Mutational analysis of the carboxy-terminal phosphorylation site of GLUT-4 in 3T3-L1 adipocytes

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This regulated movement of GLUT-4 to the cell surface in response to treatment with insulin or other agonists can be explained by two different models. In the first model, GLUT-4, together with other proteins such as the insulin-regulated membrane aminopeptidase (IRAP) (26, 35), may be packaged into a highly insulin-responsive secretory compartment where it predominantly resides under basal conditions. Insulin may stimulate the exocytosis of this compartment, resulting in a redistribution of all proteins found in these vesicles to the cell surface. The important feature of this model is that there is no sorting step between recruitment of these vesicles with insulin and fusion with the plasma membrane. In the second model, insulin might regulate the translocation of GLUT-4 and other proteins to the cell surface by directly altering the rate constants that determine their individual recycling rates. Recycling membrane proteins are differentially sequestered within endosomes as a function of the rate constants that direct their movement through this system, and these rate constants are in turn determined by the efficiency of the targeting motifs within the cytoplasmic tails of these proteins.

It has previously been reported that GLUT-4 is phosphorylated in vivo (17, 28). The site of phosphorylation in GLUT-4 has been mapped to a serine residue at position 488 within its cytoplasmic carboxy-terminal tail (27). This site is unique to GLUT-4, because no site corresponding to Ser-488 is present in other glucose transporter isoforms (27). Moreover, this residue is immediately adjacent to a dileucine motif (Leu-489Leu-490) in the carboxy terminus, which plays an important role in targeting GLUT-4 intracellularly in adipocytes (33, 55). Dileucine motifs have been shown to regulate the trafficking of numerous recycling membrane proteins, such as the T cell surface antigen (46, 47), the signal transducing component (gp130) of the interleukin-6 receptor complex (12), the CD3γ subunit of the T cell receptor (TCR) (30), the insulin-like growth factor II/mannose 6-phosphate receptor (IGF-II/MPR) (21, 32), and the cation-dependent mannose 6-phosphate receptor (CD-MPR) (20, 22). Changes in the phosphorylation state of serine residues juxtaposed to, and amino-terminal of dileucine motifs in all of these proteins have been proposed to modulate their sorting. Hence, phosphorylation and/or dephosphorylation of GLUT-4 could be involved in both of the above models, either by facilitating the exocytosis of GLUT-4 vesicles in re-
sponse to insulin or other agonists (model 1) or in modifying the intracellular sorting of GLUT-4 en route to the plasma membrane or to the intracellular GLUT-4 storage compartment (model 2). However, a direct role for GLUT-4 phosphorylation in trafficking has so far not been determined. Several labs have examined the effect of insulin on GLUT-4 phosphorylation in adipocytes, but the consensus of opinion is that it has no significant effect (17, 27, 28, 37, 45). Other agents such as isoproterenol, dibutyryl-cAMP, 8-bromo-cAMP, okadaic acid, and calcium have been shown to stimulate GLUT-4 phosphorylation (2, 17, 27, 28, 37, 39). However, their effects on GLUT-4 trafficking are somewhat variable, in part because these agents presumably influence a variety of biological parameters in adipocytes.

To address the potential role of phosphorylation in regulating the intracellular trafficking of GLUT-4, we have expressed recombinant epitope-tagged GLUT-4, in which Ser-488 has been mutated to alanine, in adipocytes. Our results show that the regulatable movement of the Ser-488 mutant to the cell surface is indistinguishable from wild-type GLUT-4 in adipocytes, demonstrating that phosphorylation does not play a major role in the regulated exocytosis of GLUT-4. However, the extent of colocalization between GLUT-4 and the γ-adaptin subunit of the Golgi adaptor complex (AP-1) was significantly increased (P < 0.05) when Ser-488 was mutated to alanine, suggesting that phosphorylation might modulate the sorting of GLUT-4 at the TGN.

**Materials and Methods**

Cell Culture

Murine fibroblasts obtained from the American Type Culture Collection (Rockville, MD) were cultured in DMEM supplemented with 10% FCS (Commonwealth Serum Laboratories, Parkville, Australia). Cells were maintained and passaged as preconfluent cultures at 37°C in a 5% CO2 humidified incubator before differentiation. 3T3-L1 fibroblasts were induced to differentiate 1 day after reaching confluency by the addition of DMEM containing 10% heat-inactivated FCS (GIBCO BRL), 4 mg/ml insulin, 0.25 mM dexamethasone, 0.5 mM IBMX, and 100 ng/ml 1,2-diiodotyrosine. After 72 h, induction medium was replaced with fresh FCS/DMEM containing 4 mg/ml insulin and 100 ng/ml 1,2-diiodotyrosine. Adipocytes were utilized 14–28 days after initiation of differentiation for experiments.

**Construction of Epitope-Tagged Transporter cDNAs**

Wild-type rat GLUT-4 cDNA cloned into pBluescript (40) was epitransfer tagged at the carboxy terminus by the addition of amino acids 485–496 from human GLUT-3 to generate the pTAG construct, as described previously (33). The Ser-488-to-Ala-488 mutant was constructed by employing a PCR site-directed mutagenesis technique described elsewhere (1) and using the pTAG construct as a template. The ~140-bp Bgl II–Xho I fragment, which includes both the point mutation and the epitope tag, was completely sequenced to ensure no PCR-generated errors and subcloned back into the pTAG backbone, generating pSAG. The insert was then removed as a Xba I–Xho I fragment and cloned into the shuttle vector to facilitate insertion into the pMEXneo expression vector downstream of the MSV-LTR promoter (5) to enable stable transfection, as described previously (33).

**Selection of 3T3-L1 Cell Lines Stably Expressing Recombinant GLUT-4**

GLUT-4 cDNA constructs subcloned into the mammalian expression vector pMEXneo were transfected into subconfluent 3T3-L1 fibroblasts using the Lipofectamine reagent, according to the manufacturer’s protocol (GIBCO BRL). Individual neomycin-resistant colonies (0.8 mg/ml G418; GIBCO BRL) were isolated using glass cloning rings and were selected for use in these studies as follows. Initially, neomycin-resistant clones were induced to differentiate, as described in Cell Culture, and only clones retaining the ability to differentiate into mature adipocytes in culture were utilized for further analysis. Total cell membranes were prepared from these adipocyte cell lines, as described previously (33), and were immunoblotted using an antibody specific for the human GLUT-3 epitope tag. This enabled us to determine which clones continued to stably express recombinant GLUT-4 after differentiation and provided a quantitative measurement of the relative expression level of each clone. Direct immunofluorescence microscopy was then employed to assess the clonality of recombinant GLUT-4 expression for each clone. Briefly, fibroblasts cultured on ethanol-washed glass coverslips were fixed with 2% paraformaldehyde, permeabilized, and immunolabeled with an antibody specific for the carboxy terminus of GLUT-4 (R280). Primary antibodies were detected with FITC-conjugated sheep anti-rabbit secondary antibody (Molecular Probes), as described elsewhere (38, 41). Cells were visualized with a ×63/1.40 Zeiss oil immersion lens using a Zeiss Axioskop fluorescence microscope (Carl Zeiss) equipped with a Bio-Rad MRC-600 laser confocal imaging system. Image data were collected directly using identical photomultiplier tube, numerical aperture, and black level and gain settings.

**Subcellular Distribution of GLUT-4 in 3T3-L1 Adipocytes**

Differential centrifugation. Subcellular membrane fractions were prepared by differential centrifugation from transfected adipocytes (2 × 100-mm plates per condition) by use of a protocol previously described in detail (33, 38). Briefly, adipocytes grown in 100-mm plates were washed three times with sterile, prewarmed PBS and incubated for 2 h at 37°C in 5 ml of Krebs-Ringer phosphate (KRP) buffer containing 2% BSA and 2.5 mM glucose. Cells were then incubated for 15 min at 37°C in KRP buffer containing either insulin (4 mg/ml), okadaic acid (10 mM), or insulin plus okadaic acid. Cells were then washed, homogenized, and fractionated at 0–4°C as described previously (33). Four membrane fractions designated as high-density microsomes, low-density microsomes (LDM), plasma membranes (PM), and mitochondria/nuclei were derived from adipocytes with this protocol. These studies utilize the PM and LDM fractions because they are enriched in cell surface membranes and intracellular membranes encompassing the GLUT-4 compartment, respectively (38, 48). Okadaic acid (ammonium salt) was purchased from Sigma and prepared as a 2 mM stock in DMSO. Okadaic acid was added to KRP buffer immediately before the incubation. DMSO was added to adipocytes incubated in the absence or presence of insulin in parallel so that the final concentration of DMSO (0.5%) was the same for all incubations.

Preparation and use of HRP-conjugated transferrin. The transferrin-horseradish peroxidase (TF-HRP) conjugate was prepared and used exactly as described previously (31). Cells

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were used for ablation experiments between 8 and 12 days postdifferentiation. Human apotransferrin and all reagents for TF-HRP synthesis were from Sigma (Poole, UK). 125I-labeled transferrin and 125I-labeled goat anti-rabbit antibody were from Du Pont-NEN.

PM lawn assay. PM fragments were prepared from basal and insulin-stimulated adipocytes as described previously (43). Briefly, adipocytes cultured on glass coverslips were sonicated using a probe sonicator (Kontes) to generate a lawn of PM fragments that remained attached to the glass. These fragments, generated from either wild-type or transfected cells, were then immunolabeled with polyclonal antibodies specific for either GLUT-4 or the human GLUT-3 epitope tag, respectively. Coverslips were visualized and imaged using a confocal laser scanning immunofluorescence microscope, as described in Selection of 3T3-L1 Cell Lines Stably Expressing Recombinant GLUT-4. PM lawns were quantitated by measuring the average pixel intensity of a minimum of three fields containing 10–20 fragments/field with NIH Image analysis software. The multiples of increase in fluorescence intensity (means ± SE) of PM lawns prepared from adipocytes treated with insulin above the average intensity of basal PM lawns were then determined for each cell line.

Electron microscopy. Intracellular vesicles were prepared from 3T3-L1 adipocyte homogenates, as described previously (34), and membrane vesicles were fixed and stored at 4°C. Immunolabeling of vesicles was performed as described previously (34). Protein A-gold was from the Department of Cell Biology, University of Utrecht, Utrecht, The Netherlands.

Electrophoresis and Immunoblotting

Equivalent amounts of protein from total cellular membranes (10 µg) or subcellular membrane fractions (10 µg) were subjected to SDS-PAGE with 7.5 or 10% polyacrylamide resolving gels. The protein concentrations of membrane fractions were determined using the bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer’s instructions. Proteins were electrophoretically transferred to polyvinylidene fluoride transfer membrane (Millipore) or nitrocellulose (Schleicher and Schuell) and immunoblotted with rabbit polyclonal antibodies specific for the carboxy terminus of GLUT-1, GLUT-4, or human GLUT-3 and the cytoplasmic domain of IAP. Primary antibodies were detected by probing with either HRP-conjugated donkey anti-rabbit secondary antibody and enhanced chemiluminescence according to the manufacturers’ instructions (Amersham; Pierce) or 125I-labeled protein A (Amersham). Autoradiograms were quantified using a model GS-670 imaging densitometer (Bio-Rad), whereas 125I-protein A blots were quantitated directly using a model GS-363 molecular imaging system (Bio-Rad). The level of GLUT-4, GLUT-1, or IAP at the PM of insulin-treated adipocytes was nominally assigned a value of 1 in these studies to normalize between independent experiments and between recombinant GLUT-4 constructs expressed by different cell lines.

Antibodies

The polyclonal antibodies generated against synthetic peptides corresponding to the 12 carboxy-terminal residues of GLUT-4 (R820), GLUT-1 (R493), or human GLUT-3 (R1697) have been characterized and described previously (15, 16, 19, 38, 43). Additional polyclonal antiserum specific for the 14 carboxy-terminal residues of human GLUT-3 (R1672) was kindly provided by Dr. Gwyn W. Gould, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, Scotland. The affinity-purified polyclonal rabbit antiserum generated against the cytoplasmic domain of the IAP was generously provided by Dr. Susan G. Keller, Department of Biochemistry, Dartmouth Medical School, Hanover, NH. Anti-γ-adaptin was the generous gift of Dr. M. S. Robinson (Addenbrooks Hospital, University of Cambridge, Cambridge, UK).

Statistical Analyses

Results are presented as means ± SE. Data were analyzed using two-tailed paired t-tests, with assumption of unequal variance when these tests were appropriate.

RESULTS

Expression of Recombinant GLUT-4 in 3T3-L1 Cells

Cell lines expressing recombinant GLUT-4 proteins were isolated and screened as described in MATERIALS AND METHODS. To discriminate between recombinant and endogenous GLUT-4 in stably transfected 3T3-L1 adipocytes, we introduced a heterologous epitope tag from human GLUT-3 at the extreme carboxy terminus of GLUT-4. Each construct when expressed in adipocytes generated a translation product of similar size to endogenous GLUT-4 (~50 kDa), suggesting that all were appropriately glycosylated and processed correctly (Fig. 1). Total cellular membranes prepared from each cell line were immunoblotted with antibodies

![Fig. 1. Expression levels of recombinant GLUT-4 stably expressed in 3T3-L1 adipocyte cell lines assessed relative to endogenous GLUT-4 expression. Total cellular membranes (10 µg) prepared from adipocytes were subjected to SDS-PAGE and immunoblotted using antibodies specific for the cytoplasmic domain of insulin-regulated membrane aminopeptidase (IAP, top), the carboxy terminus of GLUT-4 (middle), or the human GLUT-3 epitope tag (bottom). Immunoblots were labeled with 125I-labeled protein A and quantitated directly using a molecular imaging system (Bio-Rad). TAG, epitope-tagged transporter cDNAs; SAG, Ser-488 mutated to Ala-488, expressed at either high or low levels. Results are expressed as arbitrary units.](https://api.endo.physiology.org/)
specific for either the carboxy terminus of GLUT-4 (to quantify the total level of recombinant plus endogenous GLUT-4 expression; Fig. 1, middle) or the human GLUT-3 epitope tag (to quantify the relative levels of expression of recombinant GLUT-4 independently of endogenous GLUT-4; Fig. 1, bottom). Cell lines expressing SAG were selected in which the total levels of GLUT-4 expression ranged from low (comparable to that of endogenous GLUT-4 in nontransfected adipocytes) to high (where total expression was about threefold greater than endogenous GLUT-4). We have previously characterized the subcellular distribution of several different SAG-expressing 3T3-L1 adipocyte clones and found that, over a range of expression levels, the trafficking of this construct is indistinguishable from wild-type GLUT-4 (33). Thus, in the present study, we have selected one of these clones (TAG 3B1) as a control for the SAG-expressing cells. As a further control, we have immunoblotted all of our membrane fractions with an antibody specific for the cytoplasmic domain of IRAP (Fig. 1, top). IRAP is highly colocalized with GLUT-4 in adipocytes (26, 35) and so serves as a useful internal control for the fidelity of the subcellular fractionation protocol that can be monitored independently of the recombinant GLUT-4 proteins.

Subcellular Distribution of Recombinant GLUT-4 Constructs in Basal and Insulin-Stimulated Adipocytes

TAG exhibited a predominantly intracellular distribution in the absence of insulin, as assessed by Western blotting membrane fractions prepared by differential centrifugation (33) (Fig. 2). TAG, like wild-type GLUT-4, was recovered in the LDM fraction and was almost entirely excluded from the PM fraction. The basal distribution of TAG and wild-type GLUT-4 is almost identical in adipocytes, as indicated by the PM-to-LDM ratios (PM/LDM) calculated from subcellular fractionation data (0.12 and 0.16, respectively). Thus the intracellular sequestration of TAG was maintained despite a level of total GLUT-4 expression approximately eightfold greater than that observed in nontransfected adipocytes (Fig. 1). TAG exhibited a fivefold increase in the PM fraction with insulin, similar to that observed for wild-type GLUT-4 (4-fold), with a corresponding decrease in intracellular membranes (Fig. 2). These observations were further corroborated by the PM lawn technique, as we will detail.

The subcellular distributions of IRAP and GLUT-1 verified that there was little variation in subcellular fractionation between individual cell lines. In both transfected and nontransfected adipocytes, IRAP was recovered predominantly in the LDM fraction in the absence of insulin. The PM/LDM for IRAP (0.04) confirmed that it was largely absent from the PM fraction in basal adipocytes. Insulin had a similar effect on the subcellular distribution of IRAP and GLUT-4, resulting in a significant redistribution from the LDM fraction to the plasma membrane (Fig. 2), consistent with previous studies (25, 26, 35).

To confirm that the insulin-dependent translocation of GLUT-4 was not altered by mutation of Ser-488, we analyzed the cell surface levels of the protein with a completely independent subcellular fractionation procedure. This technique, referred to as the PM lawn assay, allows for a more precise determination of the extent of GLUT-4 translocation, because it yields highly purified PM fragments attached to glass coverslips, which can then be labeled with antibodies specific for GLUT-4 (Table 1) (33, 43, 44, 55). PM fragments prepared from basal adipocytes and immunolabeled with antibodies specific for either the carboxy terminus of GLUT-4 (wild-type cells) or GLUT-3 (adipocytes stably expressing TAG or SAG) exhibited minimal labeling (Fig. 3). In contrast, PM lawns isolated from insulin-treated adipocytes showed similar increases in labeling for wild-type GLUT-4, TAG, and SAG.

Intracellular Distribution of SAG

To determine whether mutation of Ser-488 to alanine significantly altered the intracellular distribution of GLUT-4, we employed two independent techniques. First, the distributions of TAG and SAG were examined using an endosomal “ablation” protocol, which selectively ablates the endosomal recycling pathway but not intracellular compartments withdrawn from the endosomal system in adipocytes (31, 34). Control experiments in which cells were incubated with Tf-HRP at 4°C showed no ablation of the transferrin receptor (Tfr), wild-type, or recombinant GLUT-4 from LDM membranes (Fig. 4). In contrast, cells incubated with Tf-HRP for 1 h at 37°C exhibited a significant peroxide-dependent loss of Tfr from the LDM, consistent with previous findings (31). The pattern of ablation exhibited by TAG was not significantly different from that of endogenous GLUT-4 in native adipocytes (Fig. 4 and Table 2) (31). SAG was distributed between the ablated (endosomal) and nonablated compartments in a similar manner to TAG and endogenous GLUT-4 in the basal
state. It is noteworthy that after a 1-h incubation with Tf-HRP at 37°C, there was slightly less ablation of SAG compared with TAG, whereas after 3 h at 37°C, the ablation efficiency was identical for both proteins.

The second technique to assess the distribution of GLUT-4 among different intracellular compartments involves whole mount electron microscopy (EM) of intracellular vesicles prepared from adipocytes. Labeling of vesicles on an EM grid with two different primary antibodies, followed by protein A tagged with differently sized gold particles, enables a comparison of the distribution of two different proteins within individual vesicles. The proportion of total vesicles that were γ-adapter positive was not significantly different between the individual cell lines (Table 3). The percentage of total vesicles labeled positively for TAG and SAG with an antibody to the epitope tag additionally reflected differences in recombinant GLUT-4 expression between cell lines (Fig. 1 and Table 3). Double-labeling revealed that both TAG and SAG were significantly colocalized with γ-adapter in intracellular vesicles. Interestingly, there was a significant increase ($P < 0.01$) in the amount of SAG present in γ-adapter-positive vesicles (determined for two different cell lines).
Table 1. Insulin-stimulated movement of wild-type and recombinant GLUT-4

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Multiples of Increase in PM Staining</th>
<th>n</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>14.12 ± 1.63</td>
<td>16</td>
</tr>
<tr>
<td>TAG</td>
<td>13.51 ± 1.69</td>
<td>15</td>
</tr>
<tr>
<td>SAG&lt;sub&gt;high&lt;/sub&gt;</td>
<td>12.03 ± 2.21</td>
<td>26</td>
</tr>
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</table>

Plasma membrane (PM) fragments generated from nontransfected or transfected adipocytes were immunolabeled with polyclonal antibodies specific for either GLUT-4 or human GLUT-3, respectively, and were imaged as described in MATERIALS AND METHODS. PM lawns were thresholded to adjust for background staining and quantified by measuring the average pixel intensity of a minimum of 3 fields containing ≥10–20 fragments/field by use of NIH Image analysis software. Multiples of increase in fluorescence intensity (means ± SE) of PM lawns prepared from adipocytes treated with insulin above the average intensity of basal PM lawns were then determined for each cell line. TAG, epitope-tagged GLUT-4 cDNA; SAG<sub>high</sub>, S-488 mutated to alanine and expressed at a high level. Results are means ± SE for ≥2 separate experiments; n, total no. of fields quantified for each determination.

Fig. 3. Plasma membrane lawns, prepared from adipocytes incubated in the absence (left) or presence (right) of insulin (4 µg/ml) for 15 min at 37°C, were immunolabeled with antibodies specific for either GLUT-4 (wild-type adipocytes) or human GLUT-3 (transfected adipocytes). Shown are plasma membrane (PM) lawns prepared from wild-type adipocytes (A), adipocyte cell lines stably expressing epitope-tagged transporter cDNAs (TAG, B), and Ser-488 mutated to alanine and expressed at a high level (SAG<sub>high</sub>, C). Fields are representative of quantitative data shown in Table 1. Bar, 50 µm.

Table 2. Effect of Tf-HRP ablation on intracellular GLUT-4 levels

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>%Signal Remaining After Ablation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 h at 37°C</td>
</tr>
<tr>
<td>Wild type</td>
<td>64 ± 1.73</td>
</tr>
<tr>
<td>TAG</td>
<td>62 ± 4.62</td>
</tr>
<tr>
<td>SAG&lt;sub&gt;low&lt;/sub&gt;</td>
<td>70 ± 2.89</td>
</tr>
<tr>
<td>SAG&lt;sub&gt;high&lt;/sub&gt;</td>
<td>72 ± 1.73</td>
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</tbody>
</table>

Duplicate sets of 100-mm plates of adipocytes were loaded with transferrin-horseradish peroxidase conjugate (Tf-HRP) for 3 h at 4°C, 1 h at 37°C, or 3 h at 37°C before and after ablation (+ and − hydrogen peroxide). LDM membranes separated by SDS-PAGE were immunoblotted with either anti-transferrin receptor (TfR), anti-GLUT-4, or anti-GLUT-3 antibodies. Note that incubating cells at 4°C with Tf-HRP did not result in any ablation of TfR from LDM membranes; in contrast, incubating cells with Tf-HRP for 1 h at 37°C resulted in a peroxide-dependent loss of TfR from LDM membranes. Similar experiments to determine effect of Tf-HRP ablation on intracellular content of each of the recombinant GLUT-4 constructs examined showed that, under the same conditions, ablation was much less pronounced. Immunoblots from ≥3 independent experiments for each cell line were quantified, and results are presented in Table 2.
GLUT-4 PHOSPHORYLATION IN 3T3-L1 ADIPOCYTES

Table 3. Percentage of total intracellular vesicles labeled for either γ-adaptin or recombinant GLUT-4

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>γ-Adaptin</th>
<th>GLUT-3</th>
</tr>
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<tbody>
<tr>
<td>TAG</td>
<td>5.4 ± 0.4</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>SAG&lt;sub&gt;low&lt;/sub&gt;</td>
<td>6.8 ± 0.8</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>SAG&lt;sub&gt;high&lt;/sub&gt;</td>
<td>7.9 ± 1.55</td>
<td>4.1 ± 0.7</td>
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</table>

Intracellular vesicles were prepared from basal 3T3-L1 adipocytes stably expressing either epitope-tagged wild-type (TAG) or mutant GLUT-4 (SAG). Results of 4 independent labeling experiments were quantified; values are means ± SE. γ-Adaptin vesicles were immunolabeled with an antibody specific for γ-adaptin followed by protein A-gold (10 nm). GLUT-3 vesicles were immunolabeled with an antibody specific for the human GLUT-3 epitope tag followed by protein A-gold (10 nm).

Expressing SAG at high and low levels) compared with TAG (Fig. 5).

Effects of Okadaic Acid on the Intracellular Distribution of GLUT-4 (Wild-Type and Mutant), IRAP, and GLUT-1

Okadaic acid stimulated the movement of SAG and wild-type GLUT-4 to the cell surface to a similar extent (3.1-fold and 2.2-fold, respectively; Fig. 6). Moreover, we observed a similar redistribution of both GLUT-1 (3-fold) and IRAP (4- to 6-fold) to the plasma membrane in response to okadaic acid treatment in both wild-type and SAG-expressing cells.

Okadaic acid treatment in the presence of insulin has been shown to inhibit the insulin-dependent translocation of GLUT-4 to the plasma membrane (9, 28, 31). Consistent with these findings, okadaic acid inhibited the insulin-dependent movement of both wild-type and epitope-tagged GLUT-4 in 3T3-L1 adipocytes. Similarly, an inhibitory effect of okadaic acid on the insulin-stimulated movement of SAG was observed, suggesting that phosphorylation of Ser-488 does not facilitate this effect. The inhibitory effect of okadaic acid on the insulin-induced movement of both IRAP and GLUT-1 (Fig. 6) indicates that this may be due to modulation of a central regulatory step in either the insulin-signaling pathway or vesicular transport.

DISCUSSION

It has previously been suggested that phosphorylation of GLUT-4 at a unique site in its carboxy terminus may play a regulatory role in the trafficking of this protein (27). In the present study, the overall distribution of GLUT-4 in which this site (Ser-488) was mutated to alanine (SAG) was not significantly different from either wild-type GLUT-4 or epitope-tagged GLUT-4 (TAG). It is efficiently excluded from the cell surface in basal adipocytes and undergoes a marked redistribution to the plasma membrane in response to insulin stimulation. Moreover, its response to an agent that has previously been shown to stimulate GLUT-4 phosphorylation, okadaic acid, is similar to endogenous GLUT-4. Notably, however, immuno-electron-microscopic analysis of intracellular vesicles prepared from these cells revealed that the extent of colocalization of SAG with the γ-adaptin subunit of AP-1 was significantly higher (P < 0.01) than that for TAG, suggesting that changes in the phosphorylation state of this site might regulate the intracellular sorting of GLUT-4 to some degree. However, in the present set of experiments, we were unable to discern a major role for phosphorylation at Ser-488 in the steady-state distribution of GLUT-4 between the cell surface and intracellular membranes. Thus, we conclude that insulin- and okadaic acid-stimulated recruitment of GLUT-4 to the plasma membrane occurs independently of GLUT-4 phosphorylation at Ser-488.

Changes in the phosphorylation state of serine residues flanking dileucine motifs within the cytoplasmic tails of CD4 (46, 47), IGF-II/MPR (29), CD-MPR (7, 36), and gp130 (12) are proposed to promote either internalization or intracellular sorting by inducing conformational changes in the relevant targeting motifs. For example, the phosphorylation of a serine residue within the cytoplasmic tail of the CD3γ subunit of the TCR facilitates the interaction of an adjacent dileucine-based internalization signal with the plasma membrane adaptor protein subunit AP-2, resulting in increased internalization of the TCR via clathrin-coated pits (11). Our analysis of the Ser-488 mutant in the present study has been confined to examining the distribution of the protein under steady-state conditions. However, as suggested by the increased colocaliza-
tion of SAG with the γ-adaptin subunit of AP-1, it is conceivable that phosphorylation may play some role in modulating the sorting of GLUT-4, but it does not appear to play a major role in regulating the steady-state distribution of this protein in adipocytes. It is worth noting that we and others have examined the effects of mutating the dileucine motif (Leu-489Leu-490) in the GLUT-4 carboxy terminus in adipocytes (33, 55). At lower expression levels, the steady-state distribution of this mutant was indistinguishable from wild-type GLUT-4, yet the internalization rate of this mutant after insulin withdrawal was significantly slower than for wild-type GLUT-4 (33, 55). A rigorous assessment of carboxy-terminal GLUT-4-targeting motifs in Chinese hamster ovary cells recently revealed that, although Ser-488 may play a modulatory role in regulating GLUT-4 endocytosis, it is relatively minor compared with that played by the dileucine motif per se (14). This supports the present finding that phosphorylation of GLUT-4 does not play a major role in the regulated movement of the protein to the cell surface.

We have observed previously that the dileucine mutant exhibited a shift in steady-state distribution only when expressed at levels approximately fourfold greater than...
endogenous GLUT-4. We have attempted to address this in the present study by examining clones expressing SAG at both low and high levels. However, although the level of overexpression achieved for SAG_{high} in the present study (3-fold) approached that observed previously for the dileucine mutant expressed at high levels (4-fold), we were still unable to observe a significant change in the steady-state distribution of this mutant. It has been shown that certain agents, such as isoproterenol, dibutyryl-cAMP, and okadaic acid, have an inhibitory effect on GLUT-4 translocation in addition to stimulating the phosphorylation of this protein (9, 28, 31, 42). These data led to the suggestion that phosphorylation may play an important role in regulating cell surface levels of GLUT-4 (27). However, on the basis of the present findings, this seems unlikely. Our data would more likely indicate that the inhibitory effect of okadaic acid may be mediated via an effect on the insulin signaling pathway. It has been shown that okadaic acid increases serine/threonine phosphorylation of insulin receptor substrate-1, which prevents its tyrosine phosphorylation and thus reduces its ability to dock phosphatidylinositol 3-kinase (23, 53, 54). Taken together, the above findings suggest that the effects of okadaic acid are primarily on the cellular machinery that facilitates the movement of GLUT-4 and other proteins to the cell surface, rather than directly on these proteins per se.

Changes in the phosphorylation state of serine residues adjacent to dileucine motifs in the cytoplasmic tails of the MPRs regulate their entry into clathrin-coated vesicles exiting the Golgi apparatus at the TGN (29, 36). Because a large proportion of GLUT-4 is proposed to recycle via the TGN in insulin-sensitive cells (49), we investigated whether a similar mode of regulation facilitates GLUT-4 exit from the Golgi. We found that significant overlap exists between TAG and SAG with the γ-adaptin subunit of the Golgi adaptor complex, AP-1, suggesting that GLUT-4 must follow a similar trafficking pathway to that of the MPRs. The localization of SAG with γ-adaptin was significantly higher (P < 0.01) than for TAG, suggesting that Ser-488 might be intimately involved in regulating GLUT-4 sorting at the TGN. Moreover, after the uptake of Tf-HRP for 1 h at 37°C, SAG was less susceptible to chemical ablation than either TAG or GLUT-4, consistent with the hypothesis that γ-adaptin-positive vesicles would be inaccessible to the endocytosed Tf-HRP conjugate after shorter incubation times. We conclude that phosphorylation/dephosphorylation events may play a role in regulating the entry of GLUT-4 into γ-adaptin-positive vesicles at the TGN. However, as is the case for other proteins such as the CD-MPR, disruption of this site is without significant effect on the regulated trafficking of GLUT-4 in adipocytes (7).

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