10- to 40-fold) increase in the number of GLUT4 molecules on the cell surface which parallels the augmentation in transport (19, 37). The more ubiquitous glucose transporter, GLUT1, is present at greater relative abundance on the plasma membrane in the basal state and increases to a lesser extent in response to insulin. Moreover, there is some evidence that GLUT1 and GLUT4 reside within different intracellular compartments in rat adipocytes, and possess within their primary structures distinct signals for intracellular trafficking (42). For example, immunofluorescence microscopy studies of the cultured adipocyte cell line 3T3-L1 as well as fibroblasts transfected with transporter cDNAs has shown the distribution of GLUT4 to be predominantly perinuclear, whereas GLUT1 is primarily present on the plasma membrane (18, 20, 33, 36).

The nature of the intracellular compartment in which GLUT4 resides in cells unexposed to insulin remains a persistent unsolved problem. One possibility is that the machinery which directs efficient intracellular sorting of GLUT4 is present in all cells, possibly as a sub-compartment of endosomes (18). Alternatively, insulin-sensitive cell types may contain a preexisting specialized organelle to which GLUT4 is specifically targeted and which is capable of hormone-stimulatable translocation (4). The latter model

suggests a striking parallel between the formation of GLUT4-containing vesicles and the process of regulated secretion, in which products destined for storage and regulated release are actively sorted into secretory vesicles in the *trans*-Golgi network (TGN)[1] (6, 17). Evidence derived from the DNA-mediated gene transfer of secretory products into heterologous cell types suggests that proteins targeted to the regulated secretory pathway contain sorting signals which are conserved among different exocrine and neuroendocrine cell types (6, 30, 31, 35). Thus, should GLUT4 be directed to a mature, insulin-responsive compartment in adipocytes by a process analogous to the biosynthesis of regulated secretory vesicles, the determinants of sorting might well be recognized as such in a neuroendocrine cell type. Though there are no data to indicate whether such signals are also used by non-secretory cells for processes other than classical regulated exocytosis, this intriguing possibility provided the rationale for the present experiments, in which GLUT4 was expressed in the neuronal-like cell line PC12. Since the rate of internalization of vesicle membrane proteins is usually too rapid to allow their detection on the plasma membrane even after stimulated exocytosis, the strategy used in these studies was to co-localize GLUT4 with well-established markers of the regulated secretory pathway (12, 28, 38, 41).

Materials and Methods

Materials

^{125}I-labeled protein A and ^{35}S-labeled sulfate were purchased from ICN-Flow (Irvine, CA), and ^{3}H-labeled norepinephrine was from New England Nuclear (Boston, MA). FITC- and rhodamine-conjugated secondary antibodies were from Tago, Inc. (Burlingame, CA), Geneticin (G418) and Lipofectin reagent were from GIBCO-BRL (Gaithersburg, MD), and rat-tail collagen was from Biomedical Technologies (Stoughton, MA). NGF was purchased from the Director of Research at the University of Michigan (Flint, MI). Magnespheres were from Promega (Madison, WI), and WGA coupled to biotin (WGA-biotin) was purchased from Vector Laboratories (Burlingame, CA). All other chemicals were obtained from Sigma Immunochemicals (St. Louis, MO).

Antisera directed against synaptophysin and secretogranin were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN) or were provided by Dr. Wieland Huttner (EMBL, Heidelberg, Germany). Antisera directed against the transferrin receptor was a gift of Dr. Roger Davis (University of Massachusetts, Worcester, MA), or Dr. Ian Trowbridge (Salk Institute, San Diego, CA) and α-GLUT1 antisera was kindly provided by Dr. William Knowles (Miles Inc., West Haven, CT).

DNA Constructs, Cell Culture, and Gene Transfer

cDNAs encoding rat GLUT4 and GLUT1 were placed downstream of a viral LTR in the expression vectors pDOJ-SM and pDOJ-GT, respectively, and were introduced into the retroviral packaging cell line ψ2 (kindly provided by Dr. C. Cepko, Harvard Medical School, Boston, MA), for production of replication-incompetent recombinant retrovirus (20, 27). PC12 cells were grown on collagen-coated plates in DME supplemented with 10% horse serum and 5% FBS in an atmosphere of 5% CO_2 at 37°C. Cells infected with retrovirus encoding *neo* resistance were grown in the same medium supplemented with 400 μg/ml G418 (active concentration). Drug-resistant colonies, which were observed 10–14 d after the initiation of selection, were pooled and maintained in media containing 400 μg/ml G418.

1. *Abbreviations used in this paper*: hGH, human growth hormone; LDCV, large dense core vesicles; PAM, peptidylglycine α-amidating monooxygenase; PNS, postnuclear supernatants; SSV, small synaptic vesicles.

Immunofluorescence

Cells plated on glass coverslips and differentiated in medium containing 50 ng/ml NGF 2–6 d before an experiment were fixed in 3% paraformaldehyde and processed for indirect immunofluorescence as described (13, 20). For synaptophysin labeling, fixation was accomplished by immersion in ice cold methanol for 5 min, followed by acetone for 30 s. For detection of GLUT4, an affinity-purified antisera directed against the carboxyl terminus of rat GLUT4 was used (3). For localization of GLUT1, either affinity purified or non-purified anti-carboxyl terminal peptide antisera (East Acres, Southbridge, MA) were used with identical results. Coverslips were mounted in Mowiol and visualized with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Subcellular Fractionation

Glycerol gradients were performed as described by Linstedt and Kelly (26) with modifications. Non-differentiated cells were triturated off two 10-cm plates into 1 ml of cold Buffer A (150 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4) and homogenized by passage 10 times through a ball-bearing homogenizer with a clearance of 0.012 μm (EMBL, Heidelberg, Germany). The homogenate was centrifuged in an Eppendorf microfuge for 5 min at 11,000 rpm, and 0.3 ml of the postnuclear supernatant was loaded onto a 5–25% glycerol gradient and centrifuged in a Sorvall AH650 rotor (Sorvall Instruments, Newton, CT) at 48,000 rpm for 50 min at 4°C. 14 fractions were collected and analyzed by Western immunoblot (20). A Molecular Dynamics phosphorimager equipped with ImageQuant software was used for quantitation.

Ficoll gradients were performed as described by Cutler and Cramer (11) with several modifications. One 10-cm plate of cells was labeled for 90 min with ^{3}H-labeled norepinephrine and a postnuclear supernatant was prepared in sucrose buffer (0.32 M sucrose, 10 mM Hepes) as described above. The homogenate was loaded onto a 1–16% Ficoll gradient and centrifuged at 30,000 rpm for 70 min in a Sorvall TH641 rotor. Fractions (1 ml) were collected in siliconized tubes, and the distribution of ^{3}H-labeled norepinephrine was determined by liquid-scintillation counting. Peak fractions were pooled, immediately loaded onto a 16–40% Ficoll gradient, and centrifuged for 20 h at 25,000 rpm in a Sorvall TH641 rotor. Fractions (1 ml) were collected, 0.3 ml of each was diluted twofold with sucrose buffer and membranes were pelleted by centrifugation for 2.5 h at 80,000 rpm in a microultracentrifuge rotor (TLA100.1; Beckman Instruments, Palo Alto, CA). The membrane pellets were resuspended in urea sample buffer (5% SDS, 0.25 M Tris, pH 6.8, 8 M urea, 20% glycerol) and analyzed by Western immunoblot.

Immunoelectron Microscopy

Non-differentiated cells were removed by trituration and fixed by addition of paraformaldehyde and glutaraldehyde to final concentrations of 4 and 0.1%, respectively. They were then incubated for 15 min on ice, centrifuged in a microfuge for 5–10 min at 15,000 rpm. The pellet was carefully overlaid with 8% paraformaldehyde, centrifuged again, and then allowed to fix overnight. Ultrathin cryosections were prepared and labeled according to the method of Tokuyasu et al. (39) with affinity-purified α-GLUT1 antibody kindly provided by Dr. G. Leinhard (Dartmouth Medical School, Hanover, NH) or B. Thorens (University of Lausanne, Switzerland), or an affinity-purified guinea pig α-GLUT4 antibody (3). The transporters were visualized with 9 nm protein A gold. For measurement of the uptake of HRP, cells were incubated at 37°C in medium containing 10 mg/ml HRP for 10 or 60 min, and processed as described above. HRP was detected on cryosections with an affinity-purified anti-HRP antibody from Jean Gruenberg (EMBL, Heidelberg), and visualized with 5 nm protein A-gold (15).

Adsorption of Glucose Transporter-containing Vesicles by Antibody or WGA-Biotin

Postnuclear supernatants (PNS) were prepared from non-differentiated PC12 cells as described above. Affinity-purified antibody (4 μg) and 100 μl of Pansorbin (Calbiochem-Behring Corp., San Diego, CA) were added to the PNS from one-half of a 10-cm plate and the incubation was allowed to proceed at 4°C overnight. The Pansorbin was pelleted and washed three times with sucrose buffer. Adsorbed proteins were eluted into urea sample buffer and submitted to Western immunoblot for the measurement of glucose transporter or secretogranin. For assay of the latter, the samples were boiled in the presence of 0.2 M DTT before electrophoresis. When indi-

cated, Triton X-100 was present during the immunoprecipitation at a final concentration of 1%.

For experiments involving the adsorption of vesicles by WGA-biotin, supernatants from homogenates immunodepleted as described above served as the starting material. Streptavidin-coupled magnetic bead suspension (1 ml) was washed three times with sucrose buffer and incubated for 3–6 h at 4°C in the same buffer containing 125 μg WGA-biotin, 1% BSA, and 0.05% sodium azide. The WGA-biotin-adsorbed magnetic beads were washed three times with sucrose buffer and incubated with immunodepleted cell homogenate overnight at 4°C. The beads were then washed three times in sucrose buffer and adsorbed membrane proteins were eluted in urea sample buffer. Both pellets and supernatants from the final precipitation were saved and analyzed by Western blot.

Metabolic Labeling with [^{35}S]Sulfate

JSM PC12 cells were incubated for 45 min at 37°C in sulfate-free medium containing 1% of the usual concentration of methionine, 1% horse serum, and 0.5% FBS. Cells were pulsed with ^{35}S-labeled sulfate (1 mCi/ml) in sulfate-free medium for 10 min followed by a 35-min chase with complete medium supplemented with twice the normal concentration of sulfate. The cells were then processed for immunoadsorption as described above. Samples were submitted to 10% SDS-PAGE, the gel was treated with Enhance (New England Nuclear), dried, and exposed to film.

Results

cDNAs encoding the rat facilitated glucose transporters GLUT4, GLUT1, or, as a control, vector alone were introduced into PC12 cells by retrovirus-mediated gene transfer, producing pools of G418-resistant colonies. The cell lines were named JSM, JGT, and DOJ, respectively. Western immunoblot with a GLUT1-specific antisera detected transporter in membranes from control DOJ cells, and infection of PC12 with the DOJ-GT virus substantially increased the levels of GLUT1 (Fig. 1 *A*). GLUT4 was not present in the control DOJ cells, but was detected as a diffuse species on Western blot of JSM cell total membranes (Fig. 1 *A*). The difference in mobility of GLUT4 in membranes from PC12 cells compared to that in rat adipocyte membranes is probably due to the variability in heterogeneous glycosylation observed among cell types (Fig. 1 *B*).

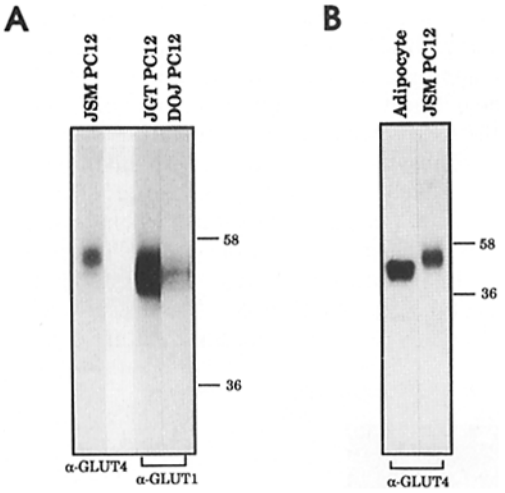

Figure 1. Western immunoblot of glucose transporter isoforms in PC12 cells. (*A*) Total membranes (10 μg) prepared from pools of JSM PC12 (GLUT4), JGT PC12 (GLUT1), or DOJ PC12 (control) were submitted to SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and probed with polyclonal antisera specific for GLUT4 or GLUT1. (*B*) Low-density microsomes (10 μg) prepared from rat adipocytes (14) and 10 μg of total membranes from JSM PC12 cells were submitted to SDS-polyacrylamide electrophoresis and Western immunoblot with antisera specific for GLUT4. Sizes of two molecular weight standards are indicated in kD.

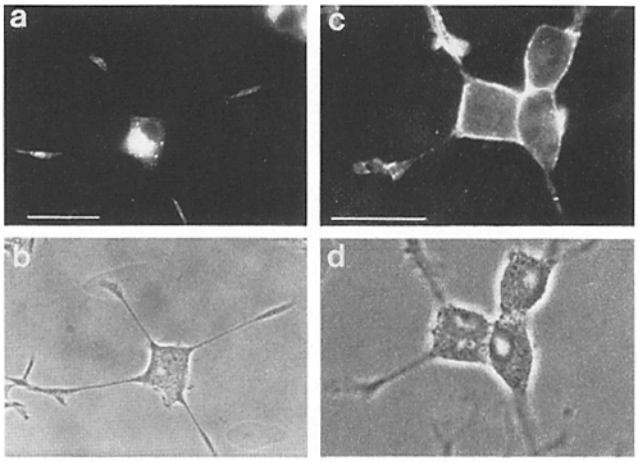

Figure 2. Immunofluorescent localization of glucose transporter isoforms in PC12 cells. Differentiated PC12 cells expressing GLUT4 (*a* and *b*) or GLUT1 (*c* and *d*) were assayed for distribution of transporter using antisera directed against GLUT1 (*a*) or GLUT4 (*c*). Phase micrographs (*b* and *d*) of the corresponding fields are shown. Bar, 20 μm.

Examination of pools of G418-resistant colonies by indirect immunofluorescence microscopy revealed that >85% of the JSM or JGT cells expressed the heterologous glucose transporter. GLUT1 overexpressed in PC12 cells resided in a peripheral distribution characteristic of targeting to the plasma membrane (Fig. 2 *C*). GLUT4, on the other hand, appeared to be excluded from the cell surface, instead localizing primarily to the perinuclear region, with some additional punctate cytoplasmic labeling (Fig. 2 *A*). In PC12 cells induced to differentiate by exposure to NGF, GLUT4 was conspicuously present in the distal neuronal processes. There was no GLUT4 detected in DOJ PC12 cells by immunofluorescence microscopy either in the presence of absence of NGF (data not shown).

The localization of GLUT4 in processes was reminiscent of the distribution of synaptic vesicle proteins. PC12 cells contain two classes of vesicles, small synaptic vesicles (SSV) and large dense core granules (LDCV), which are involved in the regulated secretion of neurotransmitters (16). Double-label immunofluorescence was performed on differentiated JSM PC12 cells using a polyclonal antiserum directed against GLUT4 and a mAb which recognizes synaptophysin (p38), a marker for SSV (32). GLUT4 and synaptophysin co-localized in the perinuclear region of the cell, and to some extent, in the processes (Fig. 3). However, the proportional intensity of GLUT4 labeling in the neurites relative to the perinuclear region was significantly greater than that of synaptophysin, suggesting that the transporter may have been targeted to an additional compartment in the distal processes. Secretogranin, a secreted polypeptide stored in LDCV (21, 34), co-localized with GLUT4 in the termini of

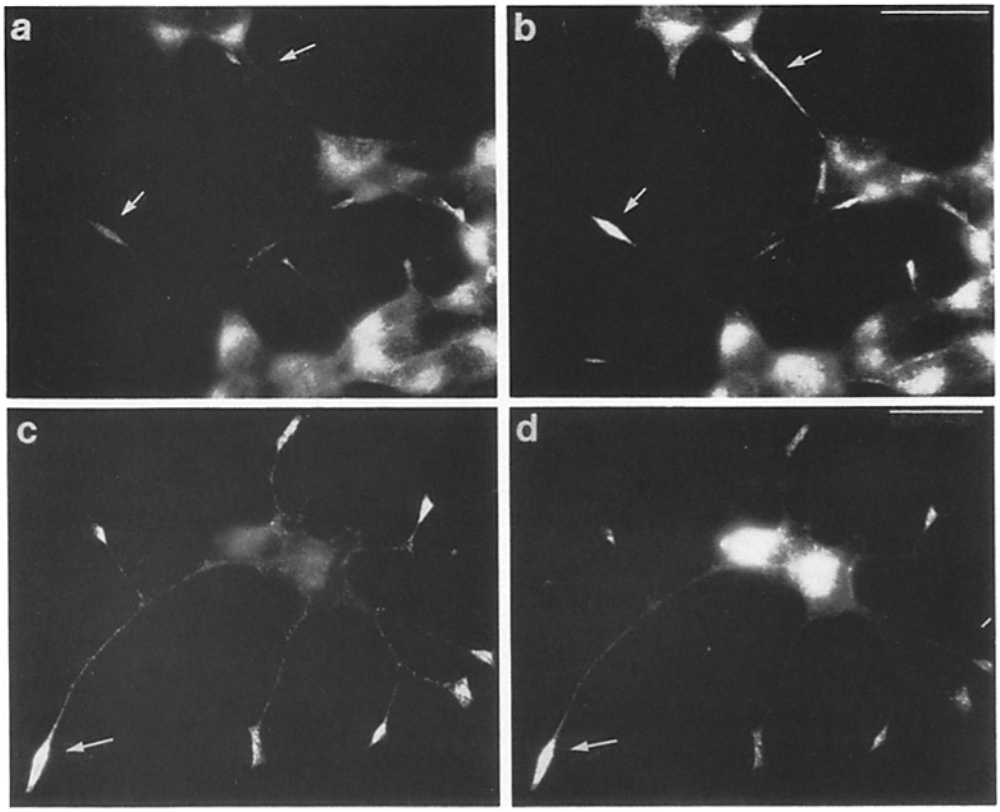

Figure 3. Immunofluorescent co-localization of synaptophysin and secretogranin with GLUT4. Pools of differentiated JSM PC12 cells were labeled with antisera against GLUT4 (*b* and *d*) and synaptophysin (*a*) or secretogranin (*c*). Arrows indicate neuronal processes where GLUT4 immunoreactivity is abundant. Bar, 20 μm.

the processes, but did not exhibit the juxtanuclear labeling pattern of the latter (Fig. 3). Since Cameron et al. (8) have shown that a significant portion of synaptophysin in PC12 cells resides in endosomes, the distribution of transferrin receptor was also examined in the GLUT4-expressing cells. At the level of light microscopy, the transferrin receptor and GLUT4 co-localized well in the perinuclear region, but again, the latter appeared to be more abundant in the distal processes (Fig. 4).

To determine whether GLUT4 was being targeted to SSV as well as endosomes, further biochemical characterization was undertaken. Velocity gradients have allowed the separation of a fraction enriched in synaptophysin as well as other SSV membrane proteins from the less buoyant endosomal structures (8, 10). JSM PC12 cells were homogenized, centrifuged in a 5–25% glycerol gradient, and the fractions submitted to SDS-PAGE and Western blot. Synaptophysin sedimented to the bottom of the gradient and as a distinct peak spanning fractions 7–9 (Fig. 5). When a sucrose "cushion" was included at the bottom of the gradient to prevent pelleting of more dense membranes, quantitation of the distribution of synaptophysin was permitted; the mid-gradient peak contained approximately 27% of the total synaptophysin, whereas the heaviest two fractions contained 62% (note that in Fig. 5, the sucrose cushion was omitted). Transferrin receptor, which resides predominantly in early endosomes, was confined exclusively to the bottom of the gradient, as reported previously (8; and data not shown). When the same gradient fractions were probed with the α-GLUT4 antiserum, transporter immunoreactivity was concentrated in the denser fractions of the gradient (Fig. 5). These results demonstrate that GLUT4 is excluded from SSV and are consis-

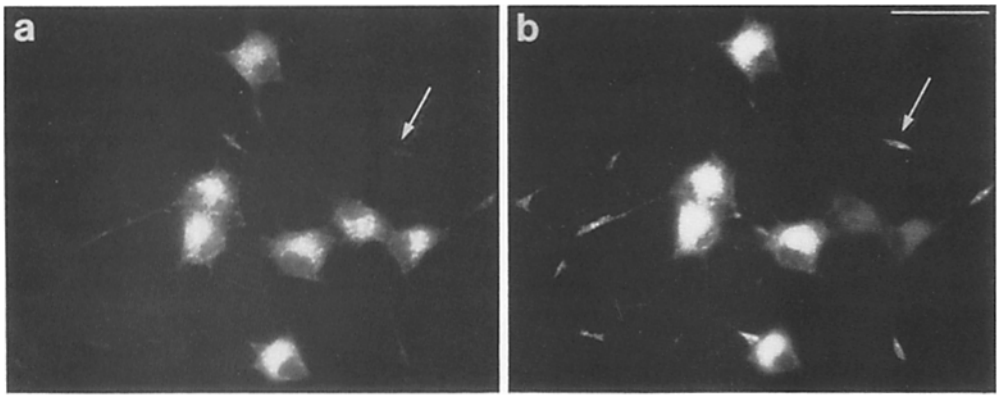

Figure 4. Co-localization of transferrin receptor and GLUT4. Pools of differentiated JSM PC12 cells were labeled with antisera against the transferrin receptor (*a*) and GLUT4 (*b*). The arrow indicates a neuronal process in which GLUT4 is abundant. Bar, 20 μm.

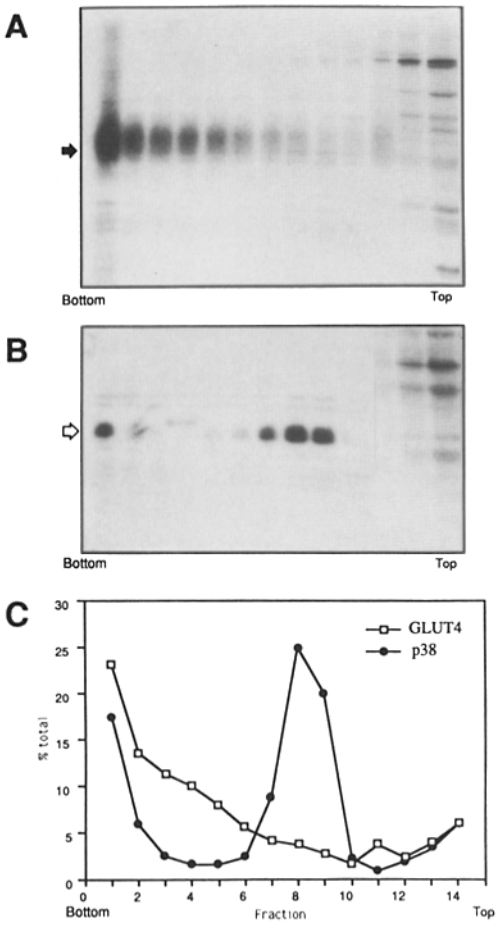

Figure 5. Velocity gradient analysis of GLUT4 and synaptophysin. Postnuclear supernatants from one confluent 10 cm plate of JSM PC12 cells were loaded onto a 5–25% glycerol velocity gradient. Fractions were collected and submitted to Western immunoblot with antisera specific for GLUT4 (*A*) or synaptophysin (*B*). The positions of GLUT4 and synaptophysin on the gels are noted by solid and open arrows, respectively. (*C*) GLUT4 (□) and synaptophysin (●) were quantitated and graphed as a percent of total immunoreactivity.

tent with the immunofluorescent microscopy suggesting targeting of the hexose carrier to endosomes.

Immunoelectron microscopy on ultrathin cryosections was carried out to more definitively establish the intracellular location of GLUT4. Sections of JSM PC12 cells incubated with an affinity-purified guinea pig antibody against the intracellular carboxyl terminus of GLUT4, followed by 9 nm protein A gold, showed specific labeling of the cytoplasmic surface of LDCVs (Fig. 6, *A* and *B*). In addition, there were a significant number of gold particles associated with tubulovesicular elements throughout the cell (Fig. 6, *C* and *D*). In a random sampling of sections, ~80% of the LDCVs were labeled with one or more gold particles, and 14% of the total gold particles counted were associated with LDCVs. When JSM PC12 cells were allowed to internalize HRP for either 10 or 60 min, much of the fluid-phase marker co-localized with GLUT4 in vesicular structures demonstrating characteristics of early or late endosomes, respectively (Fig. 7). Interestingly, the plasma membrane was relatively free of immunoreactive GLUT4, even after stimulation of the cells with carbachol or 50 mM KCl (Fig. 6; data not shown). In all experiments, the amount of nonspecific labeling was estimated to be low based on the virtual absence of gold particles overlying the nucleus. Furthermore, the control cell line DOJ PC12 did not exhibit any specific labeling when exposed to α-GLUT4 (data not shown). Immunogold localization of GLUT1 in JGT PC12 cells showed virtually all of the labeling on the plasma membrane, with occasional gold associated with tubulovesicular structures (Fig. 8). Importantly, LDCV were free of GLUT1, as determined by immunogold EM.

As an independent assessment of the presence of GLUT4 in LDCV from JSM PC12 cells, vesicles adsorbed to an antibody directed against a cytoplasmic epitope of GLUT4 were assayed for the presence of secretogranin (Fig. 9). α-GLUT4–coated Pansorbin immunoprecipitated ~80% of the transporter (see Fig. 11 *B*, lanes *3* and *4*). Western blot analysis of the α-GLUT4 immunoprecipitate with an α-secretogranin antibody showed the presence of up to 90% of the secretogranin in the adsorbed vesicle pellet (Fig. 9, lanes *7–9*). Co-immunoprecipitation was dependent on the integrity of the vesicles, as disruption with Triton X-100 before immunoadsorption prevented the precipitation of secretogranin, while only slightly reducing GLUT4 (Fig. 9, lanes *2* and *8*).

An identical experiment was performed with α-GLUT1 antiserum and extract from JGT PC12 cells. Unlike α-GLUT4, antiserum directed against the cytoplasmic carboxyl terminus of GLUT1 adsorbed a relatively minor fraction of the transporter, except when detergent was included during the immunoprecipitation (Fig. 10, lanes *1* and *2*). One possibility was that homogenization resulted in the formation of "rightside-out" plasma membrane vesicles, rendering the cytoplasmic epitope inaccessible to antibody. To test this idea, magnetic beads coated with the lectin WGA were used to precipitate plasma membrane vesicles in which the carbohydrate-rich extracellular surface was exposed. Incubation of immunodepleted JGT PC12 homogenate with WGA-coated beads resulted in precipitation of ~80% of GLUT1, as analyzed by Western immunoblot of the supernatants (Fig. 10, lanes *4*, *6*, and *10*). Secretogranin was not detectable in any of the GLUT1 vesicle pellets, even after >80% precipitation of the transporter (Fig. 10, lanes *7–9*). As expected, adsorption of α-GLUT4 immunodepleted JSM PC12 vesicles by WGA-coated magnetic beads resulted in precipitation of only small amounts of GLUT4 (Fig. 9, lane *10*).

To further ensure that immunoprecipitation of secretogranin by α-GLUT4 was not the result of co-localization in a pre-sorting biosynthetic compartment, metabolic labeling of JSM PC12 cells with ^{35}S-labeled sulfate followed by immunoprecipitation with α-GLUT4 antisera was performed. Secretogranins I and II, the major tyrosine-sulfated proteins synthesized in PC12 cells, acquire sulfate in the TGN immediately before sorting to immature secretory granules (1). The formation of immature secretory granules from the TGN is completed within 15 min (40). Thus, adsorption of vesicles containing ^{35}S-labeled sulfate–labeled secretogranins by α-GLUT4 would provide additional direct evidence for their association in LDCV. After a 10-min pulse and 35-

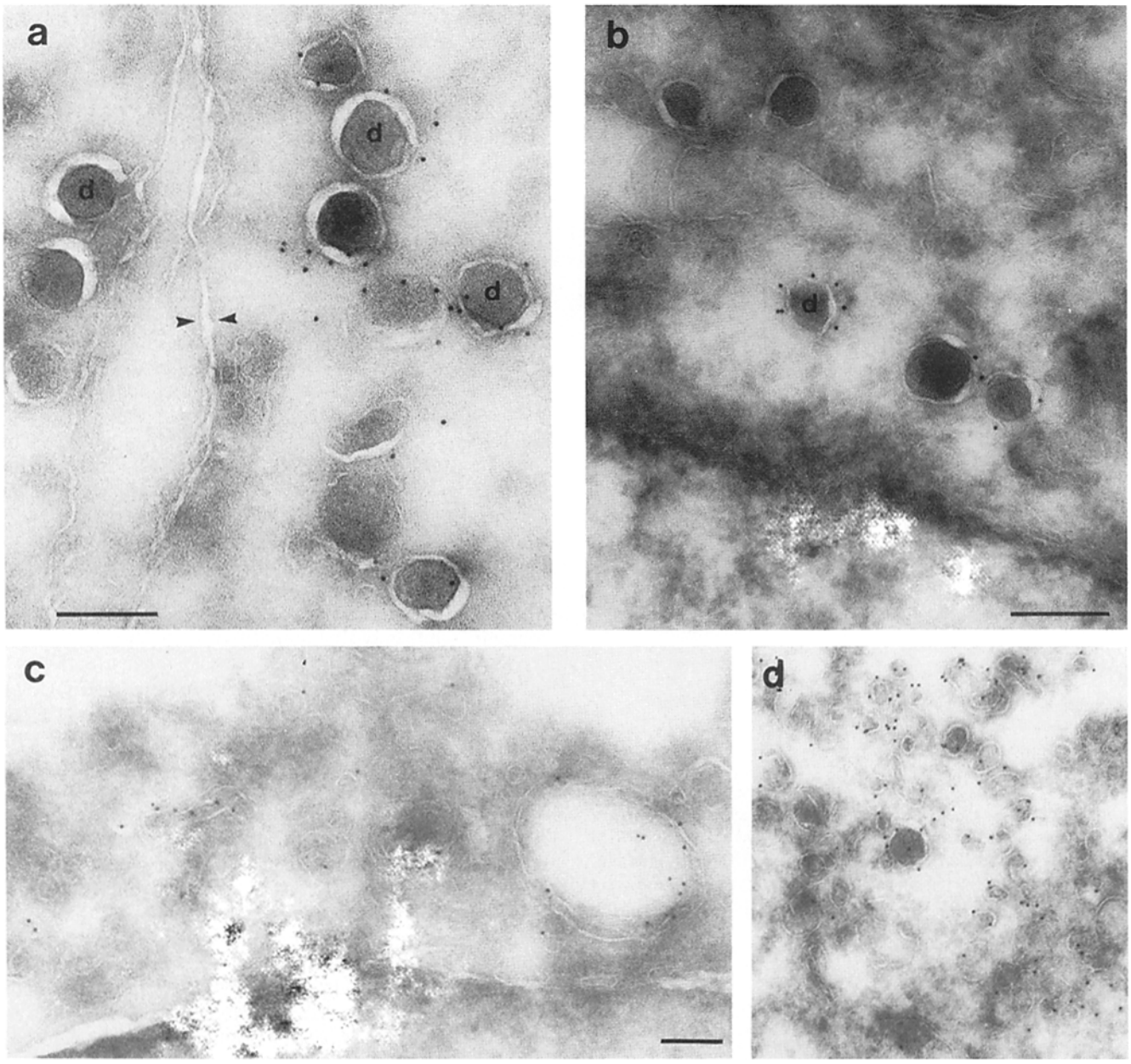

Figure 6. Immunoelectron microscopic localization of GLUT4. Pools of JSM PC12 cells were labeled with antisera directed against GLUT4; representative fields are shown in *a–d*. Facing arrowheads indicate the plasma membrane, and dense-core granules are marked with a "*d*". GLUT4 (gold particles) can be seen surrounding dense core granules (*a* and *b*), as well as in tubulo-vesicular structures (*c* and *d*). The three dense-core granules to the left in *a* and the two unlabeled granules above in *b* are members of adjacent, non-expressing cells. Bar, 200 nm.

min chase with ^{35}S-labeled sulfate, metabolically labeled secretogranins I and II were immunoprecipitated by α-GLUT4 (Fig. 11 *A*). Approximately 90% of the ^{35}S-labeled sulfate-labeled secretogranin was depleted by adsorption with α-GLUT4 (Fig. 11 *A*, lanes *3* and *4*), indicating that a substantial fraction of the newly synthesized regulated secretory vesicles contain the transporter.

As another assay for the targeting of GLUT4 to LDCV, the latter compartment was isolated from PC12-JSM cells by sequential velocity and equilibrium gradient centrifugation (11). When the gradient fractions were analyzed by Western blot, a peak of GLUT4 was visualized coincident with secretogranin, a marker specific for LDCV (Fig. 12). These data are consistent with the immunoadsorption experiments presented above, indicating the presence of GLUT4 in LDCV.

Discussion

The experiments described herein were designed to test the hypothesis that the adipose tissue/muscle-specific glucose transporter (GLUT4) shares a conserved sorting signal with

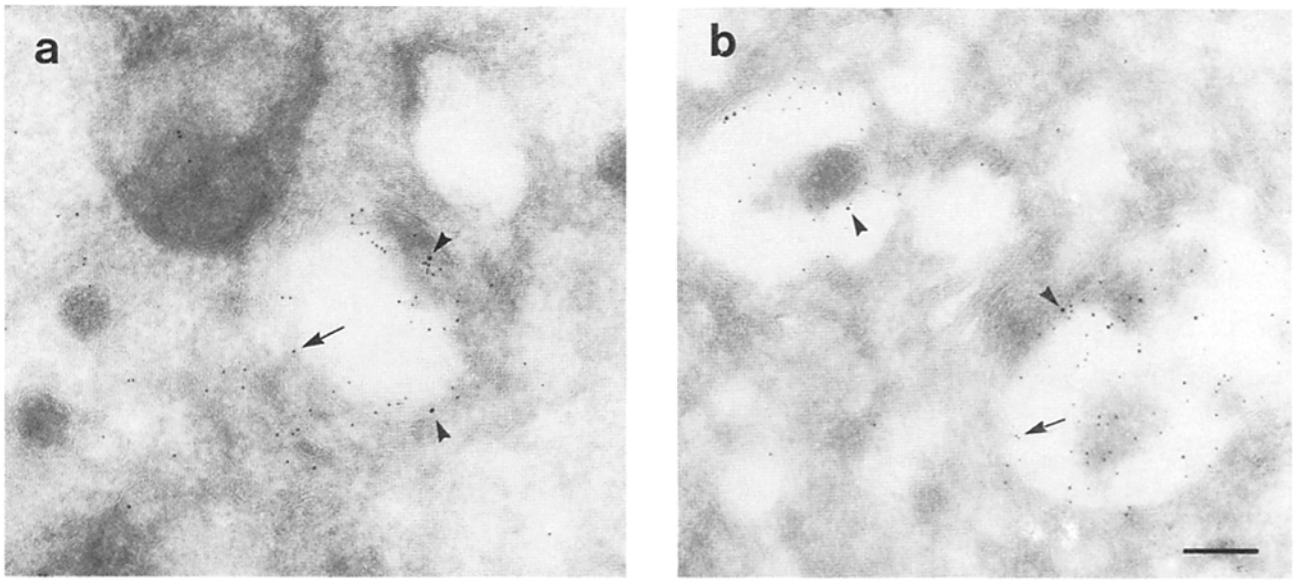

Figure 7. Immuno-electron microscopic co-localization of GLUT4 and internalized HRP. Pools of JSM PC12 cells were incubated in medium containing 10 mg/ml HRP for 10 (*a*) or 60 (*b*) min at 37°C, fixed, and labeled with antisera directed against GLUT4 followed by 9 nm gold (*arrowheads*) and antisera directed against HRP followed by 5 nm gold (*arrows*). Bar, 200 nm.

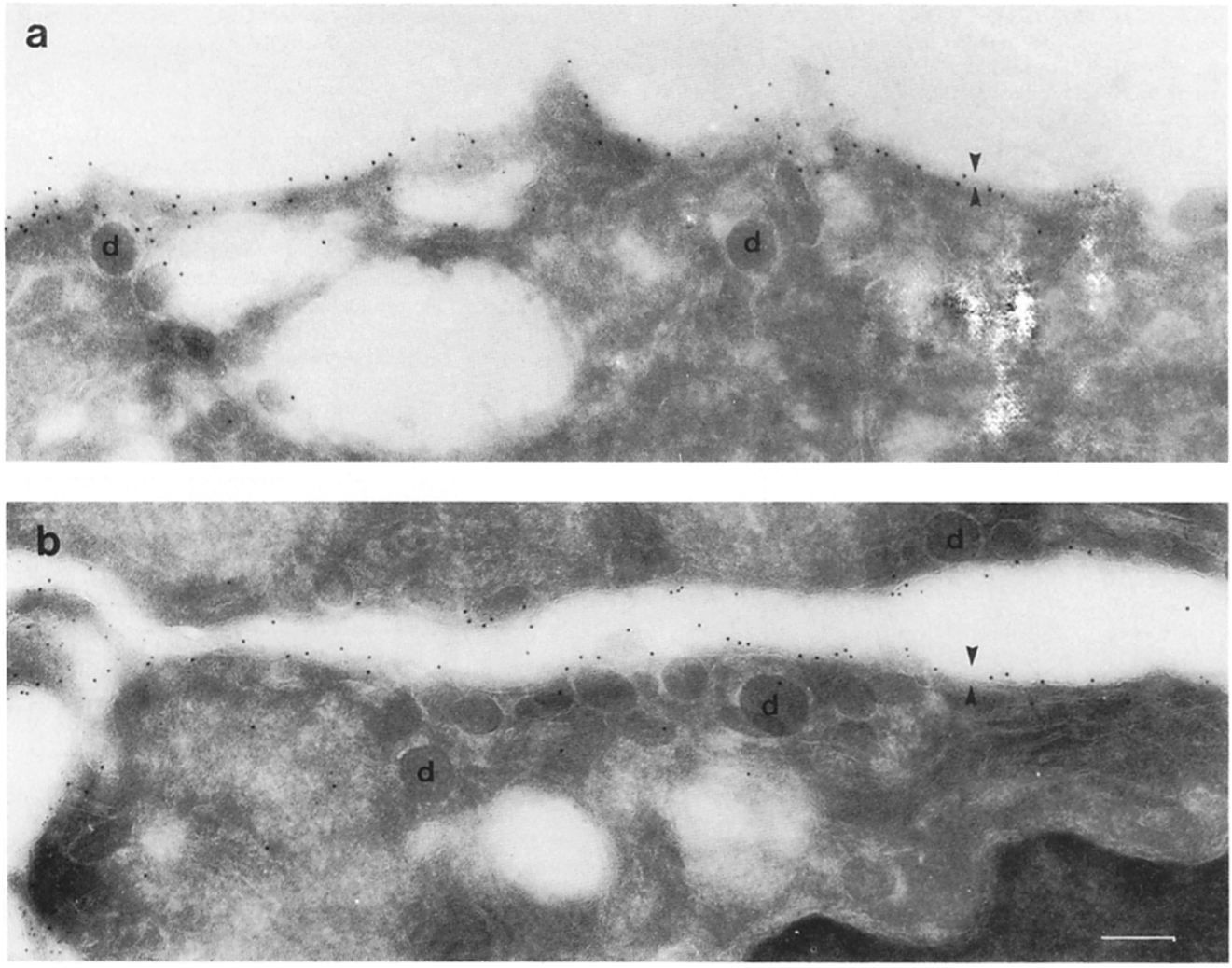

Figure 8. Immuno-gold localization of GLUT1. JGT PC12 cells were labeled with antisera directed against GLUT1; two representative fields are shown (*a* and *b*). Dense-core granules are marked with a "*d*," and plasma membrane is indicated by facing arrowheads. Bar, 200 nm.

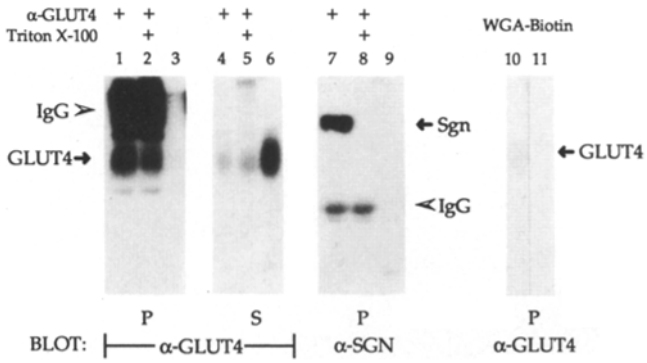

Figure 9. Adsorption of GLUT4-containing vesicles. Postnuclear supernatant from one quarter of a confluent 10-cm plate of JSM PC12 cells were immunoprecipitated with an affinity-purified antibody specific for GLUT4 (lanes *1–3* and *7–9*). Magnetic beads coated with WGA were then used to adsorb vesicles from the immunodepleted supernatant (lane *10*). WGA was omitted from the precipitation in lane *11*. The supernatants remaining after both precipitations are shown in lanes *4–6*. One third of each precipitate (*P*) and 1/20 of the final supernatant (*S*) were analyzed by Western blotting with the antisera directed against GLUT4 or secretogranin, as indicated below the blots. The presence of antiserum or 1% Triton X-100 in the precipitations is indicated above the blots. Solid arrows indicate the migration of GLUT4 and secretogranin, and the arrowheads mark the position of IgG. It should be noted that in lanes *1–6*, *10*, and *11* the samples have not been reduced, whereas in lanes *7–9* they have been treated with DTT, accounting for the apparent differences in the mobility of IgG. In a series of four experiments, α-GLUT4 depleted 30–90% of the secretogranin from the JSM PC12 homogenate.

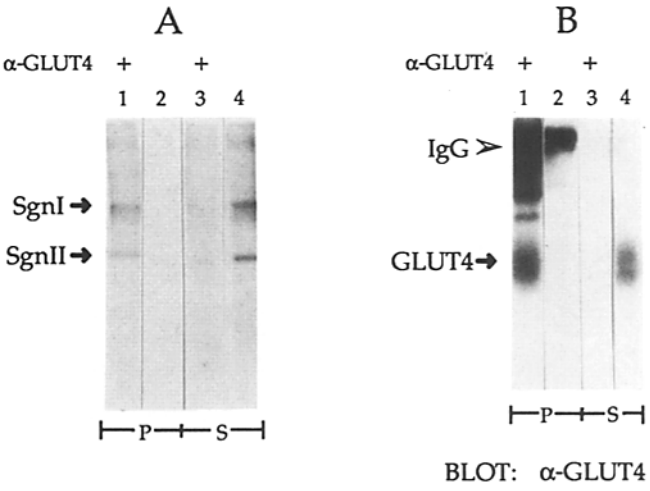

Figure 11. Immunoprecipitation of ^{35}S-labeled sulfate-labeled proteins with α-GLUT4. One confluent 10 cm plate of JSM PC12 cells was pulsed with ^{35}S-labeled sulfate for 10 min, chased for 35 min and GLUT4-containing vesicles from one quarter of the cells were incubated with Pansorbin with or without affinity-purified antibody specific for GLUT4, as indicated. One third of the precipitate (*P*) and 1/20 of the supernatant (*S*) were submitted to SDS-PAGE and analyzed by fluorography (*A*) or Western blotting with α-GLUT4 (*B*). Arrows indicate the migration of GLUT4 and secretogranins I and II, and the arrowhead marks the position of IgG.

proteins destined for the regulated secretory pathway. Our test of this model has been to express GLUT4 in the rat pheochromocytoma cell line PC12, which undergoes regulated exocytosis in response to secretagogues. The strategy of transfecting genes encoding secretory proteins into neuroendocrine cell lines has provided the experimental basis for the widely held view that the recognition motifs which dictate targeting to the regulated secretory pathway are shared by disparate exocrine, neural, and endocrine cell types (5). Thus, the above hypothesis predicts that the ectopic expression of GLUT4 in PC12 cells should result in the stable incorporation of this transporter isoform into secretory vesicles. Three independent experiments establish this to be true: first, immunoelectron microscopy studies show a significant amount of GLUT4 associated with LDCVs (Fig. 6); second, the adsorption of vesicles by GLUT4-specific antisera results in the co-immunoprecipitation of secretogra-

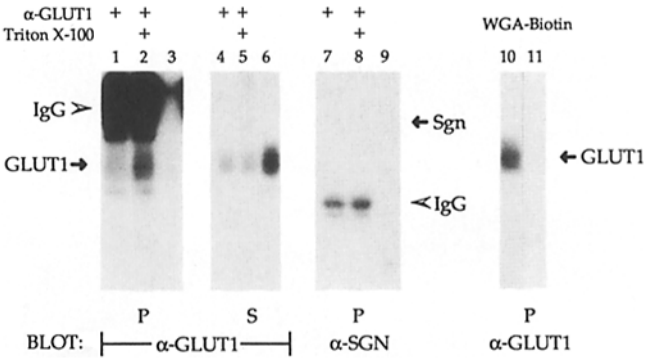

Figure 10. Adsorption of GLUT1-containing vesicles. Postnuclear supernatant from one quarter of a confluent 10-cm plate of JGT PC12 cells were immunoprecipitated with an affinity-purified antibody specific for GLUT1 (lanes *1–3* and *7–9*). Magnetic beads coated with wheat germ agglutinin (WGA) were then used to adsorb vesicles from the immunodepleted supernatant (lane *10*). WGA was omitted from the precipitation in lane *11*. The supernatants remaining after both precipitations are shown in lanes *4–6*. The experiment was performed as described in the legend for Fig. 9.

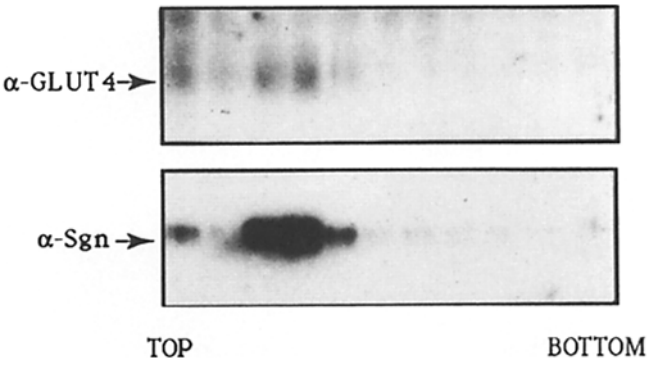

Figure 12. Gradient purification of large dense-core vesicles. One confluent 10 cm plate of JSM PC12 cells was labeled with ^{3}H-labeled norepinephrine, homogenized, and loaded onto a 1–15% Ficoll gradient. Peak [^{3}H] fractions were pooled and immediately separated on a 15–40% Ficoll equilibrium gradient. One third of each fraction was loaded on an SDS–polyacrylamide gel and analyzed by Western blotting with α-GLUT4 or α-secretogranin (α-*Sgn*).

nin, a marker specific for LDCV (Figs. 10 and 11); and third, purification of LDCVs by density and equilibrium centrifugation clearly demonstrates the co-fractionation of GLUT4 (Fig. 12). Taken together, these observations indicate that GLUT4 is specifically targeted to dense core granules.

At the outset of these experiments, PC12 cells appeared to be particularly well suited for these studies since they possess two distinct classes of regulated secretory organelles, the SSV, and the LDCVs (for review see reference 11). It has been suggested that the most probable neuronal compartment equivalent to GLUT4-containing vesicles in adipose tissue would be SSV (7, 8). SSV recycle at the nerve terminal through endosomes via a pathway strikingly similar to GLUT4 recycling in insulin-responsive cell types (10, 23). In separate experiments involving transfection into fibroblasts, both the SSV protein synaptophysin and GLUT4 are targeted to endosomes or an endosome-derived compartment (20, 23, 25). Moreover, the SSV protein VAMP has recently been shown to be a component of a fraction enriched in GLUT4-containing vesicles in adipocytes (7). Nevertheless, glycerol velocity gradients clearly indicated that whereas GLUT4 co-fractionates with synaptophysin in endosomes, it is efficiently excluded from the buoyant SSV fraction.

Several aspects of the experimental design concerned us as potentially leading to artifact. First, the possibility existed that significant overexpression of a foreign protein might result in some "non-specific missorting", particularly in a transformed cell line such as PC12. To control for this possibility, we infected PC12 cells with a retrovirus encoding the more ubiquitous facilitated hexose carrier, GLUT1. In many cell types, including classical insulin target tissues, GLUT1 resides predominantly on the plasma membrane. Thus, extending the analogy of regulated secretion to the trafficking of glucose transporters, GLUT1 might be viewed as passing through the "constitutive" pathway. More importantly, GLUT1 displays 65% amino acid identity and a predicted transmembrane topology similar to GLUT4 (3, 22). Should targeting of the latter isoform to LDCV be the result of missorting secondary to overexpression, a similar phenomenon would likely be observed for GLUT1. Examination of JGT PC12 by light or immunoelectron microscopy revealed most of the GLUT1 to be on the plasma membrane, with the LDCV essentially free of colloidal gold, and immunoadsorption experiments failed to indicate significant co-localization with secretogranin. Since both GLUT1 and GLUT4 almost certainly pass through the same biosynthetic compartments, we take the relative absence of GLUT1 in secretory vesicles as evidence against the idea that the appearance of GLUT4 in LDCV is simply a result of its high level of expression in the TGN. Additional support for the interpretation that the presence of GLUT4 in LDCV results from specific sorting is provided by the complete exclusion of GLUT4 from SSV (Fig. 5).

An additional concern was that the co-precipitation of secretogranin with GLUT4-containing membranes was a result of vesicularization of components of the biosynthetic pathway. The ability of α-GLUT4 to immunoprecipitate the majority of sulfate-labeled secretogranins disproves this, as tyrosine sulfation occurs in the TGN and by 35 min the newly sulfated secretogranins reside in an immature fraction of LDCVs (1). Moreover, purification of LDCVs by gradient centrifugation results in co-enrichment of GLUT4 in the LDCV peak fractions (Fig. 12).

In spite of the apparent specific targeting of GLUT4 to LDCV in JSM PC12 cells, a relatively modest proportion of the transporter resided in secretory vesicles under steady-state conditions. These data are quite reminiscent of that reported in the original studies in which secreted products were expressed in heterologous cell types. For example, introduction of the gene encoding human growth hormone (hGH) into PC12 cells resulted in the packaging of only ~15–30% of the exogenous hormone into the regulated secretory pathway, a value similar to that for endogenous ACTH in the pituitary cell line AtT-20 (30). Given the inefficient sorting of both exogenous and endogenous secreted protein in tissue culture cell lines, the observed steady-state subcellular distribution of GLUT4 is consistent with specific targeting of GLUT4 to the regulated secretory pathway. This is especially true considering that, unlike secreted proteins, GLUT4 is likely to continuously recycle through the endocytic pathway. GLUT4 predominantly resides in such structures, as judged by co-localization of GLUT4 with transferrin receptor and the visualization by EM of fluid phase markers in GLUT4-containing vesicles (Figs. 4, 5, and 7). That significant quantities of authentic secretory membrane proteins are present in endosomes can be inferred from recent studies on the overexpression of the membrane-associated form of the secretory protein-processing enzyme, peptidylglycine α-amidating monooxygenase (PAM), in AtT-20 cells (29). The predominant location of PAM was perinuclear, much like GLUT4 in PC12 cells, suggesting that this form of the enzyme may be distributed between endosomes and secretory vesicles even in the absence to secretagogue. In independent experiments, P-selectin, a transmembrane protein specific to α granules of platelets and Weibel–Palade bodies of endothelial cells, was expressed in AtT-20 cells and ~20% of the protein co-fractionated with ACTH-containing granules on a Percoll gradient (24). Moreover, only ~25% of synaptophysin endogenous to PC12 cells is located in SSV, with the remainder residing in endosomes (8). Thus, the presence of ~15% of GLUT4 in LDCV is entirely consistent with prior estimates of the efficiency of sorting of proteins to the regulated pathway in cultured cells, as well as the distribution of integral membrane protein between secretory vesicles and endosomes. The lack of antibody probes directed against a well-characterized rodent integral membrane protein specific to LDCV precluded a direct comparison of the intracellular location of GLUT4 to an endogenous LDCV integral membrane protein.

When exocytosis is elicited in neuronal cells, it is generally impossible to detect a change in the cell surface abundance of integral membrane proteins translocating from secretory vesicles (28, 41). Similarly, we have been unable to convincingly demonstrate a change in the amount of synaptophysin on the plasma membrane of PC12 cells following depolarization, a condition known to induce exocytosis (Hudson, A. W., and M. J. Birnbaum, unpublished observations). Presumably, these observations are a result of the exceedingly brief residence time of these proteins on the plasma membrane (9). Interestingly, when GLUT4 was introduced into fibroblastic cell types, it was internalized very rapidly, resulting in no detectable cell surface transporter

(20). For these reasons, it was necessary to establish the sorting of GLUT4 to the regulated pathway by co-localization with a marker of LDCV, rather than demonstrating translocation. Since GLUT4 co-enriches with secretogranin, which has been demonstrated in numerous studies to reside in a bona fide regulated secretory vesicle, and the immunoelectron microscopy experiments place the transporter in a morphologically characteristic electron-dense vesicle, there is little doubt that GLUT4 is present in a regulated compartment (12).

The data presented in this communication are consistent with the proposition that GLUT4 contains within its primary structure information required for targeting to a classical, regulated secretory pathway, and recycling through an endosomal compartment. The major implication of such a model is that GLUT4 would likely reside in a distinct, tissue-specific organelle in insulin-responsive cells. More conclusive proof of such an hypothesis awaits further characterization of the GLUT4-containing vesicles in adipose tissue and muscle, and the precise delineation of the relevant sorting signals.

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References

1. Baeuerle, P. A., and W. B. Huttner. 1987. Tyrosine sulfation is a *trans*-Golgi-specific protein modification. *J. Cell Biol.* 105:2655–2664.
2. Bell, G. I., T. Kayano, J. B. Buse, C. F. Burant, J. Takeda, D. Lin, H. Fukumoto, and S. Seino. 1990. Molecular biology of mammalian glucose transporters. *Diabetes Care.* 13:198–208.
3. Birnbaum, M. J. 1989. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell.* 57:305–315.
4. Birnbaum, M. J. 1992. The insulin-responsive glucose transporter. *Int. Rev. Cytol.* 137A:239–297.
5. Burgess, T. L., and R. B. Kelly. 1987. Constitutive and regulated secretion of proteins. *Ann. Rev. Cell Biol.* 3:243–293.
6. Burgess, T. L., C. S. Craik, and R. B. Kelly. 1985. The exocrine protein trypsinogen is targeted into the secretory granules of an endocrine cell line: studies by gene transfer. *J. Cell Biol.* 101:639–645.
7. Cain, C. C., W. S. Trimble, and G. E. Lienhard. 1992. Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. *J. Biol. Chem.* 267: 11681–11684.
8. Cameron, P. L., T. C. Sudhof, R. Jahn, and P. DeCamilli. 1991. Colocalization of synaptophysin with transferrin receptors: implications for synaptic vesicle biogenesis. *J. Cell Biol.* 115:151–164.
9. Ceccarelli, B., and W. P. Hurlbut. 1980. Vesicle hypothesis of the release of acetylcholine. *Physiol. Rev.* 60:396–441.
10. Clift-O'Grady, L., A. D. Linstedt, A. W. Lowe, E. Grote, and R. B. Kelly. 1990. Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. *J. Cell Biol.* 110:1693–1703.
11. Cutler, D. F., and L. P. Cramer. 1990. Sorting during transport to the surface of PC12 cells: Divergence of synaptic vesicle and secretory granule proteins. *J. Cell Biol.* 110:721–730.
12. De Camilli, P., and R. Jahn. 1990. Pathways to regulated exocytosis in neurons. *Annu. Rev. Physiol.* 52:625–645.
13. Garcia de Herreros, A., and M. J. Birnbaum. 1989. The acquisition of increased insulin-responsive glucose transport in 3T3-LI adipocytes correlates with expression of a novel transporter gene. *J. Biol. Chem.* 264: 19994–19999.
14. Garvey, W. T., T. P. Huecksteadt, S. Matthaei, and J. M. Olefsky. 1988. Role of glucose transporters in the cellular insulin resistance of type II non-insulin-dependent diabetes mellitus. *J. Clin. Invest.* 81:1528–1536.
15. Geuze, H. J., J. W. Slot, R. C. T. Scheffer, and P. A. van der Ley. 1981. Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* 89:653–665.
16. Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal cell line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.
17. Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.
18. Haney, P. M., J. W. Slot, R. C. Piper, D. E. James, and M. Mueckler. 1991. Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J. Cell Biol.* 114:689–699.
19. Holman, G. D., I. J. Kozka, A. E. Clark, C. J. Flower, J. Saltis, A. D. Habberfield, I. A. Simpson, and S. W. Cushman. 1990. Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J. Biol. Chem.* 265:18172–18179.
20. Hudson, A. W., M. L. Ruiz, and M. J. Birnbaum. 1992. Isoform-specific subcellular targeting of glucose transporters in mouse fibroblasts. *J. Cell Biol.* 116:785–797.
21. Huttner, W. B., H.-H. Gerdes, and P. Rosa. 1991. The granin (chromogranin/secretogranin) family. *Trends Biochem. Sci.* 16:27–30.
22. James, D. E., M. Strube, and M. Mueckler. 1989. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature (Lond.).* 338:83–87.
23. Johnston, P. A., P. L. Cameron, H. Stukenbrok, R. Jahn, P. De Camilli, and T. C. Sudhof. 1989. Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8: 2863–2872.
24. Koedam, J. A., E. M. Cramer, E. Briend, B. Furie, B. C. Furie, and D. D. Wagner. 1992. P-Selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. *J. Cell Biol.* 116:617–625.
25. Leube, R. E., B. Weidenmann, and W. W. Franke. 1989. Topogenesis and sorting of synaptophysin: synthesis of a synaptic vesicle protein from a gene transfected into nonneuroendocrine cells. *Cell.* 59:433–446.
26. Linstedt, A. D., and R. B. Kelly. 1991. Synaptophysin is sorted from endocytotic markers in neuroendocrine PC12 cells but not transfected fibroblasts. *Neuron.* 7:309–317.
27. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell.* 33:153–159.
28. Matteoli, M., K. Takei, M. S. Perin, T. C. Südhof, and P. De Camilli. 1992. Exoendocytic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell Biol.* 117:849–861.
29. Milgram, S. L., R. C. Johnson, and R. E. Mains. 1992. Expression of individual forms of peptidylglycine α-amidating monooxygenase in AtT-20 cells: endoproteolytic processing and routing to secretory granules. *J. Cell Biol.* 117:717–728.
30. Moore, H.-P. H., and R. B. Kelly. 1985. Secretory protein targeting in a pituitary cell line: differential transport of foreign secretory proteins to distinct secretory pathways. *J. Cell Biol.* 101:1773–1781.
31. Moore, H.-P. H., M. D. Walker, F. Lee, and R. B. Kelly. 1983. Expressing human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. *Cell.* 35:531–538.
32. Navone, F., R. Jahn, G. G. Di, H. Stukenbrok, P. Greengard, and C. P. De. 1986. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* 103: 2511–2527.
33. Piper, R. C., L. J. Hess, and D. E. James. 1991. Differential sorting of two glucose transporters expressed in insulin-sensitive cells. *Am. J. Physiol.* 260:C570–580.
34. Rosa, P., U. Weiss, R. Pepperkok, W. Ansorge, C. Niehrs, E. H. K. Stelzer, and W. B. Huttner. 1989. An antibody against secretogranin I (Chromogranin B) is packaged into secretory granules. *J. Cell Biol.* 109:17–34.
35. Schweitzer, E. S., and R. B. Kelly. 1985. Selective packaging of human growth hormone onto synaptic vesicles in a rat neuronal (PC12) cell line. *J. Cell Biol.* 101:667–676.
36. Shibasaki, Y., T. Asano, J.-L. Lin, K. Tsukuda, H. Katagiri, H. Ishihara, Y. Yazaki, and Y. Oka. 1992. Two glucose transporter isoforms are sorted differentially and are expressed in distinct cellular compartments. *Biochem. J.* 281:829–834.
37. Slot, J. W., H. J. Geuze, S. Gigengack, G. E. Lienhard, and D. E. James. 1991. Immuno-localization of the insulin-regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 113:123–135.
38. Südhof, T. C., and R. Jahn. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron.* 6:665–677.
39. Tokuyasu, K. T. 1973. A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551–565.
40. Tooze, S. A., and W. B. Huttner. 1990. Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell.* 60:837–847.
41. Torri-Tarelli, F., A. Villa, F. Valtorta, C. P. De, P. Greengard, and B. Ceccarelli. 1990. Redistribution of synaptophysin and synapsin I during α-latrotoxin-induced release of neurotransmitter at the neuromuscular junction. *J. Cell Biol.* 110:449–459.
42. Zorzano, A., W. Wilkinson, N. Kotliar, G. Thoidis, B. E. Wadzinkski, A. E. Ruoho, and P. F. Pilch. 1989. Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J. Biol. Chem.* 264:12358–12363.