Separation and Partial Characterization of Three Distinct Intracellular GLUT4 Compartments in Rat Adipocytes

SUBCELLULAR FRACTIONATION WITHOUT HOMOGENIZATION

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Insulin recruits GLUT4 from an intracellular location to the plasma membrane in rat adipocytes. The process involves multiple intracellular compartments and multiple protein functions, details of which are largely unknown partly due to our inability to separate individual GLUT4 compartments. Here, by hypotonic lysis, differential centrifugation, and glycerol density gradient sedimentation, we separated intracellular GLUT4 compartments in rat adipocytes into three fractions: plasma membrane-containing fraction T and plasma membrane-free fractions H and L. The GLUT4 contents in fractions T, H, and L were ~25, 56, and 18% of total GLUT4, respectively, in basal adipocytes and 55, 42, and 3–4% in insulin-stimulated adipocytes. The plasma membrane GLUT4 contents estimated separately further revealed that intracellular GLUT4 in fraction T amounts to ~20% in both basal and insulin-stimulated adipocytes. Organelle-specific marker and membrane traffic-related protein distribution data suggested that intracellular GLUT4 in fraction T represents sorting endosomes, whereas GLUT4 in fractions H and L represents storage endosomes and exocytic vesicles, respectively. The subcellular fractionation without homogenization described here should be useful in identifying the role of the individual GLUT4 compartments and the associated proteins in insulin-induced GLUT4 recruitment in rat adipocytes.

The uptake of glucose by muscle and adipose cells is a tightly insulin-regulated process, mediated primarily by the GLUT4 facilitative glucose transporter isoform (recently reviewed in Refs. 1–3). GLUT4 in these cells is mostly (>90%) sequestered in an intracellular pool, and insulin stimulates glucose uptake by recruiting GLUT4 from this intracellular pool to the plasma membrane (2, 4, 5). It is also known that GLUT4 constantly recycles between the plasma membrane and the intracellular pool by endocytosis and exocytosis and that insulin causes GLUT4 recruitment through modulation of these processes (6–8). Immunoelectron microscopic studies have revealed that GLUT4 in adipocytes is associated with several morphologically distinct membrane structures, including tubulovesicular elements, small vesicles, clathrin-coated vesicles, and plasma membrane invaginations (9, 10). These data support the notion that the intracellular GLUT4 pool of adipocytes is composed of several distinct compartments or organelles. We do not yet know, however, exactly what roles these various GLUT4 compartments play in GLUT4 recycling and sequestration and how they are regulated by insulin.

The intracellular GLUT4 pool in rat adipocytes has been isolated after cell homogenization by immunoadsorption of microsomal fractions with anti-GLUT4 antibodies. The GLUT4 pool thus isolated was shown to be a homogeneous population of small vesicles of uniform size, 50–70 nm in diameter (11, 12). The GLUT4-containing vesicles show significant biochemical differences from other intracellular membrane compartments such as synaptic vesicles and secretory vesicles (13). Biochemical and immunological analyses of the GLUT4-containing vesicles have revealed the identity of a number of resident proteins. These include the vesicle fusion machinery proteins such as VAMPs (14–16), carrier proteins in secretory granules and synaptic vesicles such as secretory carrier-associated membrane proteins (17, 18), endosomal markers such as the insulin-like growth factor II/Man-6-P receptor (11), and recycling endosomal markers such as the transferrin receptor (19, 20). The proteins noted above are not uniquely found together with GLUT4, but the insulin-responsive aminopeptidase appears completely co-localized with the transporter and translocates to the plasma membrane in response to insulin in a quantitatively similar manner to GLUT4 (16, 21–23). Sortilin is another integral membrane protein previously reported as a neurotensin receptor (24) that also localizes with GLUT4 to some extent (25), and there are a number of extrinsic membrane proteins that are also known to associate with GLUT4-containing vesicles. The latter include acyl-CoA synthetase-1 (26), Akt-2/protein kinase B (27, 28), Rab4 (29), PI 3-kinase (30), PI 4-kinase (31), and GDP dissociation inhibitor-2 (32). It is expected that some or most of these proteins may participate in GLUT4 recycling or sequestration, playing specific roles in distinct compartments. Identification of such compartment-specific association and roles for these proteins may be facilitated if individual GLUT4 compartments can be isolated intact and free of homogenization artifacts.

In this study, we describe a novel method that separates intracellular GLUT4 organelles of rat adipocytes into three distinct fractions (T, H, and L) with minimal vesiculation artifacts. The method uses hypotonic lysis instead of conventional mechanical homogenization, followed by differential centrifugation.
...vation and glycerol gradient velocity sedimentation. Electron microscopic and organelle-specific marker distribution data show that these fractions are largely free from vesicular artifacts. We also show that the GLUT4 compartments in these fractions are distinct not only in immunoreactivity and protein composition, but also in response to cellular insulin exposure. Furthermore, we immunopurified a major insulin-responsive intracellular GLUT4 compartment composed of small (50–70 nm) vesicles of uniform size that may be involved in GLUT4 exocytosis. This is the first purification of insulin-responsive GLUT4 exocytic vesicles as a major compartment in the itinerary of insulin-induced GLUT4 recruitment in rat adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Collagenase (type I) was obtained from Worthington. 1F8, an affinity-purified GLUT4-specific monoclonal antibody (MAIRGT 1F8) with an epitope in the carboxy-terminal 30 amino acids, was purchased from Biogenesis Ltd. (Sandown, NH). Anti-TRI monoclonal antibody (A6431) was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-phosphotyrosine (Y-1591) and anti-phosphotyrosine (Y-1594) antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). All other reagents were prepared in NaCl/HEPES buffer over 400 g for 1 h using a Beckman SW 40.2 rotor to obtain the total particulate fraction analyzed in Fig. 1. The 185,000 g pellet was resuspended in 600 μl of NaCl/HEPES buffer (150 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 2.5 mM Na2HPO4, 2.5 mM CaCl2, and 10 mM HEPES, pH 7.4) containing 1% bovine serum albumin and 2 mM glucose. Cells were then washed and lysed with hypo-osmotic medium for 15 s by floating the suspension for 15 s at 900 g. The infranatant and pellet were centrifuged in the Ti-50.2 rotor and resuspended in 600 μl of NaCl/HEPES buffer (150 mM NaCl, 10 mM HEPES, 1 mM EDTA, and 1 mM MgCl2, pH 7.4) was eluted with SDS-containing Laemmli buffer without β-mercaptoethanol for 1 h at room temperature. Recovered eluates were diluted with 1% NaCl/HEPES buffer using the pipette as described above. These pellets were fixed with 3% glutaraldehyde, pH 7.4, in PBS (134 mM NaCl, 2.6 mM KCl, 6.4 mM Na2HPO4, and 1.46 mM KH2PO4, pH 7.4) for at least 1 h, post-fixed in 1% OsO4 for 2 h, rinsed three times with PBS, and then dehydrated with a 50–100% ethanol gradient. Pellets were embedded in Epon-Araldite and dissected at a thickness of 700 Å. Uranyl acetate saturated in ethanol was used for staining, and lead citrate was used for counterstaining. Sections were examined and photographed at 80 kV using a Siemens Analytical X-Ray Instruments 101 electron microscope.

Electron Microscopy—The 900 × g pellet and two fractions of the 185,000 × g pellet after glycerol gradient fractionation were examined without sonication. Glycerol gradient fractions P, 1, and 2 of the 900 × g pellet (fraction T) or the 185,000 × g pellet (fraction H) were pooled and centrifuged at 185,000 × g for 2 h. Similarly, gradient fractions P–L were pooled and centrifuged as described above. These pellets were fixed with 3% glutaraldehyde, pH 7.4, in PBS (134 mM NaCl, 2.6 mM KCl, 6.4 mM Na2HPO4, and 1.46 mM KH2PO4, pH 7.4) for at least 1 h, post-fixed in 1% OsO4 for 2 h, rinsed three times with PBS, and then dehydrated with a 50–100% ethanol gradient. Pellets were embedded in Epon-Araldite and dissected at a thickness of 700 Å. Uranyl acetate saturated in ethanol was used for staining, and lead citrate was used for counterstaining. Sections were examined and photographed at 80 kV using a Siemens Analytical X-Ray Instruments 101 electron microscope.

RESULTS

Hypotonic Lysis Introduces Minimal Vesiculation in Artifacts—We prepared rat adipocyte lysates by conventional homogenization and by hypotonic lysis as described under “Experimental Procedures” and the tendency of the resulting membranes to artificial vesiculation was assessed by glycerol gradient velocity sedimentation (Fig. 1). The distribution of total protein was not notably different between lysates prepared by either method (data not shown). Both methods resulted in two distinct GLUT4 pools upon gradient centrifugation: a relatively sharp, rapidly sedimenting pool (fractions P, 1, and 2) and a broad, slowly sedimenting pool (fractions 3–11). However, the relative amount of the slowly sedimenting GLUT4 pool was clearly different between the two lysates. The slowly sedimenting GLUT4 pool was as large as 60% of total...
GLUT4 in the homogenized lysate, whereas it was <20% in the hypotonically prepared lysate. Using homogenized adipocyte lysates, Herman et al. (13) have already shown that the slowly sedimenting pool seen here represents a pool of uniformly small vesicles with an estimated diameter of 50–100 nm, similar to the size of synaptic vesicles (50–70 nm). Apparently, homogenization, due to shearing forces inherent to the method, may have caused vesiculation of large organelles, and thus, a significant portion of the slowly sedimenting, small GLUT4 vesicles observed in the homogenized lysate may be artifacts. On the other hand, hypotonic lysis, which would produce little or no shearing forces, would have produced little or no artificial vesiculation. Electron microscopic studies have indicated that a significant portion (as high as 40%) of GLUT4 in adipocytes exists as small vesicular structures (9, 10). The slowly sedimenting GLUT4 pool detected in the lysates prepared by hypotonic lysis may therefore represent a part of these preexisting small (50–70 nm) vesicular GLUT4 compartments.

Fractionation of Hypotonically Lysed Adipocyte Membranes into Fractions T, H, and L—We separated hypotonically lysed adipocytes into three particulate fractions (T, H, and L) by differential centrifugation and glycerol gradient velocity sedimentation as described under “Experimental Procedures.” A low-speed centrifugation step separated the lysate into the 900 × g pellet (fraction T) and its supernatant. Fraction T was originally described by Rodbell (39) as delipidated adipocyte ghosts, a preparation of plasma membrane sheets with other organelles, typically mitochondria and nuclei. On the other hand, the 900 × g supernatant contained, in addition to cytosol, those intracellular membrane structures and organelles that were released from plasma membrane boundaries during hypotonic lysis. The organelles and membrane structures in this 900 × g supernatant were quantitatively recovered in a high-speed pellet (185,000 × g pellet). Subsequent glycerol gradient velocity sedimentation separated the 185,000 × g pellet and its GLUT4 content into two distinct pools: a relatively sharp, rapidly sedimenting one (fraction H) and a broad, slowly sedimenting one (fraction L) (Fig. 2).

In contrast, glycerol gradient velocity sedimentation of fraction T showed only a rapidly sedimenting GLUT4 pool, with no slowly sedimenting GLUT4 pool (Fig. 2). However, sonication changed the sedimentation characteristics of fraction T; it induced a small but distinct amount of the slowly sedimenting GLUT4 pool, (T,) in fraction T (Fig. 2 and Table I), with a slight reduction in the rapidly sedimenting GLUT4 pool, (T,) (please note that we use parentheses to distinguish sonically disrupted samples from those not disrupted by sonication throughout this study). The (T,) GLUT4 pool constituted as much as 5% of total cellular GLUT4 in basal adipocytes, and it was increased to 11–12% in insulin-treated cells. The (T,) GLUT4 pool size was not appreciably increased by extended sonication time or intensity (data not shown). The exact origin of the (T,) GLUT4 pool is not known. It may represent in part a population of authentic GLUT4-containing vesicles that were structurally trapped in or otherwise associated with the plasma membrane sheets in fraction T. It is important to emphasize that the membrane structures in these sonically disrupted fractions may not represent native compartments: sonically disrupted samples were used only for the purpose of biochemical and immunological characterization of GLUT4 compartments (see below).

Electron microscopic examination revealed that fractions T, H, and L are made of morphologically distinct membrane structures (Fig. 3). Fraction T (900 × g pellet without sonication) showed intact nuclei and mitochondria sequestered in cell boundaries with large membranous structures similar to rough and smooth endoplasmic reticula. Fraction H showed only large membranous structures with relatively few and variously sized vesicular structures. In fraction L, however, only small vesicular particles uniform in size (estimated to be 50–100 nm in diameter) were seen, together with ribosome-like structures and abundant amorphous protein fragments.

Relative Distribution of GLUT4 in Fractions T, H, and L and Its Sensitivity to Insulin—We next investigated the relative GLUT4 pool sizes of fractions T, H, and L and how they are affected by insulin treatment. Adipocytes were treated for 20 min with or without insulin (100 nM) and then subjected to hypotonic lysis in the presence or absence of insulin, respectively. The lysates were fractionated into fractions T, H, and L as described above, and their respective protein and GLUT4 contents were assayed (Fig. 4 and Table I). Insulin had no appreciable effect on the amount of protein in each fraction, whereas the GLUT4 content in each of these fractions was affected by insulin in a characteristic manner (Table I). GLUT4 in fraction L was particularly sensitive to insulin treatment. It amounted to −18% of total cellular GLUT4 in basal adipocytes and was reduced to <4% in insulin-stimulated adipocytes. Insulin also significantly reduced GLUT4 content in fraction H. Fraction H contained 56% of total cellular GLUT4 in basal adipocytes, and this was reduced to 41% in insulin-stimulated adipocytes. In contrast, insulin increased GLUT4 content in fraction T or (T,) plus (T,), Fraction T contained 26% of total cellular GLUT4 in basal adipocytes, and this was increased to 55% after insulin treatment (Fig. 4 and Table I).

Relative Distribution of Organelle-specific Markers in Fractions T, H, and L—We next assessed the relative enrichments of specific organelle markers in fractions T, H, and L by Western blot analysis (Fig. 5A). Integrin β1, a plasma membrane

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**Fig. 1.** Subcellular distribution of GLUT4 revealed by glycerol gradient velocity sedimentation of rat adipocyte total particulates. A total particulate fraction was prepared from 5 ml of packed adipocytes as described under “Experimental Procedures.” This was fractionated over a 5–30% glycerol gradient centrifuged at 60,000 × g for 1 h at 4 °C. Fractions were collected from the bottom to the top (pellet (P) and fractions 1–12). One-fortieth of each fraction was analyzed by immunoblotting using anti-GLUT4 antibody and detected using horseradish peroxidase-labeled protein A. The data shown are representative of three independent experiments.

**Fig. 2.** Distribution of GLUT4 in fractions T, (T,), H, and L. Hypotonically lysed adipocyte total particulates were subjected to fractionation by glycerol gradient velocity sedimentation as described under “Experimental Procedures.” 900 × g pellets were separately prepared before (fraction T) and after (T,) and (T,) sonication (2 h in a bath-type sonicator). One-fortieth of each fraction was used for immunoblotting. The data shown are representative of four independent experiments.
Three Intracellular GLUT4 Compartments in Rat Adipocytes

Percent distributions of protein and cellular GLUT4 in the absence or presence of insulin

| Protein | GLUT4 |
|---------|-------|
| Basal | Insulin |
| % | % |
| 900 × g | 185,000 × g |
| T | 55.3 ± 5.8 | 45.2 ± 4.2 |
| (T_H) | 22.6 ± 3.9 | |
| (T_L) | 32.7 ± 1.4 | |
| H | 24.7 ± 2.2 | 74.3 ± 2.9 |
| L | 20.3 ± 0.3 | 15.1 ± 1.3 |
| 59.4 ± 6.3 | 41.8 ± 3.9 |
| 55.0 ± 4.1 | 45.1 ± 2.2 |

Values represent mean ± S.D. from three independent experiments.

The fractions were prepared from hypotonically lysed adipocytes and fixed for microscopy as described "under Experimental Procedures." The scale of magnification is shown as a thick bar in the photograph. Arrowheads in the right panel indicate typical resolution of small vesicles ~50–80 nm in diameter. Similar results were obtained from three different preparations.

FIG. 3. Electron microscopy of three subcellular fractions (T, H, and L). The fractions were prepared from hypotonically lysed adipocytes and fixed for microscopy as described "under Experimental Procedures." The scale of magnification is shown as a thick bar in the photograph. Arrowheads in the right panel indicate typical resolution of small vesicles ~50–80 nm in diameter. Similar results were obtained from three different preparations.

FIG. 4. Effect of insulin on GLUT4 subcellular distribution revealed after separation by glycerol gradient sedimentation of hypotonically lysed adipocytes. Adipocytes (5 ml of packed cells) were incubated for 20 min in the absence (Basal) or presence (Insulin) of 100 nM insulin prior to hypotonic lysis. The entire lysis procedure was carried out in the presence of 100 nM insulin. 900 × g pellets were sonicated for 2 h in a bath-type sonicator prior to glycerol gradient fractionation to separate (T_H) and (T_L) (parentheses signify sonicated samples). 185,000 × g pellets were subjected to glycerol gradient fractionation without sonication to obtain fractions T and H. Western blotting was performed as described in the legend Fig. 1. The data shown are representative of six independent experiments. P, pellet.

The integrin signal was localized almost exclusively (95% or greater) in (T_H), with <5% found in fraction H. The integrin signal was absent in (T_L), indicating that sonication did not produce vesiculation of plasma membranes in fraction T. Thus, plasma membranes are almost exclusively in (T_H), and a trace, if any, is found in fraction H. Fraction L is totally devoid of plasma membranes. Cytochrome oxidase, a mitochondrial marker, was distributed in (T_H), (T_L), and H with relative abundance of ~70, 25, and 5%, respectively, and was absent in fraction L. This would indicate that 90–95% of mitochondria are in fraction T, with a small portion in fraction H. This is consistent with our electron microscopic findings (Fig. 3), where mitochondria were seen only in fraction T. 20% of this marker in (T_L) indicates that sonication of fraction T caused significant vesiculation of mitochondria. α-Adaptin, a subunit of the clathrin adaptor complex acting selectively on the plasma membrane (40), was abundant in T_H (75%) and T_L (20%), although a slight amount (~5%) was detectable in fraction H. There was no α-adaptin in fraction L. On the other hand, γ-adaptin, which is known to act on TGN membranes (41), was particularly abundant (>85%) in fraction H, although a small but significant portion was found in all other fractions, including fraction L. Furthermore, γ-adaptin in fractions H and L was reduced in insulin-stimulated adipocytes, a pattern similar to the insulin-induced GLUT4 reduction in these fractions. TGN38, a specific marker for TGN, was found only in fraction H, indicating that fractions T and L are free of TGN contamination. Rab5, an early endosomal marker, was found in (T_H), (T_L), and H with relative abundance of 60, 10, and 30%, respectively, but not in fraction L.

We also assessed, by Western blotting, the distributions in fractions T, H, and L of several proteins that may participate in the regulation of GLUT4 movement (Fig. 5B). It is interesting to note that insulin-responsive aminopeptidase, a resident protein in immunopurified GLUT4 vesicles, showed a distribution pattern similar to that of GLUT4 throughout all fractions. Both the aminopeptidase and GLUT4 were also affected similarly by insulin treatment; insulin increased the pool sizes in (T_H), (T_L), and reduced the pool sizes in fractions H and L. TR, a recycling endosomal marker, also showed a distribution pattern similar to those of GLUT4 and insulin-responsive aminopeptidase throughout the fractions and was affected similarly by insulin treatment. On the other hand, PI 3-kinase was broadly distributed in (T_H), (T_L), H, and L with relative abundance of ~40, 10, 30, and 20%, respectively, and insulin slightly but significantly increased the amount of this enzyme in every fraction (Fig. 5B). Akt-2/protein kinase B was predominantly (~85–90%) in fraction T (T_H plus T_L), although a small amount was found in fractions H and L. Protein kinase Cζ was predominantly (85–90%) in fraction T (T_H plus T_L), although a small amount was found in fractions H and L. Protein kinase Cζ was also found in (T_L), but not in fraction L. Myosin-II (42) and actin (43), the cytoskeletal proteins with an implied GLUT4 regulatory role, were abundant in (T_H) and H, found slightly in (T_L), but were absent in fraction L.
under similar experimental conditions (data not shown). Interestingly and as shown in Fig. 6, GLUT4 in fraction H became readily immunoadsorbable after sonication, and 50–60% of GLUT4 in (H) of basal adipocytes and practically all (90% or more) of GLUT4 in (H) of insulin-stimulated adipocytes were now immunoadsorbed with 1F8. Sonication also improved in part GLUT4 immunoadsorbability in fraction T, and this was related to production of a slowly sedimenting pool, (TL) mentioned above. More than 90% of GLUT4 in (T_L) was readily immunoadsorbed. On the other hand, GLUT4 in (T_H) was totally resistant to immunoadsorption.

The relative enrichment of some known GLUT4 vesicle-associated proteins in 1F8-adsorbed vesicles in (T_L), (H), and L was similarly assessed by Western blotting using appropriate antibodies (Fig. 6). Insulin-responsive aminopeptidase was found in every fraction with relative intensities quite similar to those of GLUT4, both in the presence and absence of insulin. TIR was also co-immunoadsorbed with GLUT4 in all fractions, and it was notably abundant in (TL). VAMP2 was seen primarily in immunoadsorbed vesicles from fraction L, and this was decreased dramatically after insulin treatment. Akt-2 occurred in all fractions, but co-immunoadsorbed with GLUT4 only in (H). Interestingly, Akt-2 was not co-immunoadsorbed with GLUT4 in insulin-treated (H).

Proteins in 1F8-immunoadsorbed and unadsorbed vesicles obtained from (T_L), (H), and L were studied after resolving them by 8% SDS-polyacrylamide gel electrophoresis. Typical results of 1F8-immunoadsorbed proteins visualized by silver staining are illustrated in Fig. 7. For each fraction, very little protein was immunoadsorbed with nonimmune IgG (NI), whereas several distinct protein bands were visible in 1F8-adsorbed vesicles. Proteins specific to 1F8-adsorbed vesicles were only a very small portion of the total protein species for each of the fractions (data not shown). The 1F8-immunoadsorbed proteins differed between fractions and insulin treatment (Fig. 7), even though unadsorbed supernatants had no difference with or without insulin for NI or 1F8 (data not shown). For (T_L), these included 280-, 230-, 120-, 110-, 92-, 86-, 75-, 67-, 62-, 55-, 46-, and 42-kDa proteins (Fig. 7, Pt.). Of these, the 110-, 92-, and 86-kDa proteins appeared to be increased after insulin treatment. Practically all of the protein bands seen in 1F8-adsorbed vesicles in (T_L) were also seen in 1F8-adsorbed vesicles of (H); there was no clear difference in component protein species between these two immunoadsorbates. However, the insulin responsiveness of some of these component proteins differed significantly between the (T_L) and (H) adsorbates. Thus, in the (H) adsorbates, insulin increased the 67- and 42-kDa protein contents in addition to the 92- and 86-kDa protein contents, decreased the 280- and 62-kDa protein contents, and did not affect the 110-kDa protein content. On the other hand, the 1F8 immunoadsorbates of fraction L showed a constituent protein pattern significantly different from (TL) and (H).

FIG. 5. Relative distributions of various organelle-specific markers (A) and selected GLUT4 pool-associated proteins (B) in fractions T, H, and L as assessed by immunoblotting. Fraction T was separated into (T_H) and (T_L) after sonication and used in immunoblotting. Fractions were prepared from adipocytes before (−) and after (+) insulin treatment. Insulin (100 nM) treatment was carried out as described in the legend to Fig. 4. One-fortieth of each fraction was loaded on each lane for SDS-polyacrylamide gel electrophoresis and blotted using various antibodies as described under “Experimental Procedures.” The data shown are representative of three independent experiments. CytOx, cytochrome oxidase; IRAP, insulin-responsive aminopeptidase; PI3K, PI 3-kinase; PKCζ, protein kinase Cζ.

FIG. 6. Insulin effects on the relative enrichment of selected GLUT4 pool-associated proteins in GLUT4 compartments of (T_L), (H), and L as assessed by Western blotting. GLUT4 compartments were purified by immunoadsorption using 1F8 for each fraction (100 μg of protein), and the resulting immunoadsorbed materials (Pt.) and unadsorbed supernatants (Sup.) were immunoblotted using appropriate antibodies as described under “Experimental Procedures.” One-fifth of adsorbed materials and one-fiftieth of unadsorbed supernatants were used for analysis. The data shown are representative of four independent experiments.
from those of (T<sub>L</sub>) and (H). The L adsorbates showed a highly selective protein profile, typically abundant in higher molecular mass species, namely 280-, 250-, 230-, 160-, 120-, 110-, 92-, 86-, 62-, and 59-kDa proteins. Of these, the 280-, 250-, 160-, and 110-kDa proteins were reduced greatly after insulin treatment. The identities of the 280- and 250-kDa proteins are unknown, although the 250-kDa protein could be the insulin-like growth factor II/Man-6-P receptor. The 160- and 110-kDa proteins are most likely insulin-responsive aminopeptidase and sortilin, respectively. It appears that the L adsorbates were abundant in higher molecular mass protein species, whereas the (T<sub>L</sub>) and (H) adsorbates were abundant in lower molecular mass protein species. Some of these component proteins may be peripheral membrane proteins, and immunopurified vesicles from fraction L (G4L), where no sonic disruption was involved, may include cargo proteins such as adipin or leptin (44, 45).

**DISCUSSION**

**Hypotonic Lysis Allows Separation of Intracellular GLUT4 Compartments Close to Their Native States**—Like that of many recycling membrane proteins and receptors (reviewed in Refs. 46 and 47), the itinerary of GLUT4 recycling in rat adipocytes appears to involve multiple intracellular organelles, including endocytosed vesicles; endosomes for sorting, storage, and/or recycling; and exocytic vesicles (2, 4, 5). Evidence indicates that a number of specific proteins participate in this process, each playing a specific role in designated compartments. GLUT4-containing vesicles immunopurified from homogenized cell lysates have been instrumental to the biochemical identification of many GLUT4 pool-associated proteins as noted in the Introduction. However, compartment-specific assignment of these proteins in the GLUT4 itinerary requires subcellular fractionation and isolation of intracellular organelles close to their native states. The GLUT4-containing vesicles immunopurified after conventional cell homogenization, because of vesiculation due to mechanical shearing, could have lost much of their information on compartment-specific protein functions.

In this study, we have demonstrated that hypotonic lysis followed by differential centrifugation and glycerol velocity gradient sedimentation separates intracellular GLUT4 compartments of rat adipocytes into three distinct fractions: T, H, and L. The intracellular GLUT4 compartments in fractions T, H, and L (G4T, G4H, and G4L, respectively) are close to their native states, with little evidence for the existence of homogenization artifacts. The following findings support this interpretation. In lysates obtained by hypotonic lysis, <20% of cellular GLUT4 was in a slowly sedimenting vesicular pool as analyzed by glycerol gradient sedimentation analysis, whereas in lysates obtained by mechanical homogenization, as much as 60% of GLUT4 was in a similarly sedimenting vesicular pool (Figs. 1 and 2). Because insulin treatment further reduced this slowly sedimenting vesicular pool of GLUT4 to as low as 4% in hypotonically lysed adipocytes (Fig. 4), the slowly sedimenting vesicular pool is not likely to be an artifact. Electron microscopic examination (Fig. 3) showed that mitochondria in fraction T are largely intact, with no apparent fragmentation. Organelle-specific marker distribution data (Fig. 5A) revealed that very little (5% or less) integrin β<sub>1</sub>, calnexin, cytochrome oxidase, or α-adaptin occurs in fraction H, and they are totally absent in fraction L, the only vesicular fraction, indicating that hypotonic lysis produces little membrane and organelle vesiculation.

**Fraction L Contains an Insulin-sensitive GLUT4 Exocytic Compartment**—Of the three fractions of GLUT4 compartments we separated here, the one in fraction L (G4L) seems relatively straightforward to identify. This GLUT4 compartment (G4L) was readily and quantitatively immunoadsorbed by 1F8 with no additional experimental manipulations after lysis. We propose that G4L is the insulin-responsive GLUT4 exocytic vesicle population. The following findings support this contention. Electron microscopy (Fig. 3) showed that fraction L is a homogeneous population of uniformly small vesicles (50–100 nm in diameter). Fraction L was free of integrin β<sub>1</sub>, calnexin, TGN38, cytochrome oxidase, and Rab5 (Fig. 5), ruling out contamination of vesiculated plasma membranes, endoplasmic reticulum, TGN, mitochondria, and recycling endosomes in this fraction and in G4L. On the other hand, fraction L contained PI 3-kinase, Akt-2, and γ-adaptin in addition to GLUT4 and insulin-responsive aminopeptidase (Fig. 5B). Similar to fraction L (Fig. 5B), G4L is enriched not only in insulin-responsive aminopeptidase and TfR, but also in VAMP2, a v-SNARE protein found in exocytic vesicles (Fig. 6). Interestingly, G4L was devoid of Akt-2. Furthermore, G4L was exceptionally sensitive to insulin stimulation. It accounted for as much as 18% of total cellular GLUT4 in basal adipocytes, which was reduced to <4% in insulin-stimulated adipocytes, a 75–80% reduction in compartment size, or a reduction corresponding to 14% cellular GLUT4, after insulin treatment (Fig. 4 and Table II). Insulin typically recruited 30% of cellular GLUT4 from the intracellular pool to the plasma membrane in our experiments (data not shown). Thus, G4L is a major insulin-responsive intracellular compartment accounting for as much as one-half of the insulin-induced GLUT4 recruitment in adipocytes.

The role of G4L in insulin-induced GLUT4 recruitment and the molecular mechanism by which insulin reduces this compartment are yet to be identified. One interesting possibility is that docking and fusion of these presumably GLUT4 exocytic vesicles with the plasma membrane are regulated by insulin. Fusion machinery proteins such as v- and t-SNAREs have been shown to participate in insulin-stimulated GLUT4 translocation (2, 48, 49) and may be targets of insulin regulation in this compartment, as might be the recently identified syntaxin 4-interacting protein, Synip (50).

**GLUT4 Compartment in Fraction H Represents a Major Insulin-sensitive Storage Pool**—The GLUT4 compartment in fraction H (G4H) is by far the largest intracellular GLUT4 compartment, and it is also insulin-sensitive. G4H accounted for 55% of total GLUT4 in basal adipocytes and 40% of total GLUT4 in insulin-stimulated adipocytes. Insulin thus reduced
this compartment size by 25–30%. Compared with G4L, which was reduced by insulin by 75–80%, the insulin effect on G4H is relatively modest. Nevertheless, because G4H is much larger than G4L in size, the two compartments contribute equally (each providing 14–15% cellular GLUT4) to insulin-induced GLUT4 recruitment.

Although the exact role of G4H is not known, we propose as a working hypothesis that G4H is the putative GLUT4 recycling (or sequestration) endosomal compartment. The following findings support this possibility. Fraction H is particularly enriched in TGN38 and γ-adaptin (Fig. 5). G4H is abundant in TIR (Fig. 6), and TIR recycling endosomes are known to sediment in this fraction (13). A substantial co-localization of GLUT4 in the recycling endosomal compartment has been indicated (5). The large pool size (~55% of cellular GLUT4) and its limited sensitivity to insulin are also consistent with this possibility.

Intracellular GLUT4 Compartment in Fraction T Is Largely Insulin-insensitive—Fraction T contains plasma membrane sheets and trapped intracellular organelles such as nuclei and mitochondria (Fig. 3). Fraction T contained >95% of integrin β1 and α-adaptin (Fig. 5), indicating that this fraction contains practically all of the plasma membrane. This fraction contained 26 and 55% of total GLUT4 in basal and insulin-treated adipocytes, respectively (Table I). On the other hand, the amount of plasma membrane GLUT4 in our adipocyte preparations determined by cell-surface labeling amounted to 6 and 36% of total cellular GLUT4 in basal and insulin-stimulated adipocytes, respectively (6). Thus, by a simple subtraction, one realizes that the intracellular GLUT4 compartment in fraction T (G4T) was not affected in size by insulin treatment, amounting to ~20% of total cellular GLUT4 in both basal and insulin-stimulated adipocytes.

GLUT4 compartments in fraction T, like those in fraction H, were not immunoadsorbed by 1F8 unless they were sonicated. Sonication separated fraction T into two subfractions, a rapidly sedimenting fraction, (T1H), and a slowly sedimenting fraction, (T1L), in glycerol gradients. GLUT4 in (T1H), like that in fraction T, was not immunoadsorbable by 1F8. In contrast, GLUT4 in (T1L) was readily and almost quantitatively immunoadsorbed by 1F8. (T1L) accounted for ~4 and 12% of cellular GLUT4 in basal and insulin-stimulated adipocytes, respectively (Table I). The exact relationship of GLUT4 in (T1L) to that in fraction T has yet to be determined. It may be in part a portion of G4T such as insulin-responsive endocytic vesicles and/or vesiculated recycling endosomes. Alternatively, it may be a sonication artifact of plasma membrane sheets, although the lack of integrin speaks against this possibility. GLUT4 in (T1L) corresponded to 25 and 60% of G4T and 60 and 40% of plasma membrane GLUT4 in basal and insulin-stimulated adipocytes, respectively.

Possible Role of G4T, G4H, and G4L in the GLUT4 Itinerary in Rat Adipocytes: A Model—The itinerary of GLUT4 recycling must involve not only membrane dynamics such as membrane budding and fusion, but also a series of intracellular compartments (collectively known as endosomal compartments), including early, sorting, sequestering, and recycling endosomes. GLUT4 internalization appears to occur via clathrin-coated endocytosis (9, 51–54), the pathway shared by a number of membrane proteins, including TIR and insulin-like growth factor II receptors (46, 47). GLUT4 is nevertheless regulated by insulin selectively from other recycling proteins, indicating the presence of a compartment where GLUT4 is sorted from these receptors (16, 55). The presence of a GLUT4 storage compartment has also been indicated by a large intracellular GLUT4 pool apparently not directly involved in insulin-induced recruitment (2, 9).

The possible identities of the intracellular GLUT4 compartments (G4T, G4H, and G4L) to the organelles or endosomal compartments involved in GLUT4 recycling are illustrated in Fig. 8. G4T, with its modest size, which is not affected by insulin, is most likely to represent the putative sorting compartment. On the other hand, G4H, the largest GLUT4 compartment, supplying 50% of insulin-induced GLUT4 recruitment, is likely the recycling or storage compartment. This compartment contains abundant TIR, with little or no VAMP2 (Fig. 6), and most likely corresponds to the tubulovesicular structure (Fig. 3), as seen in electron microscopy (9), from which exocytic vesicles may bud. G4L may be an authentic vesicular compartment of GLUT4 exocytic vesicles in “ready to go state” for fusion to the plasma membrane. Insulin reduces this compartment much more drastically than G4H. This suggests this compartment, specifically at the SNARE-mediated GLUT4 vesicle fusion step, as a major control point in insulin-induced GLUT4 recruitment.

In conclusion, the separation of G4T, G4H, and G4L described here would allow us to identify compartment-specific association and the role of many known GLUT4 vesicle resident proteins. Furthermore, our immunopurified G4L is the first preparation available for detailed biochemical and functional characterization of a GLUT4 compartment in rat adipocytes close to native states.
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