Vesicle-associated Membrane Protein 2 Plays a Specific Role in the Insulin-dependent Trafficking of the Facilitative Glucose Transporter GLUT4 in 3T3-L1 Adipocytes*

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Vesicle-associated membrane protein 2 (VAMP2) has been implicated in the insulin-regulated trafficking of GLUT4 in adipocytes. It has been proposed that VAMP2 co-localizes with GLUT4 in a postendocytic storage compartment (Martin, S., Tellam, J., Livingstone, C., Slot, J. W., Gould, G. W., and James, D. E. (1996) J. Cell Biol. 134, 625–635), suggesting that it may play a role distinct from endosomal v-SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) such as cellubrevin that are also expressed in adipocytes. The present study examines the effects of recombinant glutathione S-transferase (GST) fusion proteins encompassing the entire cytoplasmic tails of VAMP1, VAMP2, and cellubrevin on insulin-stimulated GLUT4 translocation in streptolysin O permeabilized 3T3-L1 adipocytes. GST-VAMP2 inhibited insulin-stimulated GLUT4 translocation by ~35%, whereas GST-VAMP1 and GST-cellubrevin were without effect. A synthetic peptide corresponding to the unique N terminus of VAMP2 also inhibited insulin-stimulated GLUT4 translocation in a dose-dependent manner. This peptide had no effect on either guanosine 5′-3-O-(thio)triphosphate-stimulated GLUT4 translocation or on insulin-stimulated GLUT1 translocation. These results imply that GLUT4 and GLUT1 may undergo insulin-stimulated translocation to the cell surface from separate intracellular compartments. To confirm this, adipocytes were incubated with a transferrin-horseradish peroxidase conjugate to fill the intertial endocytic system after which cells were incubated with H$_2$O$_2$ and diaminobenzidine. This treatment completely blocked insulin-stimulated movement of GLUT1, whereas in the case of GLUT4, movement to the surface was delayed but still reached similar levels to that observed in insulin-stimulated control cells after 30 min. These results suggest that the N terminus of VAMP2 plays a unique role in the insulin-dependent recruitment of GLUT4 from its intracellular storage compartment to the cell surface.

The molecular machinery that regulates the docking and fusion of intracellular vesicles with their appropriate target membranes is currently under intense investigation. From studies in the mammalian synapse it has been revealed that membrane proteins referred to as v-SNAREs, 1 located in transport vesicles, bind in a highly specific manner to t-SNAREs located in the target membrane (1). The high affinity and specificity of this interaction ensures that transport vesicles dock and fuse with their correct target membrane. Vesicle-associated membrane protein 2 (VAMP2 or synaptobrevin 2) has been shown to be the predominant v-SNARE that targets small synaptic vesicles (SSVs) to the presynaptic plasma membrane by interacting with its cognate t-SNAREs, syntaxin 1 and synaptosome-associated protein of 25 kDa (SNAP-25) found on the target membrane (reviewed in Refs. 2–4).

Several observations suggest that SNAREs play a pivotal role in vesicular transport. First, these proteins are the major substrates for tetanus toxin and various serotypes of botulinum toxins (reviewed in Ref. 5). For example, VAMP2 is cleaved at distinct sites by tetanus toxin and botulinum toxins B, D, F, and G. These cleavage sites are found within the central domain of VAMP2, which constitutes the t-SNARE binding domain (6–10). Treatment of cells with toxins specific for these binding domains disrupts vesicle transport (reviewed in Ref. 5). Second, large gene families of v- and t-SNAREs have been identified in eucaryotic cells. From genetic studies in yeast, it appears that unique combinations of v- and t-SNAREs regulate vesicle transport events between distinct intracellular organelles. Furthermore, mutations in these proteins result in compartment-specific vesicle transport defects (reviewed in Refs. 2–4).

Several years ago, it was reported that VAMP2 is expressed in adipocytes (11). This was of interest because these cells, like neurons, also possess a regulated secretory system (reviewed in Ref. 12). This system responds to the metabolic needs of the adipocyte to increase the surface levels of a variety of proteins including the facilitative glucose transporter GLUT4 (13, 14) and an amino peptidase Vp165 (15, 16). These proteins are sequestered in the absence of insulin within an intracellular compartment, referred to here as intracellular GLUT4 storage vesicles (GSVs), which morphologically resemble SSVs in synapses.

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1 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; DAB, 3,3′-diaminobenzidine; GSV, GLUT4 storage vesicle; HRP, horseradish peroxidase; LDM, low density microsome(s); FM, plasma membrane(s); SSV, small synaptic vesicle; Tf, transferrin; Tf-HRP, transferrin-HRP conjugate; VAMP, vesicle-associated membrane protein; SNAP, synaptosome-associated protein; DMEM, Dulbecco’s modified Eagle’s medium; SLO, streptolysin; GTPyS, guanosine 5′-3-O-(thio)triphosphate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
apases (17). Insulin stimulates the exocytosis of the GSVs resulting in a >10-fold increase in the levels of GLUT4 and vpl65 proteins at the plasma membrane. Several lines of evidence suggest a role for VAMP2 in the regulated movement of this compartment. First, VAMP2 co-localizes with GLUT4 and vpl65 in GSVs (18, 19). Second, VAMP2 translocates from this intracellular compartment to the cell surface in response to insulin (19, 20). Third, tetanus toxin (21) as well as botulinum neurotoxin D and a GST-VAMP2 fusion protein (22) block the insulin-stimulated translocation of GLUT4 in permeabilized adipocytes. Finally, syntaxin 4 is specifically bound to VAMP2 (7) and is localized to the cell surface in adipocytes (23, 24). The insulin-stimulated translocation of GLUT4 in permeabilized or microinjected adipocytes was blocked by syntaxin 4-specific antibodies or a GST fusion protein encompassing the cytoplasmic tail of syntaxin 4 (23, 24).

VAMP2 shares a high degree of amino acid homology with two other v-SNAREs, VAMP1 and cellubrevin (25), which are also expressed in insulin-sensitive cells (20, 23). VAMP1 is expressed at much lower levels than VAMP2 in adipocytes (23). However, cellubrevin is abundantly expressed and, like VAMP2, undergoes insulin-dependent movement to the cell surface (20, 23). Furthermore, it has been reported that cellubrevin co-localizes with intracellular GLUT4 (20), although this may reflect the presence of both of these proteins in endosomes. Cellubrevin is also cleaved by tetanus toxin (26) and, like VAMP2, binds to syntaxin 4 with reasonable affinity (23). Cellubrevin and VAMP2 are almost identical in amino acid sequence within the central coiled-coil t-SNARE binding domain that spans residues 15–68 in cellubrevin (25). Hence, it is not surprising that both of these proteins have an overlapping t-SNARE binding specificity. However, VAMP2 differs from cellubrevin within its N terminus, sharing little amino acid identity as well as possessing an additional 13 amino acids (25).

Nevertheless, in view of the overall similarities between these two proteins and the available data concerning their role in insulin action, it is difficult to determine if only one, or both, of these proteins is involved in the insulin-stimulated movement of GLUT4 in adipocytes.

In the present study, we have attempted to dissect the potential role of VAMP1, VAMP2, or cellubrevin in GLUT4 trafficking by introducing recombinant fusion proteins or synthetic peptides comprising different domains of these proteins into permeabilized adipocytes and assessing their effects on insulin action. Our results suggest that VAMP2 is specifically involved in the insulin-dependent movement of GLUT4 to the cell surface from its intracellular storage compartment (GSV) but that it may not be involved in the insulin-dependent recruitment of other proteins such as GLUT1. This implies that insulin may stimulate exocytosis of distinct intracellular compartments, one being endosomes containing GLUT1 and the other being a separate postendocytic GLUT4 compartment. Using a chemical ablation technique following uptake of a Tf-HRP conjugate, we show that GLUT4-containing vesicles can still fuse with the cell surface following ablation of endosomes. This supports the notion that GLUT4 is partitioned into a separate intracellular compartment in adipocytes that can dock and fuse directly with the cell surface. We postulate that VAMP2 plays a specific role in the docking and fusion of GSVs with the cell surface in response to insulin.

**Materials and Methods**

**Cell Culture**

3T3-L1 fibroblasts (ATCC, Rockville, MD) were grown in DMEM containing high glucose, 1-glutamine, and sodium pyruvate (Life Technologies, Inc.) supplemented with 10% bovine calf serum (Commonwealth Serum Laboratory, Parkville, Australia). Confluent cells were induced to differentiate into adipocytes by the addition of DMEM containing 10% fetal calf serum, 4 μg/ml insulin, 0.25 mM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, and 100 ng/ml n-biotin (27). After 72 h, induction medium was replaced with DMEM containing 2.5% fetal calf serum, insulin, and biotin. Adipocytes were used for experiments 10–14 days following initiation of differentiation. Prior to experiments, cell monolayers were washed with serum-free DMEM to remove all serum-derived insulin and transferrin and incubated with serum-free DMEM for a minimum of 2 h.

**Streptolysin O (SLO) Permeabilization and Cell Treatment**

3T3-L1 adipocytes were permeabilized with SLO (28, 29) as described previously (30) with minor modifications. Cells were washed with cold intracellular buffer (ICB; 140 mM potassium glutamate, 20 mM Hepes, 1 mM MgCl₂, 5 mM NaH₂PO₄, pH 7.4), permeabilized with cold ICB containing 0.5 μg/ml SLO on ice for 10 min. Cells were again washed with ICB before incubation and treatment at 37 °C. Modified ICB containing 1 mg/ml BSA, 1 mM dithiothreitol, and an ATP-regeneration system (40 μM IUTP, creatine phosphokinase, 5 mM creatine phosphate, and 1 mM ATP) was used as the incubation buffer in all experiments. SLO-treated cells were permeabilized by an initial 10-min incubation at 37 °C in the presence or absence of test protein. The cells were then incubated for an additional 15 min in modified ICB containing the test protein with or without stimulus (either 1 μM insulin or 100 μM GTP-γ-S).

**Subcellular Fractionation**

To determine the relative levels of different proteins at the cell surface, two different subcellular fractionation techniques were employed.

**The Plasma Membrane Lawn Assay**—The plasma membrane lawn assay is a technique previously described by our laboratory to generate highly purified plasma membrane (PM) fragments (30, 31). Quantification of the level of different proteins in this membrane was achieved using antibodies specific for cytoplasmic epitopes combined with immunofluorescence microscopy. Cells cultured on glass coverslips were treated as described in the figure legends. At the conclusion of the experiment, cells were rapidly washed in phosphate-buffered saline (PBS) followed by a 30-s treatment in PBS containing 0.5 mg/ml poly-L-lysine (Sigma). The cells were swollen by three rapid washes in hypotonic buffer (1/2 × buffer A), transferred to buffer A (70 mM KCl, 30 mM Hepes, 5 mM MgCl₂, 3 mM EGTA, pH 7.4), and sonicated using a probe sonicator (Kontes, Vineland, NJ) to generate a lawn of FM fragments attached to the glass. The membranes were then fixed to the coverslips for 15 min using freshly prepared 2% formaldehyde (Probing and Structure, Thuringowa, Queensland, Australia) in buffer A. Cells were then washed three times with PBS and incubated for 10 min in PBS containing 100 mM glycerol followed by an additional 30-min incubation in PBS containing 5% milk powder. Cells were washed with PBS three times, washed three times over a 10-min period. Coverslips were then sonicated with primary antibodies diluted in 0.5% milk for 1 h at room temperature. Coverslips were washed three times over a 10-min period with PBS. The coverslips were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:500; Molecular Probes) for 1 h. Cells were washed three times with PBS prior to mounting and viewing. Coverslips were viewed using × 40 and × 83/1.40 Zeiss oil immersion objectives on a Zeiss Axiosvert fluorescent microscope (Carl Zeiss, Germany) equipped with a Bio-Rad MRC-600 laser confocal imaging system. For each experiment, samples were run in duplicate, and 10 random representative images were collected from each. Fluorescence was quantified using NIH Image analysis software.

**A Differential Centrifugation Procedure—**A differential centrifugation procedure was also used to determine the subcellular distribution of proteins in permeabilized adipocytes as described previously (32, 33). Briefly, cells (2 × 10⁶ plates/condition) were resuspended in 20 mM HEPES (pH 7.4), and homogenized in HES buffer containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 250 μg/ml PMSF) (2 ml/plate) by 10 passes through a 27-gauge needle. The homogenate was centrifuged at 17,300 × g in a Sorvall high speed centrifuge for 20 min at 4 °C. The pellet was washed and layered onto a 1.12 m sucrose cushion (20 mL HEPES, pH 7.4, 1 mL EDTA, 11.2 m sucrose) and centrifuged at 100,000 × g in an SW41 rotor (Beckman, Fullerton, CA) for 60 min at 4 °C. The membranous layer above the cushion contains highly enriched PMs as described previously (32, 33). The supernatant from the initial spin was subsequently centrifuged at 38,700 × g for 20 min to remove endoplasmic reticulum and smaller pieces of the plasma mem-
bran. The resulting supernatant was centrifuged at 180,000 × g for 75 min to generate the low density microsomal pellet (LDM) that is enriched in the intracellular GLUT4 storage vesicles (32). Membrane fractions were resuspended in HES buffer containing protease inhibitors (50–100 μl). The protein concentration of these subcellular membrane fractions was determined using the bicinchoninic acid (BCA) assay (Pierce).

Antibodies, GST Fusion Proteins, and Synthetic Peptides

Rabbit polyclonal antibodies raised against synthetic peptides encompassing the last 12 amino acids of GLUT1 and GLUT4 have been described previously (13, 32). These antibodies were used for immunofluorescence microscopy at dilutions of 1:200 and 1:500, respectively. A polyclonal antibody raised against a recombinant GST fusion protein encompassing the entire cytoplasmic tail of v165 was kindly provided by Dr. Susanna Keller (Dartmouth Medical School). GST fusion proteins comprising the cytoplasmic tails of VAMP2, VAMP1, and cellubrevin were kindly provided by Dr. Richard Scheller (Stanford, CA). VAMP2 peptides (human/murine VAMP2-(1–15) and rat VAMP2-(1–25)) were synthesized by Chiron Mimotopes (Melbourne Australia). Irrelevant peptide used as control was as follows: H-MPSGAGQIGSED-OH.

Electrophoresis and Immunoblotting

Equivalent amounts of subcellular fractions (10 μg of protein) were prepared in Laemmli sample buffer prior to SDS-PAGE using a 10% resolving gel. Proteins were electrophoretically transferred to nitrocellulose membranes (Amersham, Little Chalfont, Buckinghamshire, UK), which were incubated with 5% milk powder in PBS for 60 min at room temperature and then with polyclonal antibodies diluted in PBS and 1% milk powder at dilutions of 1:500 for 60 min at room temperature. Membranes were washed three times over 30 min with 0.1% Tween 20 in PBS and then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Pierce). Immunoreactive bands were visualized using SuperSignal chemiluminescent substrate stable peroxide solution (Pierce).

Endosome Ablation Using HRP-conjugated Transferrin

Transferrin conjugated to horseradish peroxidase (TR-HRP) was prepared by the carbodiimide method and purified as described (34). The conjugate was aliquoted and stored at −80 °C until use. A sample was assayed for protein and iron-loaded as described (34). After a 2-h incubation in serum-free DMEM, adipocytes were incubated with 20 μg/ml TR-HRP for the times indicated to fill the transferrin itinerary. Cells were thereafter chilled by washing in ice-cold isotonic citrate buffer (150 mM NaCl, 20 mM sodium citrate, pH 5.0) and kept on ice to prevent any further vesicle trafficking during the DAB cytochemistry reactions (see below). Cell surface-attached TR-HRP was removed by acid washing for three consecutive 10 min washes in ice-cold isotonic citrate buffer followed by a single wash in ice-cold PBS, pH 7.4. TR-HRP (diluted as a 0.4 mg/ml stock and 0.22 mm-filtered) was added at 100 μg/ml to all cells, and H2O2 was added to 0.02% (v/v) to one of each pair of wells. After a 60-min incubation at 4 °C in the dark, the reaction was stopped by washing in PBS containing 5 mg/ml BSA. This was then aspirated and replaced with 1 mg/ml BSA and 1 ml of 1 M sodium hydroxide was added to each plate. Cells were triturated, and the radioactivity associated with the cells was determined by γ-counting. As a measure of nonspecific binding, duplicate sets of plates were incubated with hot transferrin in the presence of 10 μM unlabeled transferrin and treated exactly as described above. The radioactivity associated with the cells under these conditions was regarded as nonspecific association, and this value was subtracted from measurements of cell-associated counts from all other conditions.

Statistical Analysis

Data are presented as mean ± S.E. Data were normalized to the insulin response detected in the same experiment and are expressed as a percentage of the average insulin effect observed. The significance of various treatments was determined by using analysis of variance with a Tukey-Kramer multiple comparison post hoc test of significance.

RESULTS

Effects of GST-VAMP Fusion Proteins on GLUT4 Trafficking—To determine if VAMP2 plays a specific role in insulin-stimulated GLUT4 translocation, we utilized SLO-permeabilized 3T3-L1 adipocytes. This system has previously been used by our laboratory to reconstitute the insulin-dependent movement of GLUT4 to the cell surface (30). We showed that an exogenous source of ATP was required subsequent to permeabilization to restore insulin-dependent GLUT4 translocation. Nonhydrolyzable GTP analogs such as GTPγS had a similar effect to insulin in stimulating cell surface levels of GLUT4 (30). The pores generated by SLO are of sufficient size to allow the passive entry of large molecules into adipocytes (28). Hence, this system can be effectively used to transiently access the intracellular milieu with otherwise impermeant reagents.

Recombinant fusion proteins comprising the cytoplasmic tail of VAMP2 have previously been shown to block insulin-stimulated GLUT4 trafficking in adipocytes (22). However, in view of the high degree of amino acid homology in the cytoplasmic tails of VAMP2 and other v-SNAREs, these studies did not definitively distinguish a unique role for this protein. In an effort to address this issue, we examined, by immunofluorescence of PM lawns, the effects of VAMP1, VAMP2, and cellubrevin cytoplasmic tail GST fusion proteins on cell surface levels of GLUT4 in the absence and presence of insulin. In the absence of insulin, cell surface levels of GLUT4 were very low (Fig. 1) and were unaffected by incubation with the GST fusions of VAMP1, VAMP2, cellubrevin, or GST alone (data not shown). Insulin containing 0.2% (v/v) fish tail gelatin and 1% (v/v) goat serum and then washed a further three times in filtered PBS prior to mounting and viewing under immunofluorescence conditions. Coverslips were viewed using a × 40 objective lens on a Zeiss Axiosvert microscope operated in Laser Scanning Confocal mode. Samples were illuminated at 488 nm, and images were captured at 600 nm. Fluorescent images were quantified using Imaris software running on a Silicon Graphics Indy computer. Intensities of three images at each time point were averaged to give mean ± S.D. Data are expressed as percentage of fluorescence intensity observed in the basal state.
increased cell surface levels of GLUT4 by 6.8-fold in permeabilized cells. Similarly, in cells incubated with GST-VAMP1, GST-cellubrevin, or GST alone, insulin increased surface levels of GLUT4 by 6.8–7.6-fold (Fig. 1). However, in the presence of 10 μM GST-VAMP2 insulin-stimulated GLUT4 translocation was reduced by 34% (p < 0.001, n = 5).

Effects of VAMP2 Synthetic Peptides on GLUT4 Translocation—The data shown in Fig. 1 suggest that VAMP2 plays a unique role in insulin-dependent GLUT4 trafficking compared with either VAMP1 or cellubrevin. Sequence comparison of these different v-SNAREs indicates that the predominant heterogeneity in amino acid sequence between these different proteins occurs within the first 20–30 amino acids at the N terminus (see Table I). To determine if this domain conferred the inhibitory effect of VAMP2 in 3T3-L1 adipocytes, we tested the effect of a synthetic peptide, comprising the first 25 amino acids of VAMP2, on GLUT4 translocation in permeabilized 3T3-L1 adipocytes. At a concentration of 10 μM, this peptide blocked the insulin-stimulated increase in cell surface levels of GLUT4 by 34% (n = 2), whereas a control peptide of similar length was without effect (data not shown). To further map this inhibitory motif, we next examined the effects of a synthetic peptide spanning the first 15 amino acids of VAMP2. This peptide also significantly inhibited (p < 0.01, n = 5–7 experiments) insulin-stimulated GLUT4 translocation in a dose-dependent manner (Fig. 2). Assuming that we had achieved a maximal inhibitory effect at a peptide concentration of 100 μM, the highest concentration tested, the half-maximal inhibitory concentration was 100 μM.

TABLE I
Sequence comparison of the N termini of different v-SNAREs

| v-SNARE   | Sequence                  | Reference |
|-----------|---------------------------|-----------|
| Rat cellubrevin | MSTGVPSSAATGSNRRL | 25        |
| Rat VAMP1   | MSAPAQPGEQPPEGPSPPNNTSNRR | 43        |
| Rat VAMP2   | MSATA ATVPAAPAGGGPPAPPNLTSNRRL | 43        |
| Aplysia VAMP| MSAGPGFGQGMOPPREQSRR | 26        |

FIG. 1. VAMP2-GST specifically inhibits insulin-stimulated GLUT4 translocation. SLO-permeabilized 3T3-L1 adipocytes were pretreated for 10 min with buffer alone or buffer containing GST fusion proteins comprising the entire cytoplasmic tails of VAMP1, VAMP2, or cellubrevin (Ceb) at a final protein concentration of 10 μM. GST alone was used as a negative control. Cells were then incubated for an additional 15 min, in the presence of fusion proteins, with (shaded bars) or without (unshaded bars) 10 μM insulin. Plasma membrane lawns were prepared, and the extent of GLUT4 translocation relative to the plus insulin value minus fusion protein (100%) was determined using scanning laser confocal immunofluorescence microscopy. Data are expressed as GLUT4 translocation as a percentage of the maximal insulin response (mean ± S.E.; n = 5). *, p < 0.001 compared with insulin control.

FIG. 2. Effect of N-terminal VAMP2-peptide on insulin-stimulated GLUT4 translocation SLO-permeabilized 3T3-L1 adipocytes were incubated with different concentrations of a synthetic VAMP2 peptide corresponding to the N-terminal 15 amino acids, as described in the legend to Fig. 1. Cells were then incubated for an additional 15 min with peptide alone or peptide plus 10 μM insulin. Plasma membrane lawns were prepared, and the extent of GLUT4 translocation was determined using scanning laser confocal immunofluorescence microscopy. Data are expressed as GLUT4 translocation as a percentage of the maximal insulin response. The dose response plus insulin represents the mean ± S.E. for five separate experiments and without insulin for two separate experiments.
Role of VAMP2 in GLUT4 Trafficking

FIG. 3. N-terminal VAMP2-peptide inhibits insulin stimulated GLUT4 but not GLUT1 translocation. SLO-permeabilized 3T3-L1 adipocytes were pretreated for 10 min with buffer alone or buffer containing 10 μM VAMP2 or 10 μM control (CON) peptide followed by an additional 15-min incubation with peptide with (shaded bars) or without (unshaded bars) 10 μM insulin. Plasma membrane lawns were prepared, and the extent of glucose transporter movement was determined using scanning laser confocal immunofluorescence microscopy. Data are expressed as GLUT translocation as a percentage of the maximal insulin response (mean ± S.E.; n = 7). **, p < 0.001 compared with insulin control.

Effect of VAMP2 Peptide on GLUT1 Translocation—In addition to GLUT4, adipocytes also express another facilitative glucose transporter isoform, GLUT1, which is 63% identical to GLUT4 at the amino acid level (13, 14). However, in contrast to GLUT4, there is a relatively small increase in surface levels of GLUT1 in response to insulin (32). To determine if the inhibitory effect of the VAMP2 N terminus was specific to GLUT4, we studied the effects of the VAMP2-(1–15) synthetic peptide on GLUT1 translocation in permeabilized cells (Fig. 3). In agreement with previous studies (32), insulin increased cell surface levels of GLUT1 by 2.4-fold, which contrasted with the 5.5-fold increase in surface GLUT4 levels. The VAMP2 synthetic peptide (10 μM) inhibited insulin-stimulated GLUT4 translocation by 33.5% (p < 0.001, n = 7 experiments), whereas a control 15-mer peptide had no significant effect (Fig. 3). In contrast, neither the control peptide nor the VAMP2 peptide affected cell surface levels of GLUT1 in either the absence (data not shown) or presence of insulin (Fig. 3).

Effect of VAMP2 Peptide on vp165 Translocation—An amino peptidase (vp165) has recently been identified in adipocytes, and this protein co-localizes almost identically with GLUT4 in insulin-sensitive cells (15, 16, 18). Furthermore, in contrast to other recycling proteins such as GLUT1, the insulin-dependent movement of GLUT4 but also impacts on other proteins such as vp165 that presumably traffic through the same intracellular pathway as GLUT4.

Effect of VAMP2 Peptide on GTPγS-stimulated GLUT4 Translocation—It has previously been shown that nonhydrolyzable GTP analogs such as GTPγS markedly stimulate GLUT4 translocation in 3T3-L1 adipocytes (30, 37). However, it has been suggested that the mode of action of GTPγS may differ from that observed with insulin (38). Hence, it was of interest to determine if the VAMP2 inhibitory effect on GLUT4 translocation was general or somewhat specific to insulin-dependent movement. As indicated in Fig. 5, the 15-mer VAMP2 peptide had no significant effect (p > 0.05, n = 4) on GTPγS-stimulated GLUT4 translocation in permeabilized adipocytes over the full dose response range of the peptide (1–100 μM).

The Effect of Endosomal Ablation on GLUT1, GLUT4, and...
Transferrin Receptor Trafficking in 3T3-L1 Adipocytes—The studies described above are consistent with a unique role for VAMP2 in GLUT4 trafficking in adipocytes. This is consistent with our previous studies using the TF-HRP ablation technique where we were able to segregate intracellular GLUT4 into two compartments, one of which was enriched in cellubrevin and the other VAMP2 (19). Collectively, these data support a model whereby GLUT4 is targeted to recycling endosomes and separate intracellular storage vesicles (GSVs). Within each of these locations, the presence of different v-SNAREs, capable of interacting with the t-SNARE syntaxin 4 expressed at high levels on the adipocyte plasma membrane (23, 24), is consistent with the idea that endosomes and GSVs can independently dock and fuse with the cell surface. This interaction may account for the selective effect of the VAMP2 N-terminal peptide on insulin-stimulated GLUT4 trafficking in permeabilized cells (Figs. 2–5). To further test this model, we have attempted to determine if insulin-stimulated GLUT4 trafficking remains intact after endosomes have been ablated with TF-HRP. This technique relies on the ability of TF-HRP to bind to transferrin receptors on the cell surface and to traffic throughout the recycling endosomal pathway (39). The HRP enzyme activity can then be used to cross-link the proteins resident within the recycling system by employing diaminobenzidine and H$_2$O$_2$. By initiating the enzymatic activity of HRP within the living cell, it is possible to achieve a compartment-specific ablation of proteins that co-localize with the conjugate presumably in the recycling endosome. In the present studies, we have allowed adipocytes to endocytose TF-HRP for 1 h at 37 °C, after which endosomes were ablated at 4 °C as described previously (34). The cells were then reincubated at 37 °C in the absence or presence of insulin to determine the effects of endosomal ablation on subsequent protein trafficking.

Initially, we examined recycling of transferrin in ablated cells as a marker of the constitutive recycling pathway. Cells were loaded with radiolabeled transferrin plus TF-HRP to fill the itinerant recycling pathway. Cell surface Tf and TF-HRP were stripped by incubating cells in low pH medium and then incubated with DAB (with or without H$_2$O$_2$) to ablate the endosomal system. Cells were then rapidly washed at 37 °C, and the amount of cell-associated radiolabeled Tf was followed over time as a measure of transferrin recycling. There was a rapid loss of radiolabeled Tf from cells that had been incubated with TF-HRP and DAB in the absence of H$_2$O$_2$ (Fig. 6), indicating that the recycling pathway was still intact under these conditions. However, when ablation was followed by the addition of H$_2$O$_2$, there was no reduction in cell-associated radiola-beled Tf, indicating that the endosomal recycling pathway had been effectively disrupted (Fig. 6).

We next determined if insulin was still capable of stimulating GLUT4 translocation in adipocytes following endosomal ablation. In these experiments, duplicate plates of adipocytes were exposed to TF-HRP and DAB, but only one of each pair received hydrogen peroxide. After DAB cytochemistry was performed, cells were rapidly washed at 37 °C and incubated with or without insulin for the times indicated. Insulin stimulated the time-dependent movement of GLUT4 to the plasma membrane even after ablation of the recycling system. However, the time course of this translocation was slower than that observed in nonablated cells (Fig. 7). Irrespective, the cell surface levels
of GLUT4 observed after 30 min of insulin stimulation were not different between ablated and nonablated cells.

As described above, insulin also stimulates the translocation of other recycling proteins to the cell surface in adipocytes including GLUT1. Several observations including the lack of an inhibitory effect of the VAMP2 N-terminal peptide on insulin-stimulated GLUT1 trafficking (Fig. 3) led us to propose that GLUT1 predominantly resides in endosomes and that insulin stimulates recycling through the endosomal pathway. If this model is correct, then in contrast to what we have seen for GLUT4 (Fig. 7), endosomal ablation using Tf-HRP should effectively inhibit insulin-stimulated recruitment of GLUT1 to the plasma membrane. As shown in Fig. 8, even after 20 min of incubation with insulin, there was no change in the cell surface levels of GLUT1, whereas there was a marked increase in surface levels of GLUT4 under the same conditions.

**DISCUSSION**

The comprehensive block in neurotransmission that results from the proteolytic cleavage of VAMP2 by clostridial neurotoxins together with the observation that VAMP2 forms a stable ternary complex with two presynaptic plasma membrane proteins, syntaxin 1A and SNAP-25, has provided compelling evidence in favor of a specific role for VAMP2 in the docking and fusion of SSVs with the cell surface (reviewed in Ref. 2–4). In the present studies, we have provided evidence to suggest that VAMP2 may play a similar role in the regulation of GLUT4 trafficking in adipocytes. A specific role for VAMP2 in this process is implicated by the observation that a recombinant fusion protein comprising the cytoplasmic tail of VAMP2 blocked the insulin-dependent accumulation of GLUT4 at the cell surface (Fig. 1). A similar effect was not observed using fusion proteins encompassing the entire cytoplasmic tails of either VAMP1 or cellubrevin (Fig. 1). Each of these fusion proteins contains the syntaxin binding domain, and we have previously shown that both the VAMP2 and cellubrevin fusion proteins are not different between ablated and nonablated cells.

As described above, insulin also stimulates the translocation of other recycling proteins to the cell surface in adipocytes including GLUT1. Several observations including the lack of an inhibitory effect of the VAMP2 N-terminal peptide on insulin-stimulated GLUT1 trafficking (Fig. 3) led us to propose that GLUT1 predominantly resides in endosomes and that insulin stimulates recycling through the endosomal pathway. If this model is correct, then in contrast to what we have seen for GLUT4 (Fig. 7), endosomal ablation using Tf-HRP should effectively inhibit insulin-stimulated recruitment of GLUT1 to the plasma membrane. As shown in Fig. 8, even after 20 min of incubation with insulin, there was no change in the cell surface levels of GLUT1, whereas there was a marked increase in surface levels of GLUT4 under the same conditions.
proteins used in these studies bind with similar avidity to syntaxin 4, the presumed adipocyte t-SNARE (23). Further evidence in favor of a specific role for VAMP2 was provided by the observation that a synthetic peptide encompassing the unique N terminus of VAMP2 had a similar inhibitory effect on insulin-stimulated GLUT4 translocation (Fig. 2–4) to that observed with the entire VAMP2 cytoplasmic tail (amino acids 1–94). These results suggest that the unique role of VAMP2 in GLUT4 trafficking may be confined to this N-terminal domain.

Previous studies in Aplysia neurons have used a strategy similar to that described here to map functional domains in VAMP2 involved in SSV exocytosis (40). A variety of synthetic peptides spanning the cytoplasmic tail of VAMP2 were micro-injected into neurons to assess their effects on acetylcholine release. Similar to that reported in the present study, these peptides had an inhibitory effect on exocytosis, and the minimal effective domain was confined to the extreme N-terminal 19 amino acids. Based on an alignment of the VAMP2 sequence from different species, a proline-rich motif (PAAPXGXXPP in human VAMP2) was proposed to constitute the effective inhibitory domain. In contrast to the Aplysia studies, however, we observed that an N-terminal peptide (VAMP2-(1–15)) that only partially overlaps with this proline-rich region was as effective in blocking insulin-stimulated GLUT4 translocation as a longer peptide (VAMP2-(1–25)) that completely encompassed this motif. This is of interest, because this shorter peptide only comprises part of the motif suggested to be required for inhibition of exocytosis in neurons (see Table I). This may reflect differences in regulation between GSV and SSV exocytosis or differences in amino acid sequence between Aplysia and mammalian VAMP homologs (Table I).

This study and the previous studies in Aplysia neurons (40) suggest an important role for the VAMP N terminus in exocytosis. As described above, the binding sites for both SNAP-25 and syntaxin have been mapped to the central coiled-coil domain in VAMP (6–10). Hence, it is unlikely that the N terminus plays a direct role in the formation of the ternary complex. However, the N terminus of VAMP2 has been shown to be necessary for the interaction between VAMP2 and synaptophysin (41, 42). The interaction between VAMP2 and synaptophysin in vitro is blocked by an N-terminal VAMP2 fragment (residues 1–32) but not by a similar domain from VAMP1 (7, 8, 10). As yet, the role of synaptophysin in neuronal exocytosis has not been clearly established. Furthermore, a synaptophysin homolog has not been reported in insulin-sensitive cells. Clearly, identification of a protein that interacts with the N terminus of VAMP2 in adipocytes may provide important clues about the unique regulation of GLUT4 trafficking by insulin in these cells.

The use of the N-terminal VAMP2 peptide in permeabilized adipocytes enabled us to uncouple the insulin-dependent trafficking of two different proteins, GLUT1 versus GLUT4. Whereas this peptide inhibited the insulin-dependent movement of GLUT4 by ~35%, it had no significant effect on insulin-stimulated translocation of GLUT1 (Fig. 3). The most plausible explanation for these data is that insulin stimulates the exocytosis of two distinct intracellular compartments in adipocytes. We suggest that one of these is a postendocytic storage compartment for GLUT4 and vp165 that we have referred to as GSV,\(^2\) and the other is the constitutive recycling endosomal system. It is highly probable that cellubrevin regulates the docking and fusion of recycling endosomes with the cell surface, whereas VAMP2 may be involved predominantly in the regulation of GSVs. Several observations support this model. Both immunofluorescence microscopy and vesicle immunoadsorption studies have provided evidence for the intracellular segregation of GLUT1 and GLUT4 in adipocytes (31, 32, 44). Moreover, using the Tf-HRP endosomal ablation technique in 3T3-L1 adipocytes, it has been shown that GLUT1 and cellubrevin are predominantly targeted to recycling endosomes, whereas GLUT4, VAMP2, and vp165 are excluded from this compartment (18, 19, 34). Importantly, GLUT4 and vp165 are not completely excluded from recycling endosomes in the absence of insulin (18, 19, 34, 45, 46), and so this may explain the inability of either GST-VAMP2 or the N-terminal VAMP2 peptides to completely block insulin-stimulated GLUT4 translocation (Figs. 1–3). This may also account for the inability of these reagents to inhibit GTP\(_{\gamma}\)S-stimulated GLUT4 translocation (Fig. 5), which may selectively target the recycling endosomal pool as opposed to GSVs. GTP\(_{\gamma}\)S also stimulates GLUT1 translocation to the cell surface in permeabilized adipocytes,\(^3\) supporting this hypothesis.

The intracellular segregation of GLUT4 and vp165 from the constitutive recycling pathway and the potential involvement of VAMP2 in the former but not the latter raises some important points concerning insulin regulation of glucose transport in adipocytes. First, it suggests that these compartments are separate entities within the cell. Second, these compartments may dock and fuse directly with the cell surface, thus providing the basis for their autonomous regulation. Third, it implies that an important site of insulin action is the molecular machinery that regulates the movement of these vesicles and/or their subsequent docking and fusion with their relevant target membrane. Several observations lend support to these concepts. Growth factors such as platelet-derived growth factor and epidermal growth factor and protein kinase C agonists stimulate the translocation of GLUT1 but not GLUT4 in adipocytes (47–49), indicating that the constitutive recycling pathway can be regulated independently of GSVs in these cells. Ablation of endosomes using Tf-HRP inhibited transferrin recycling (Fig. 6) as well as insulin-stimulated GLUT1 translocation (Fig. 8). Despite this block in endosomal recycling, insulin was still capable of eliciting a significant increase in surface levels of GLUT4 (Fig. 7). We noted, however, that there was a significant delay in insulin-stimulated arrival of GLUT4 at the surface under these conditions. This delay raises two interesting possibilities to explain the insulin-regulated trafficking of GLUT4 in adipocytes. The first possibility is that GSVs fuse directly with the cell surface independently of the recycling pathway. The delay we have observed would be explained if insulin normally stimulates movement through the endosomal system more rapidly than GSV exocytosis. Hence, in ablated cells the reduction in GLUT4 at the surface at early times may be accounted for by loss of endosomal GLUT4. The second possibility is that GLUT4 joins the endosomal pathway prior to its arrival at the cell surface, and this effect is blocked early after ablation but not at later times when endosomes have had a chance to reform. We are currently undertaking studies to distinguish between these two possibilities.

The similarity between the regulation of GLUT4 or GSVs in adipocytes and SSV exocytosis in neurons is quite striking. In particular, like SSVs, the intracellular GLUT4 compartment utilizes the v-SNARE VAMP2 and lends further support to the notion that the GSV may dock and fuse directly with the cell surface. For this to be the case, adipocytes should express a t-SNARE at the cell surface that is capable of binding to VAMP2. Syntaxin 4, a t-SNARE that binds avidly to VAMP2, is

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\(^2\) Rea, S., and James, D. E., (1997) *Diabetes* **46**, 1667–1677.

\(^3\) L. B. Martin, A. Shewan, C. A. Millar, G. W. Gould, and D. E. James, unpublished observation.
expressed in adipocytes, and it is predominantly targeted to the plasma membrane (23, 24). Furthermore, a specific role for syntaxin 4 in GLUT4 trafficking has been documented with the use of specific antibodies or recombinant syntaxin 4 fusion proteins (23, 24).

Syntaxin 4 also binds with high affinity to cellubrevin (23), so it is conceivable that it is a t-SNARE for recycling endosomes as well as GSVs. We have recently identified a member of the Sec1p family in adipocytes that binds with high affinity to syntaxin 4 (23). This protein, Munc-18c, inhibits the ability of syntaxin 4 to bind to both VAMP2 and cellubrevin. Thus, by regulating the interaction between Munc-18c and syntaxin 4, it may be possible to manipulate the docking and fusion of both GSVs and recycling endosomes with the plasma membrane. This step may reflect a common step in both pathways. This would leave VAMP2 as a central player in mediating some of the unique regulatory aspects of GSVs and GLUT4 trafficking by insulin in adipocytes. Determining the nature of these functions and in particular the role of the VAMP2 N terminus are major challenges for the future.

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