GLUT4 Overexpression or Deficiency in Adipocytes of Transgenic Mice Alters the Composition of GLUT4 Vesicles and the Subcellular Localization of GLUT4 and Insulin-responsive Aminopeptidase

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The majority of GLUT4 is sequestered in unique intracellular vesicles in the absence of insulin. Upon insulin stimulation GLUT4 vesicles translocate to, and fuse with, the plasma membrane. To determine the effect of GLUT4 content on the distribution and subcellular trafficking of GLUT4 and other vesicle proteins, adipocytes of adipose-specific, GLUT4-deficient (aP2-GLUT4−/−) mice and adipose-specific, GLUT4-overexpressing (aP2-GLUT4-Tg) mice were studied. GLUT4 amount was reduced by 80–95% in aP2-GLUT4−/− adipocytes and increased ~10-fold in aP2-GLUT4-Tg adipocytes compared with controls. Insulin-responsive aminopeptidase (IRAP) protein amount was decreased 35% in aP2-GLUT4−/− adipocytes and increased 45% in aP2-GLUT4-Tg adipocytes. VAMP2 protein was also decreased by 60% in aP2-GLUT4−/− adipocytes and increased 2-fold in aP2-GLUT4-Tg adipocytes. IRAP and VAMP2 mRNA levels were unaffected in aP2-GLUT4-Tg, suggesting that overexpression of GLUT4 affects IRAP and VAMP2 protein stability. The amount and subcellular distribution of syntaxin4, SNAP23, Munc-18c, and GLUT1 were unchanged in either aP2-GLUT4−/− or aP2-GLUT4-Tg adipocytes, but transferrin receptor was partially redistributed to the plasma membrane in aP2-GLUT4-Tg adipocytes. Immunogold electron microscopy revealed that overexpression of GLUT4 in adipocytes increased the number of GLUT4 molecules per vesicle nearly 2-fold and the number of GLUT4 and IRAP-containing vesicles per cell 3-fold. In addition, the proportion of cellular GLUT4 and IRAP at the plasma membrane in unstimulated aP2-GLUT4-Tg adipocytes was increased 4- and 2-fold, respectively, suggesting that sequestration of GLUT4 and IRAP is saturable. Our results show that GLUT4 overexpression or deficiency affects the amount of other GLUT4-vesicle proteins including IRAP and VAMP2 and that GLUT4 sequestration is saturable.

Insulin-stimulated glucose transport is essential for the maintenance of glucose homeostasis in mammals. The GLUT4 glucose transporter mediates insulin-regulated glucose transport (1–5). GLUT4 is expressed primarily in adipose tissue and skeletal and cardiac muscle (1–5), although expression has also been reported in kidney (2) and brain (6). In the absence of insulin, GLUT4 resides primarily in intracellular vesicles (1–5). The binding of insulin to its receptor initiates a cascade of tyrosine phosphorylation events that promote translocation, docking, and fusion of GLUT4-containing vesicles with the plasma membrane, promoting glucose uptake by the cell (7). This process plays a key role in normal physiology, and dysregulation of GLUT4 expression or trafficking is a major pathogenic factor in the insulin resistance seen in obesity and type 2 diabetes (8, 9).

Evidence suggests that, in addition to trafficking through the endosomal recycling pathway, GLUT4 vesicles consist of a unique intracellular storage compartment, separate from this pathway (10, 11). In 3T3-L1 adipocytes and primary rat adipocytes, GLUT4 is targeted to intracellular vesicles that are distinct from those containing GLUT1 (12). Ectopic expression of GLUT4 in neuroendocrine PC12 cells shows that GLUT4 is targeted to neither synaptic vesicles nor secretory granules, suggesting that GLUT4 forms a distinct class of storage vesicles (13, 14). In addition, diaminobenzidine treatment of adipocytes specifically ablates transferrin receptor (TR)3 and cellobrevin-containing endosomes, without eliminating a large portion of GLUT4-containing endosomes (15). An insulin-responsive aminopeptidase (IRAP) was the first protein identified that co-localizes with GLUT4 specifically in its unique intracellular compartment (16, 17). Like GLUT4, IRAP is ex-
pressed in adipocytes and skeletal muscle (17) and in other tissues. IRAP translocates with GLUT4 to the cell surface in response to insulin (16). The function of IRAP and its relationship to GLUT4 are unknown, although a recent study shows that IRAP-null mice have decreased GLUT4 expression in muscle and adipocytes (18).

Incorporation of vesicles into target membranes is mediated by a high affinity interaction between a ligand on the vesicle SNARE and receptors on the target membrane (target SNARE) which mediate docking and fusion (11, 19). Two vesicle SNAREs, VAMP2 (vesicle associated membrane protein 2) and VAMP3 (cellubrevin), are expressed in adipocytes (20). Unlike VAMP2, a large portion of adipocyte VAMP2 is targeted to the GLUT4 vesicle, suggesting a role for VAMP2 in the mediating GLUT4 trafficking (15). Adipocytes express two different target SNAREs, syntaxin4 (21, 22) and SNAP23 (23, 24), which together bind VAMP2 and provide the necessary target SNARE complex for vesicle fusion at the plasma membrane (25). Interaction between syntaxin4 and Munc-18c, which inhibits insulin-stimulated GLUT4 translocation by blocking binding of syntaxin4 and VAMP2, may also be required for proper incorporation of the GLUT4 vesicle into the plasma membrane (26). Although the involvement of VAMP2, SNAP23, syntaxin4, and Munc-18c in GLUT4-vesicle trafficking in normal adipocytes has been established (11, 19), many questions remain regarding the nature and regulation of GLUT4 vesicle number, size, protein content, and trafficking. Adipocytes from transgenic mice in which GLUT4 has been markedly reduced or overexpressed provide unique tools to learn more about the regulation of GLUT4 vesicles.

We hypothesized that altering adipocyte GLUT4 content may alter the protein composition, number, and/or sequestration of GLUT4-containing vesicles but not affect plasma membrane proteins mediating GLUT4 trafficking or other proteins trafficking through endosomes in response to insulin. We showed previously (27) in adipocytes from GLUT4-overexpressing mice that the polypeptide composition of immunoabsorbed GLUT4 vesicles was similar to control except for increased GLUT4 and a modest increase in aminopeptidase activity (presumably IRAP). We hypothesized that there were either more GLUT4 per vesicle or more vesicles with GLUT4. However, we did not assess the distribution of GLUT4 or other vesicle proteins between the plasma membrane and intracellular membranes nor the effect of GLUT4 deficiency on vesicle characteristics.

Here we employ complementary techniques to determine whether adipocyte GLUT4 content changes the characteristics of GLUT4 vesicles, using adipocytes of adipose-specific, GLUT4-deficient (aP2-GLUT4/−/−) mice (28) or adipose-specific, GLUT4-overexpressing (aP2-GLUT4-Tg) mice (29). We show that overexpression or deficiency of GLUT4 in adipocytes causes a corresponding change in IRAP and VAMP2 protein levels. IRAP and VAMP2 mRNA levels are unaltered by GLUT4 overexpression, suggesting that increased GLUT4 may alter the turnover of these proteins. Levels of plasma membrane proteins syntaxin4, SNAP23, and Munc-18c, which mediate GLUT4-vesicle docking and fusion with plasma membranes, are unchanged. Levels of GLUT1, which traffics to the plasma membrane primarily from a different intracellular compartment than GLUT4, are unchanged. Interestingly, the subcellular distribution of TIR, an early endosomal protein, is altered by high level GLUT4 overexpression, whereas the distribution of GLUT1 is not affected. Immunogold EM analysis shows that overexpression of GLUT4 increases the amount of GLUT4 per vesicle as well as the number of GLUT4- and IRAP-containing vesicles per cell. In addition, GLUT4 overexpression results in an increased proportion of GLUT4 at the plasma membrane in the absence of insulin, supporting the notion that GLUT4 sequestration is saturable. This could occur at the level of retention, endocytosis, and/or recycling of GLUT4 from the plasma membrane to intracellular stores. Our results also indicate that GLUT4 is a major determinant of GLUT4 vesicle net biogenesis and sequestration in vivo.

**Experimental Procedures**

*Mice—* Adipose-specific GLUT4−/− mice (28) and adipose-specific GLUT4-overexpressing mice (29) were housed at 22 °C with a 12-h light/dark cycle and fed standard rodent chow ad libitum. Mice were genotyped as described previously (28, 29). All studies were carried out in mice 12-16 weeks of age in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Similar results were observed in adipocytes from male and female mice.

**Isolation of Adipocytes and Determination of Glucose Transport and Cell Size and Number—*Perigonadal fat pads (1-2 g) from fed male or female mice sacrificed by CO2 inhalation (~10 per genotype for each experiment) were weighed, finely minced in Krebs buffer containing 20 mm Heps, 2.5% bovine serum albumin (Interneer, New York), and 200 mm adenosine, pH 7.4, at 37 °C, and digested for 30-40 min at 37 °C with constant shaking by addition of collagenase (1 mg/ml, Worthington). Following digestion, cells were passed through a 200-μm mesh (Braun, Melsungen, Germany) and washed four times. Cell suspensions of cells were incubated in the absence or presence of 100 nm regular porcine insulin ( Lilly) for 30 min with constant shaking at 37 °C (29, 30).

Uptake of [U-14C]glucose into isolated adipocytes (20% cell suspensions) was measured in the presence or absence of 100 nm porcine regular insulin for 30 min at 37 °C as described previously (29, 30). Aliquots of adipocytes were fixed in osmic acid and counted in a Coulter counter (31). To determine cell size, adipocyte mass was determined in quadruplicate by dividing the lipid content of the cell suspension by the cell number (31).

**Preparation of Total Membranes from Adipose Tissue and Isolated Adipocytes—** Adipose tissue (from either perigonadal or subcutaneous fat depots) or isolated adipocytes were homogenized with a Polytron (“Tissue Tearer” homogenizer; Biospec Products, Inc., Bartlesville, OK) in 10 nm Heps, 5 mM EDTA, 250 mm sucrose with 10 μg/ml aprotinin and 10 μg/ml leupeptin. Homogenates were centrifuged at 12,000 × g for 10 min at 4 °C to remove insoluble material. The supernatant was centrifuged at 225,000 × g for 90 min at 4 °C. The resulting pellet was resuspended to a concentration of ~2 μg/μl in homogenization buffer, and total membrane proteins were stored at ~80 °C.

**Preparation of Subcellular Membrane Fractions from Isolated Adipocytes—** Isolated adipocytes (20% cell suspensions), after a 30-min incubation at 37 °C in the absence or the presence of 80 mm insulin, were pelleted twice with TES buffer (20 mm Tris, 1 mm EDTA, 255 mm sucrose, pH 7.4 at 20 °C) at 15–18 °C and homogenized in TES at 4 °C using 15 strokes of a Potter drill per sample. Aliquots of homogenates were collected and stored at ~80 °C. Remaining homogenates were centrifuged at 16,000 × g for 15 min at ~4 °C, and surface solidified fat was removed. High density microsomal membranes and low density microsomal membranes (LDM) were isolated from supernatants, and plasma membranes (PM) were isolated from the pellets. Pellets containing PM were resuspended in TES, using 10 strokes of a Potter drill, and centrifuged at 16,000 × g for 20 min. Pellets were resuspended in TES, layered on a 1.12 sucrose cushion in 20 nm Tris-HCl, 1 mm EDTA, and centrifuged at 100,000 × g for 20 min at 4 °C. Plasma membranes at the interface between the sucrose cushion and buffer were collected, resuspended in TES, and centrifuged at 100,000 × g for 30 min at 4 °C. Plasma membrane pellets were resuspended to ~2 μg/μl in TES.

For preparation of microsomal membrane fractions, supernatants were centrifuged at 4 °C for 20 min at 50,000 × g. LDM was isolated from supernatants, and high density microsomal membranes were isolated from pellets. Pellets were resuspended in TES and centrifuged at 4 °C for 20 min at 50,000 × g and resuspended at ~1 μg/μl. Supernatants containing LDM were centrifuged at 200,000 × g for 90 min 4 °C; LDM pellets were resuspended at ~2 μg/μl. All membranes were kept at ~4 °C until use.

**Purity, Enrichment, and Recovery of Subcellular Fractions—** Purity of PM fractions was determined by immunoblotting for Ras, which is located exclusively in PM (32). Purity of LDM fractions was determined by immunoblotting for β-catenin protein (β-COP), a marker for LDM (33). Cross-contamination of fractions was negligible by immunoblot-
GLUT4 Amount Affects GLUT4-Vesicle Composition/Localization

### Table I

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body weight†</th>
<th>Fat pad weight†</th>
<th>n†</th>
<th>Adipocyte size†</th>
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<tr>
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<tr>
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<td>0.320 ± 0.024</td>
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<tr>
<td>aP2-GLUT4-Tg</td>
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<td>28.43 ± 0.91</td>
<td>4</td>
<td>0.351 ± 0.03</td>
</tr>
</tbody>
</table>

† indicates the number of experiments for adipocyte size. Each experiment was performed on pools of adipocytes from 6–10 mice per genotype. Results are expressed as counts/min per gel slice and corrected for the number of cells in each genotype.

GLUT4 Amount Affects GLUT4-Vesicle Composition/Localization

A quantitative real-time PCE—IRAP and VAMP2 mRNA from isolated adipocytes of control and aP2-GLUT4-Tg mice were quantified by quantitative real-time reverse transcriptase-PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Total RNA was extracted from isolated adipocytes using Trizol (Invitrogen). Total RNA concentrations were quantified in triplicate, and reverse transcriptase-PGRs for IRAF and 18 S RNA or VAMP2 and 18 S RNA were done in duplicate using 100 ng of total RNA for each sample. Sense 5′-GAAATTGGATTTGGTGGCCA-3′ and antisense 5′-TCCGAAGGAGCAGCCCG-3′ primers and probe 5′-FAM-GTCCTGACTCTTG-GAAGGGACGGGACATGGAAAAC-TAMRA-3′ specific for the mouse IRAP nucleotide sequence were used for cDNA synthesis and PCR amplification. Sense 5′-CTGGCAGCTCCTCTCACCAC-3′ and antisense 5′-CACCCACTCTCACCTGG-3′ primers and probe 5′-FAM-CTAG-TAACAGGAGACTGCAGCAGACCG-TAMRA-3′ specific for the mouse VAMP2 nucleotide sequence were used for cDNA synthesis and PCR amplification. Total RNAs were reverse-transcribed and amplified using the Taqman One-step PCR Master Mix Reagent (Applied Biosystems) following the manufacturer’s instructions. Reverse transcription was carried out at 48 °C for 30 min and terminated at 95 °C for 10 min. Reactions were amplified for 35 cycles at 95 °C for 15 s and 60 °C for 60 s.

**Results**

Effect of GLUT4 Amount on Fat Pad Weight, Adipocyte Size, and Glucose Transport in Adipocytes—Adipose-specific GLUT4-overexpressing (aP2-GLUT4-Tg) mice have increased perigenadal fat pad weight (Table I) due to adipocyte hyperplasia (28). Fat pad weight (Table I) and adipocyte cell number (28) are normal in adipose-specific, GLUT4-deficient (aP2-GLUT4−/−) mice. Adipocyte size in perigenadal fat pads of aP2-GLUT4−/− mice and aP2-GLUT4-Tg mice is similar to controls (Table I) as reported previously (28, 29).

In aP2-GLUT4−/− adipocytes, basal glucose transport tended to be decreased by 57% compared with controls (Fig. 1, left panel). Insulin-stimulated glucose transport was decreased by 78%, as reported previously (28). The residual glucose transport is probably due to the presence of GLUT1 and to GLUT4 remaining in a small subset of adipocytes that escaped Cre recombinase-mediated deletion.

In aP2-GLUT4-Tg adipocytes, basal glucose transport was increased ~30-fold and insulin-stimulated glucose transport ~10-fold compared with control (Fig. 1, right panel), as observed previously (29). The increase in glucose transport with insulin stimulation was 4.8-fold for control adipocytes but only 1.7-fold for aP2-GLUT4-Tg adipocytes. However, the increment in glucose transport above basal (insulin-stimulated minus basal) was 8-fold higher in aP2-GLUT4-Tg than in control adipocytes.

**GLUT4 Deficiency or Overexpression Causes Concordant Changes in IRAP and VAMP2—Immunoblots of total membrane proteins from isolated adipocytes of control, aP2-GLUT4−/−, and aP2-GLUT4-Tg mice showed that GLUT4 protein levels in aP2-GLUT4−/− adipocytes were approximately one-tenth the level of control littermates (Fig. 2A, left
Subcellular Distribution of GLUT4 and IRAP Changes with Increased GLUT4 Content—To determine whether changes in GLUT4 amount altered the subcellular distribution of GLUT4 and other proteins in the GLUT4-vesicle, GLUT4, IRAP, and VAMP2 levels in PM and LDM fractions of control, aP2-GLUT4−/−, and aP2-GLUT4-Tg adipocytes were determined by immunoblotting (Fig. 3A). Similar numbers of adipocytes from each genotype were used for subcellular fractionation to facilitate comparison. Proteins from PM and LDM fractions were immunoblotted with antisera specific for Ras, a plasma membrane marker (β-COP), a Golgi-related protein and LDM marker (33), to determine the purity of subcellular fractions. Cross-contamination of subcellular fractions was negligible using this method (data not shown). PM enrichment with Ras averaged 9 ± 4-fold greater than in homogenates. LDM enrichment with β-COP averaged 20 ± 6-fold greater than in homogenates. Recoveries and enrichment of PM and LDM proteins from subcellular membrane fractionation experiments were similar in control, aP2-GLUT4−/−, and aP2-GLUT4-Tg adipocytes (data not shown). Thus, the distribution of proteins among fractions could be directly compared. The difference in enrichment/purity of PM compared with LDM may account for the fact that the sum of GLUT4 in PM plus LDM in the basal state does not appear to equal the sum of GLUT4 in PM plus LDM in the insulin-stimulated state (Fig. 3B).

In control adipocytes in the basal state, 8% of GLUT4 was PM-associated and 92% of GLUT4 was LDM-associated (Fig. 3B). This was calculated by taking into account the concentration of GLUT4 per mg of membrane protein and the total amount of membrane protein for each fraction (37, 38). Upon insulin stimulation, 52% of GLUT4 was PM-associated and 48% of GLUT4 was LDM-associated (Fig. 3B). In aP2-GLUT4−/− adipocytes, a similar distribution of GLUT4 was observed, although the overall amount of GLUT4 was reduced 5-fold, similar to the reduction in GLUT4 in total adipocyte membranes (Fig. 2). In the basal state, 4.7% of GLUT4 was PM-associated and 95% of GLUT4 was LDM-associated (Fig. 3B). In insulin-stimulated aP2-GLUT4−/− adipocytes, 52% of GLUT4 was in the PM, whereas 48% of GLUT4 was in the LDM (Fig. 3B). The similar distribution of GLUT4 in control

panel), indicating an ~90% “knockout” of GLUT4 (28). Most interesting, IRAP and VAMP2 protein levels were also reduced ~35 and ~60%, respectively, in adipocytes of aP2-GLUT4−/− mice compared with controls, whereas expression of the serine/threonine kinase Akt did not change (aP2-GLUT4−/− Akt 129 ± 23% of WT) (Fig. 2, A and B, left panels). Similar changes in GLUT4, IRAP, and VAMP2 were obtained from adipose tissue of WT and aP2-GLUT4−/− mice (data not shown). This suggests that GLUT4 content may influence the synthesis or turnover of other GLUT4 vesicle proteins.

In aP2-GLUT4-Tg isolated adipocyte membranes, GLUT4 was overexpressed 9–12-fold compared with control littermates (Fig. 2A, right panel), as reported previously (29). IRAP and VAMP2 protein levels were also increased by ~45 and ~100% in aP2-GLUT4-Tg adipocytes compared with control, whereas expression of Akt was unchanged (aP2-GLUT4-Tg Akt 78 ± 10% of WT) (Fig. 2, A and B, right panels). Similar changes in GLUT4, IRAP, and VAMP2 were obtained from adipose tissue of WT and aP2-GLUT4-Tg mice (data not shown). Thus, overexpression of GLUT4 in aP2-GLUT4-Tg adipocytes results in concordant increases in IRAP and VAMP2. The reciprocal changes in IRAP in adipocytes of mice overexpressing or deficient in GLUT4 strengthens the hypothesis that GLUT4 content may regulate IRAP protein levels and/or the generation of GLUT4-IRAP-containing vesicles.

Fig. 1. Glucose transport into isolated adipocytes of control and adipose-specific GLUT4−/− (knockout, KO) (left panel), and control and adipose-specific GLUT4-overexpressing (TG) mice (right panel). Results are means ± S.E. of three separate experiments, each performed on pools of adipocytes from 4 to 10 mice per genotype. *, p < 0.05 compared with the corresponding control condition.

Fig. 2. IRAP and VAMP2 protein levels in isolated adipocytes change in parallel with GLUT4 levels. Total membrane proteins were prepared from isolated adipocytes of control and adipose-specific GLUT4−/− (KO) mice (left panels) and control and adipose-specific GLUT4-overexpressing (TG) mice (right panels). Membrane proteins were immunoblotted with anti-GLUT4, anti-IRAP, anti-VAMP2 or anti-Akt antisera, as indicated. A, representative autoradiograms of immunoblots. Each lane contains isolated adipocyte membrane protein from an individual mouse of the specified genotype. B, IRAP and VAMP2 immunoblots were quantitated by densitometry. Results are means ± S.E. of control (Con, n = 7) and knockout (KO, n = 8) (left) or control (Con, n = 17) and transgenic (TG, n = 15) (right) mice. Similar results for GLUT4, IRAP, and VAMP2 were obtained with adipose tissue (not shown), *, p < 0.05 compared with control.
GLUT4 Amount Affects GLUT4-Vesicle Composition/Localization

and aP2-GLUT4/−/− adipocytes was not unexpected. Residual GLUT4 in aP2-GLUT4/−/− adipocytes is from a small number of cells that escape Cre recombinase-mediated GLUT4 deletion thus retaining their full complement of GLUT4. In contrast, in basal aP2-GLUT4-Tg adipocytes, 31% of GLUT4 was PM-associated and 69% of GLUT4 was LDM-associated (Fig. 3B). In insulin-stimulated aP2-GLUT4-Tg adipocytes, 58% of GLUT4 was in the PM, whereas 42% of GLUT4 was in the LDM (Fig. 3B). The increased proportion of GLUT4 in the PM in both basal and insulin-stimulated states suggests that intracellular sequestration of GLUT4 is saturable. When data were expressed as total amount of GLUT4 per fraction in the basal or insulin-stimulated state, the results were the same.

The subcellular distribution of IRAP corresponded to GLUT4 distribution in control, aP2-GLUT4−/−, and aP2-GLUT4-Tg adipocytes. In control adipocytes, 11% of IRAP was PM-associated, and 89% of IRAP was LDM-associated (Fig. 3B). Upon insulin stimulation, 31% of IRAP was PM-associated, and 69% of IRAP was LDM-associated (Fig. 3B). In aP2-GLUT4/−/− adipocytes, a similar distribution of IRAP was observed, although the overall amount of IRAP was reduced 55%, similar to the reduction of GLUT4 observed in the total membrane preparation (Fig. 2B). In the basal state, 4% of IRAP was PM-associated and 96% of IRAP was LDM-associated (Fig. 3B). In insulin-stimulated aP2-GLUT4−/− adipocytes, 50% of IRAP was in the PM, and 50% of IRAP was in the LDM (Fig. 3B). In aP2-GLUT4-Tg adipocytes, the 1.7-fold increase in IRAP was associated with altered subcellular distribution, similar to what was observed for GLUT4. In the basal state in aP2-GLUT4-Tg adipocytes, 24% of IRAP was PM-associated, and 76% of IRAP was LDM-associated in the basal state (Fig. 3B). In insulin-stimulated aP2-GLUT4-Tg adipocytes, 49% of IRAP was in the PM, and 51% of IRAP was in the LDM (Fig. 3B). Thus, not only was there a small increase in the overall cellular content of IRAP but the percentage of IRAP localized to the PM in the basal state was also increased, similar to what was observed for GLUT4.

Overexpression or reduction in GLUT4 also resulted in concordant changes in VAMP2 in all subcellular membrane fractions. However, unlike for IRAP, there was no effect on VAMP2 subcellular distribution (Fig. 3B), presumably because VAMP2 is present in many intracellular vesicles and not unique to the GLUT4-vesicle compartment. GLUT4 overexpression did not alter the amount or distribution of several other proteins thought to be involved in GLUT4 trafficking. Syntaxin4, SNAP23, Munc-18C, and GLUT1 were not altered by GLUT4 deficiency (Fig. 4A) or overexpression (Fig. 4B). In contrast, there was a disproportionate increase in TIR in the PM relative to the LDM in aP2-GLUT4-Tg adipocytes (Fig. 4B), suggesting that overexpression of GLUT4 may impact trafficking of some proteins through early endosomes (39).

GLUT4 Overexpression Increases GLUT4 in the Plasma Membrane of Intact Adipocytes in Basal and Insulin-stimulated States—Recent studies have delineated several steps in the trafficking of GLUT4 vesicles to the PM from intracellular stores (19). GLUT4 vesicles first dock at the PM prior to fusing with the membrane (19). GLUT4 detected in PM fractions may be either in GLUT4 vesicles which have docked, but not yet fused with the PM, or GLUT4 fully inserted in the PM (11). To determine whether the increased GLUT4 detected in PM fractions of aP2-GLUT4-Tg adipocytes is from vesicles that have fused with the PM, we photolabeled cell surface GLUT4 in intact adipocytes before and after treatment with insulin. Glucose transport was performed in aliquots of the same cells.

In intact control adipocytes, insulin increased cell surface GLUT4 2.1-fold (Fig. 5A). In aP2-GLUT4-Tg adipocytes, basal plasma membrane GLUT4 is 4-fold higher, and insulin-stimulated plasma membrane GLUT4 is 2.4-fold higher compared with control (Fig. 5A). In basal and insulin-stimulated aP2-GLUT4-Tg adipocytes, increased cell surface GLUT4 detected by photolabeling correlates with increased glucose transport (Figs. 1 and 5B) and increased GLUT4 in plasma membrane fractions (Fig. 3). Taken together, these data show that high level overexpression of GLUT4 in adipocytes increases the proportion of GLUT4 that is functionally inserted in the PM of basal and insulin-stimulated cells, presumably due to saturation of the intracellular retention mechanism for GLUT4, although a defect in endocytosis cannot be ruled out. The saturation may involve multiple steps in GLUT4 trafficking, including retention of GLUT4 intracellularly as well as recycling of GLUT4 from PM to intracellular storage pools.

Overexpression of GLUT4 in Adipocytes Increases the Number of GLUT4 and IRAP-containing Vesicles and the Amount of GLUT4 per Vesicle—We assessed the amount of GLUT4 per vesicle...
vesicle, the number of GLUT4-containing vesicles per cell and the percent of intracellular vesicles containing GLUT4, IRAP, and VAMP2 in control and ap2-GLUT4-Tg adipocytes by immunogold EM. Representative micrographs are shown in Fig. 6A, and the quantification of >200 vesicles per genotype is shown in Fig. 6, B–E. Immunogold EM shows 10.4 ± 1.9% of intracellular vesicles staining for GLUT4 in control mice (Fig. 6B). In ap2-GLUT4-Tg mice, 29.8 ± 4.4% of intracellular vesicles stain for GLUT4 (Fig. 6B), demonstrating that overexpression of GLUT4 results in a 3-fold increase in the number of vesicles per cell that contain GLUT4. The percent of ap2-GLUT4-Tg adipocyte vesicles that contain IRAP is also increased 3-fold compared with controls (Fig. 6D).

The percentage of vesicles that contain IRAP and also GLUT4 was similar in control (74 ± 8) and ap2-GLUT4-Tg (86 ± 5), and GLUT4 vesicles containing IRAP were comparable in control (35 ± 9) and ap2-GLUT4-Tg (40 ± 9). Additionally, a similar percentage of control (25 ± 14) or ap2-GLUT4-Tg (25 ± 11) GLUT4 vesicles contained VAMP2. However, 55% of control VAMP2 vesicles contained GLUT4, but nearly all (86%) ap2-GLUT4-Tg VAMP2 vesicles contained GLUT4, an increase of 60% (Fig. 6C). Overall, these results demonstrate that overexpression of GLUT4 results in an increased number of IRAP-containing vesicles and in GLUT4 localization to an increased percentage of VAMP2 vesicles, suggesting that GLUT4 intracellular trafficking may be saturated.

The amount of GLUT4 per intracellular vesicle is increased ~80% in ap2-GLUT4-Tg mice compared with control (Fig. 6D). The average diameter of ap2-GLUT4-Tg adipocyte GLUT4 vesicles was unchanged compared with control (Fig. 6E). Thus, overexpression of GLUT4 in adipocytes increases the amount of GLUT4 per vesicle as well as the number of vesicles containing GLUT4 and IRAP per cell.

GLUT4 Overexpression Does Not Change IRAP or VAMP2 mRNA Levels in Adipocytes—To determine whether the alterations in IRAP and VAMP2 protein content observed in ap2-GLUT4-Tg adipocytes were due to changes in mRNA levels, steady-state levels of IRAP and VAMP2 mRNA were quantitated by real-time reverse transcriptase-PCR. IRAP and VAMP2 mRNA levels were similar in adipose tissue of control and ap2-GLUT4-Tg (IRAP, 93 ± 20% of control; VAMP2, 81 ± 23% of control) mice. Also, in isolated adipocytes the values were similar. Thus, GLUT4 may regulate IRAP and VAMP2 protein amount either by translational regulation or by protein sorting/turnover.

DISCUSSION

Intracellular Sequestration of GLUT4 Is Saturable in Adipocytes—A major finding of this study is that GLUT4 overexpression in adipocytes causes subcellular redistribution of GLUT4. Changes in GLUT4 subcellular distribution upon GLUT4 overexpression paralleled increases in glucose transport. Data are consistent using three independent techniques as follows: subcellular fractionation, photolabeling, and immunogold EM. Taken together, these results demonstrate that intracellular sequestration of GLUT4 in adipocytes is saturable. We showed previously GLUT4 is targeted to specific vesicles in adipocytes of ap2-GLUT4-Tg mice, which have a similar composition to control adipocytes (27). However, we examined only intracellular vesicles, and we could not rule out that GLUT4 might also be present in other structures that are more likely to target to the plasma membrane. In this study we show that the proportional increase in GLUT4 in the PM is greater than in the LDM. Our results suggest that this apparent saturation of intracellular retention may be due, at least in part, to the fact that high level GLUT4 overexpression alters intracellular targeting of GLUT4. Our immuno-EM studies show that GLUT4 overexpression in adipocytes increases the amount of GLUT4 per vesicle as well as the number of GLUT4-containing vesicles. Thus, GLUT4 overexpression not only increases plasma membrane GLUT4 but also redistributes intracellular GLUT4 among intracellular vesicles.

We have shown previously (27) that GLUT4-containing vesicles from both WT and ap2-GLUT4-Tg adipocytes exclude GLUT1, a glucose transporter that resides in a different vesicle population than GLUT4 and traffics to the PM more constitutively. These data are consistent with the lack of effect of GLUT4 overexpression on GLUT1 subcellular distribution (Fig. 4). However, the modest redistribution of TIR in the ap2-GLUT4-Tg adipocytes compared with WT (Fig. 4) indicates

**Fig. 4.** Syntaxin4, SNAP23, Munc-18C, GLUT1, and TIR protein levels in adipocyte subcellular membrane fractions from of GLUT4−/− and TG mice. Plasma membranes (PM) or low density microsomal membranes (LDM) were prepared from (A) control and adipose-specific GLUT4−/− (KO) adipocytes and (B) control and adipose-specific GLUT4-overexpressing (TG) adipocytes (n = 10–24 mice per genotype, four separate experiments). Adipocytes from control, GLUT4−/−, and TG mice were incubated in the absence (−) or presence (+) of 100 nM insulin for 30 min prior to fractionation. PM and LDM proteins were immunoblotted with anti-syntaxin4, anti-SNAP 23, anti-Munc-18c, anti-GLUT1, or anti-TIR antiserum as indicated.

**Fig. 5.** Overexpression of GLUT4 increases cell surface GLUT4 in basal and insulin-stimulated adipocytes. A, photolabeled cell surface GLUT4, and B, glucose transport in isolated adipocytes of control and adipose-specific GLUT4-overexpressing (TG) mice before (−) and after (+) treatment with 100 nM insulin. Results are means ± S.E. for three separate experiments. * p < 0.05 compared with the corresponding control condition.
GLUT4 Amount Affects GLUT4-Vesicle Composition/Localization

Fig. 6. Overexpression of GLUT4 increases the amount of GLUT4 per vesicle and the number of GLUT4 and IRAP containing vesicles per cell. Intracellular membrane vesicles were isolated from control (Con) or adipose-specific GLUT4-overexpressing (TG) adipocytes, fixed, immunosorbed with antibodies to GLUT4, IRAP, or VAMP2 and gold-labeled secondary antibodies. Vesicles were visualized by staining with uranyl acetate and methylcellulose. A, representative micrographs of vesicles from control and TG adipocytes stained for GLUT4 (small gold particle) and VAMP2 (large gold particle). B, percentage of intracellular vesicles containing GLUT4, IRAP, or VAMP2. C, percentage of VAMP2 vesicles with GLUT4. D, number of GLUT4 molecules per vesicle, and E, GLUT4 vesicle diameter. Results are means ± S.E. for 3–5 separate experiments. Two hundred vesicles were examined per genotype in each experiment. *, p < 0.05 compared with control.

there is some effect of GLUT4 overexpression on the constitutive endocytic pathway.

Data regarding whether overexpression of GLUT4 saturates its sequestration are conflicting. In a study with rat adipocytes overexpressing hemagglutinin-tagged GLUT4, increases in plasma membrane GLUT4 were proportional to the overall increase in GLUT4 expression, suggesting that GLUT4 sequestration is not saturable (40). However, our results are consistent with several earlier studies. Overexpression of a carboxyterminal 43-amino acid GLUT4 peptide increased basal plasma membrane GLUT4 in rat adipocytes in vitro (41). Interestingly, overexpression of a 27-amino acid peptide of IRAP similarly redistributed GLUT4 to the plasma membrane in unstimulated 3T3-L1 adipocytes (42). Another study in which GLUT4 was overexpressed by its own promoter demonstrated disproportionately increased plasma membrane GLUT4 in adipocytes and cardiac myocytes in the basal state (43). Transgenic overexpression of GLUT4 in muscle showed that overexpression of GLUT4 saturates its intracellular compartment resulting in increased GLUT4 associated with the plasma membrane in the basal state (44–46), although changes in glucose transport were modest in some studies. These results, together with our results with transgenic adipocytes overexpressing GLUT4 in vivo, indicate that intracellular sequestration of GLUT4 is saturable. This may involve saturation of multiple steps in GLUT4 trafficking, including retention of GLUT4 intracellularly as well as recycling of GLUT4 from PM to intracellular sites. The fact that overexpression of either a GLUT4 (41) or an IRAP (42) peptide redistributes intracellular GLUT4 to the plasma membrane suggests both proteins are targeted to a common intracellular compartment by the same mechanism, consistent with data in IRAP−/− mice (18).

GLUT4 Content Regulates IRAP and VAMP2 Protein Levels

Post-transcriptionally—A second finding of the current study is that GLUT4 content alters the steady-state levels of other proteins localizing, at least in part, to the GLUT4 vesicle, including IRAP and VAMP2. In GLUT4−/− adipocytes, IRAP and VAMP2 levels were decreased (Fig. 2), whereas in GLUT4-overexpressing adipocytes, levels of these proteins were increased (Fig. 2). These changes were evident in total adipocyte membranes (Fig. 2) as well as subcellular membrane fractions (Fig. 3). Changes in IRAP are in agreement with the modest increase in aminopeptidase activity that we detected previously in intracellular membranes from GLUT4 vesicles of aP2-GLUT4-Tg adipocytes (27). In addition, in the current study the number of IRAP-containing vesicles was increased in GLUT4-overexpressing adipocytes as detected by immunogold electron microscopy (Fig. 6B). The reciprocal alterations in IRAP and VAMP2 protein levels in adipocytes overexpressing or deficient in GLUT4 suggest that GLUT4 may regulate steady-state IRAP and VAMP2 protein levels by regulation of the biogenesis or half-life of the GLUT4-IRAP compartment. Our data show that in GLUT4 overexpressors, this regulation is post-transcriptional because steady-state IRAP and VAMP2 mRNA levels do not change with increased GLUT4 content and may involve changes in mRNA translation rates and/or changes in protein stability.

Interestingly, decreases of 50–80% in GLUT4 content were recently reported in muscle, heart, and adipocytes of IRAP−/− mice (18). Taken together with our data, these results suggest that IRAP and GLUT4 can coordinately regulate steady-state levels of each other and imply that both proteins influence development of the unique GLUT4/IRAP insulin-responsive intracellular compartment. As GLUT4 and IRAP are likely targeted to their shared compartment by a common mechanism (47), it is not surprising that they may regulate each other.

In contrast to our data, Jiang et al. (48) reported IRAP levels are decreased 50% in muscle and heart but increased 2-fold in adipocytes of GLUT4-null mice, and Zhou et al. (49) reported that denervation down-regulates GLUT4 in muscle, but IRAP levels remain unchanged. The discrepancy between IRAP levels in adipocytes of GLUT4 null mice and in adipose-specific GLUT4-deficient mice may relate to decreased fat cell size of GLUT4 null mice (50). GLUT4-null mice have virtually no adipose tissue as well as other metabolic abnormalities (50), which may be complicating factors in interpreting these results. We did not observe changes in fat cell size in either aP2-GLUT4−/− or ap2-GLUT4-Tg mice (Table I). The discrepancy between IRAP levels in adipocytes of adipose-specific GLUT4−/− mice and denervated muscle may be due to tissue-specific differences or other changes in gene expression that would occur with denervation.

Our findings, together with those of others (41, 43–45), further support a model in which GLUT4 (and potentially IRAP
GLUT4 Amount Affects GLUT4-Vesicle Composition/Localization

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GLUT4 Overexpression or Deficiency in Adipocytes of Transgenic Mice Alters the Composition of GLUT4 Vesicles and the Subcellular Localization of GLUT4 and Insulin-responsive Aminopeptidase
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