Vp165 and GLUT4 share similar vesicle pools along their trafficking pathways in rat adipose cells

Daniela Malide[a,*], Jean-François St-Denis[a], Susanna R. Keller[b], Samuel W. Cushman[a]

[a] *Experimental Diabetes, Metabolism, and Nutrition Section, Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA*
[b] *Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, USA*

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Abstract vp165 (or gp160) is an aminopeptidase that has been identified as one of the major proteins of the GLUT4-containing vesicles. In the present study we have determined the degree of co-localization between vp165 and GLUT4 in rat adipose cells and used perturbation by wortmannin to assess the exocytic and endocytic steps along the translocation and recycling pathways of GLUT4 in the absence and presence of insulin. Western blots of subcellular membrane fractions demonstrate very similar distributions of vp165 and GLUT4. Confocal microscopy of whole cells provides direct evidence that these proteins share the same vesicle populations moving both towards and from the plasma membrane. These data are consistent with the presence of a distinct insulin-sensitive compartment that sequesters both GLUT4 and vp165 and suggest similar trafficking routes through the recycling compartments.

Key words: GLUT4; vp165; Adipose cell; Wortmannin; Confocal microscopy; Insulin action

1. Introduction

Insulin stimulates glucose transport in rat adipose cells by inducing the translocation of the GLUT4 glucose transporter from an intracellular compartment to the plasma membrane. This mechanism is supported by both biochemical and immunocytochemical analyses [1–4]. However, many questions regarding the localization and trafficking pathways of GLUT4 remain unanswered. It is unclear, for example, whether in the absence of insulin, GLUT4 storage vesicles represent a separate, distinct class of vesicles. Support in favor of an unique intracellular GLUT4 compartment in insulin-responsive cells comes from very recent data using in situ biochemical ablation of the endosomal system in 3T3-L1 adipocytes [5] and confocal microscopy in whole rat adipose cells [6]. These studies suggest that in the absence of insulin, GLUT4 is segregated from the compartment containing the recycling receptors. Furthermore, previous in vitro studies of the protein composition of GLUT4-containing vesicles reveals the presence of synaptobrevins, vesicle associated membrane protein 2 (VAMP2) [5,7–9] and cellubrevin (VAMP3) [5,9,10]; secretory carrier membrane proteins (SCAMP) [11]; phosphatidylinositol 4-kinase [12]; and the GTP-binding protein Rab4 [13,14]. In addition, a protein designated vp165 for vesicle protein of MW 165 kDa or gp160 for glycoprotein of MW 160 kDa has been purified from GLUT4-containing vesicles by two laboratories and has been identified as a zinc-dependent aminopeptidase [15–18]. By immunoblotting of subcellular fractions it was shown that vp165 redistributes to the cell surface upon insulin stimulation in rat adipose cells [15,17], 3T3-L1 adipocytes [19], and L6 muscle cells [20] to a similar extent as GLUT4 and that vp165 colocalizes with GLUT4 to the same vesicles obtained in vitro by cell homogenization [19,20].

However, the questions of whether vp165 and GLUT4 are entirely co-localized in vesicles within intact cells and translocate in a quantitatively similar way in response to insulin have not been addressed in rat adipose cells. Moreover, a morphological analysis of trafficking events during the re-internalization of vp165 and GLUT4 has not been described. Thus, we decided to take advantage of the effects of the fungal metabolite wortmannin (WT). Several biochemical kinetic studies in rat adipose cells and 3T3-L1 adipocytes have previously demonstrated that wortmannin inhibits the stimulatory effect of insulin on glucose transport by blocking the translocation of GLUT4 to the plasma membrane [21–24]. More recently, we have performed a morphological study in rat adipose cells of the effects of wortmannin on the subcellular distributions of GLUT4 and several membrane proteins used as 'compartment markers' [25]. Briefly, our data demonstrate that WT affects both the exocytic and endocytic limbs of the GLUT4 trafficking pathway. When added before insulin, WT blocks the insulin-stimulated GLUT4 translocation and conserves the basal GLUT4 compartment. When added after insulin, WT induces a redistribution of GLUT4 into endosomal-derived large phase-lucent vacuoles.

In the present report, we performed combined studies, using both double immunofluorescence confocal microscopy and biochemical approaches, to analyze GLUT4 and vp165 distributions in an insulin target cell of physiological significance. We further investigated by WT treatment the morphological details of exocytosis and endocytosis along the translocation and recycling pathways of vp165 and GLUT4. Our data have led us to conclude that in rat adipose cells vp165 and GLUT4 share the same vesicle populations moving both towards and from the plasma membrane. These data are consistent with the existence of a distinct insulin-sensitive compartment that sequesters both GLUT4 and vp165. Interestingly, these results also provide evidence that vp165 and GLUT4 follow similar traffic routes through the recycling compartments.

*Corresponding author. EDMNS/DB/NIDDK/NIH, Bldg. 10, Room 5N102, 10 Center Drive MSC 1420, Bethesda, MD 20892-1420, USA. Fax: (301) 402-0432.
E-mail: danielam@bdg10.niddk.nih.gov

Abbreviations: VAMP2, vesicle-associated membrane protein 2; SCAMP, secretory carrier membrane protein(s); vp165, vesicle protein 165; PM, plasma membrane(s); LDM, low-density microsome(s); HDM, high-density microsome(s); FITC, fluorescein isothiocyanate; LRSC, lissamine rhodamine sulfonyl chloride; PAGE, polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Preparation and incubation of the adipose cells

Adipose cells were isolated by collagenase digestion from the epididymal fat pads of 180–250 g male Sprague-Dawley rats as previously described [1,26]. Isolated white adipose cells ($2–4 \times 10^6$ cells/ml) were incubated without or with 700 nM insulin (Eli Lilly, Indianapolis, IN) at 37°C for 45 min. In experiments investigating the effects of WT (Sigma, St. Louis, MO), the drug was dissolved in dimethyl sulfoxide (DMSO) and added to the cells at a final concentration of 100 nM.

2.2. Glucose transport

Glucose transport activity was assessed by the tracer [U-^{14}C]glucose uptake method [27].

2.3. Preparation of subcellular membrane fractions

Homogenization and subcellular fractionation of adipose cells were carried out according to the method of Simpson et al. [1]. Briefly, cells were washed twice with TES buffer (25 mM Tris-HCl, 250 mM sucrose, 2 mM EDTA, pH 7.4) containing 0.12 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 18°C, and homogenized with a Potter-Elvehjem teflon pestle. Subcellular membrane fractions, plasma membranes (PM), high-density microsomes (HDM), and low-density microsomes (LDM), were obtained by differential centrifugation.

2.4. Antibodies

For GLUT4, we employed a rabbit polyclonal antiserum kindly provided by Hoffmann-La Roche (Nutley, NJ) and a mouse monoclonal antibody, F-27, kindly provided by Dr. P.N. Jorgensen of Novo Nordisk (Bagsvaerd, Denmark). Both antibodies are directed to C-terminal sequences of GLUT4 (of 20 and 14 amino acid residues, respectively) and their specificities were previously demonstrated by immunochemical and immunocytochemical techniques [6,28–30]. For vp165 we employed an affinity-purified rabbit polyclonal IgG raised against the cytoplasmic domain of vp165, previously used in both immunochemical and immunofluorescence studies on 3T3-L1 adipocytes [16,19]. Fluorescein isothiocyanate (FITC)- and lissamine rhodamine sulfonyl chloride (LRSC)-conjugated antibodies specific for rabbit or mouse immunoglobulins (Ig), used as secondary antibodies in immunofluorescence experiments, were obtained from Jackson ImmunoResearch (West Grove, PA).

2.5. Gel electrophoresis and Immunoblotting

Membrane proteins were separated by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). 12% and 8% polyacrylamide gels were used to resolve GLUT4 and vp165, respectively. Proteins were then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in 25 mM Tris-HCl, 192 mM glycine, 20% methanol buffer, pH 8.6, at 300 mA for 16 h. Membranes were blocked with 5% non-fat dry milk in 10 mM Tris-HCl, 0.9% NaCl buffer, pH 7.4. GLUT4 and vp165 were detected with specific antibodies, a rabbit polyclonal anti-GLUT4 (1:4000 dilution from whole serum) and an affinity-purified rabbit polyclonal anti-vp165 (3 μg/ml), respectively, followed by ^{125}I-labeled protein A as previously described [9]. Immunoreactive band intensities were quantified using a Molecular Dynamics computing densitometer (Molecular Dynamics, Sunnyvale, CA).

2.6. Immunocytochemistry

Single and double immunofluorescence experiments were performed using 4% paraformaldehyde-fixed adipose cells in suspension following the protocol described in detail elsewhere [6]. The cells were incubated sequentially in two steps with pairs of the specific primary followed by the corresponding secondary antibodies. Thus, the monoclonal anti-GLUT4 antibodies (1 μg/ml) in conjunction with the rhodamine-conjugated goat anti-mouse antibodies were followed by the polyclonal anti-vp165 antibodies (5 μg/ml) in conjunction with the FITC-conjugated goat anti-rabbit antibodies. The immunostained cells were viewed by the Nikon Optiphot 2 fluorescence microscope equipped with a Bio-Rad MRC1024 confocal laser scanning imaging system (CLSM) from Bio-Rad Labs (Hercules, CA). This system utilizes a mixed argon/krypton laser ($\lambda_1 = 488$ nm, blue line for FITC; $\lambda_2 = 568$ nm, yellow line for rhodamine) and Lasersharp image analysis software. Specimens were viewed using a planapochromat ×60/1,4NA oil immersion objective. For each experimental condition, 8–10 images/cell were recorded from at least 10–15 cells. Series of optical sections were collected sequentially at 0.5-μm intervals along the *Z*-axis for the two fluorochromes in the double-labeling experiments using Kalman averaging at an optical zoom setting of 1–2.5 [31]. Co-localization was assessed throughout the cell by examination of merged images and was expressed as 'high' when the overlap was present in all focal planes, 'partial' when the overlap was present in 1–3 focal planes, and 'absent' when no significant overlap was observed. For presentation, images were further enhanced digitally using the Adobe Photoshop 3.0 program from Adobe Systems (Mountain View, CA) and printed with a Kodak 8650 PS digital printer (Eastman Kodak, New Haven, CT).

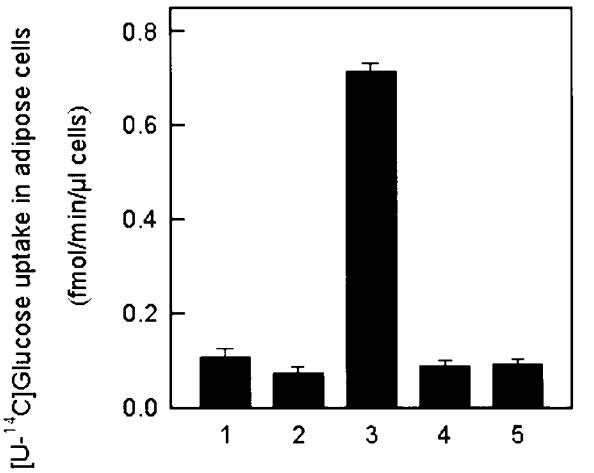

Fig. 1. Glucose transport activity in rat adipose cells. [U-^{14}C]Glucose uptake was measured as described in Section 2 in cells that were either (1) untreated (DMSO 0.1% alone), (2) treated with WT for 45 min, (3) stimulated with insulin for 45 min, (4) stimulated with insulin for 15 min followed by addition of WT for the next 30 min in the continuous presence of insulin, or (5) treated simultaneously with insulin and WT for 45 min. Results are the means ± SD of triplicate determinations obtained in a representative experiment ($n = 5$).

3. Results and discussion

Recent biochemical studies clearly show that the subcellular distributions of vp165 and GLUT4 are concordant, and thus we were prompted to analyze this co-localization in situ in adipose cells under experimental conditions which allow dissection of both the exocytic and endocytic steps of their trafficking pathways. Although the biochemical analysis of immunoisolated GLUT4-containing vesicles has contributed much to characterizing the protein composition of the GLUT4 compartments, the precise localization of these various proteins in intact cells under steady-state conditions is needed as a basis to clarify further their functional roles.

Fig. 1 illustrates the glucose transport activity of isolated rat adipose cells under several experimental conditions. WT slightly decreases basal glucose transport activity consistent with previous data in 3T3-L1 adipocytes [24], while insulin stimulates a 7-fold increase in glucose transport activity compared to basal values. In the presence of insulin, WT completely reverses the stimulation of glucose transport activity when added 15 min after insulin and completely prevents the stimulation of glucose transport activity when added concomitantly with insulin.

The subcellular distributions of vp165 and GLUT4 between

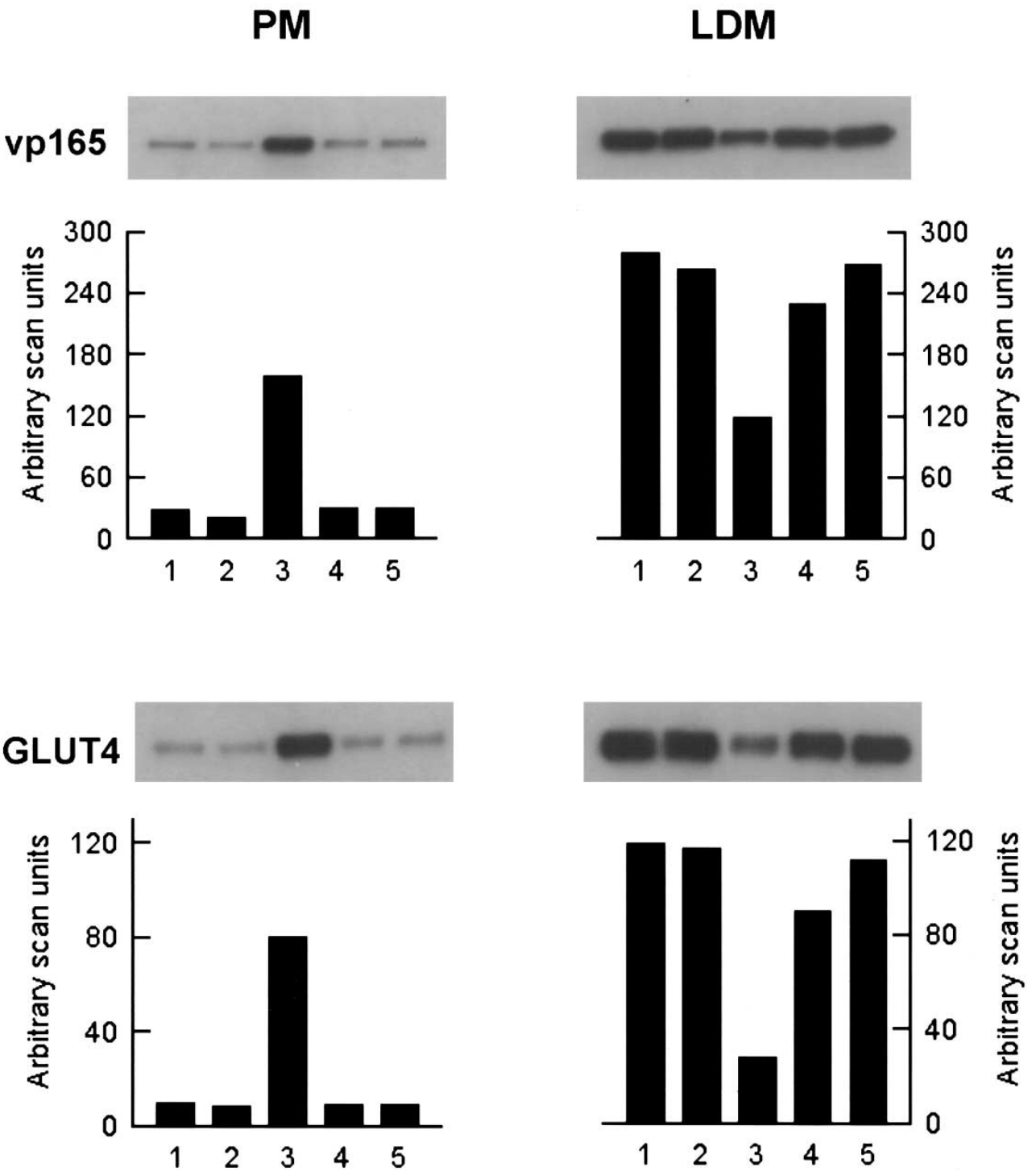

Fig. 2. Subcellular distributions of vp165 and GLUT4 in rat adipose cells. Plasma membrane (PM) and low-density microsome (LDM) fractions were prepared from samples of cells from the experiment shown in Fig. 1, as described in Section 2. Membrane proteins (20 μg/lane) were resolved by SDS-PAGE (8% for vp165 and 12% for GLUT4), transferred to nitrocellulose paper, and probed with vp165 and GLUT4 polyclonal antibodies in conjunction with ^{125}I-labeled protein A. Western blot data were quantified as phosphorimager relative units per 20 μg of membrane protein in PM and LDM. Because of the consistency of the current results for the basal and insulin-stimulated states with published data, only this one Western blotting experiment was carried out.

PM and LDM prepared from adipose cells treated as just described are shown in Fig. 2. In PM from WT-treated cells, vp165 and GLUT4 both appear to be slightly decreased compared to PM from basal cells. In the corresponding LDM of WT-treated cells, vp165 and GLUT4 do not change significantly compared to the basal patterns. In PM from insulin-stimulated cells, vp165 and GLUT4 show parallel increases compared to PM from basal cells (5.3-fold and 7.6-fold, respectively). This redistribution to the plasma membrane is accompanied by parallel decreases of vp165 and GLUT4 in

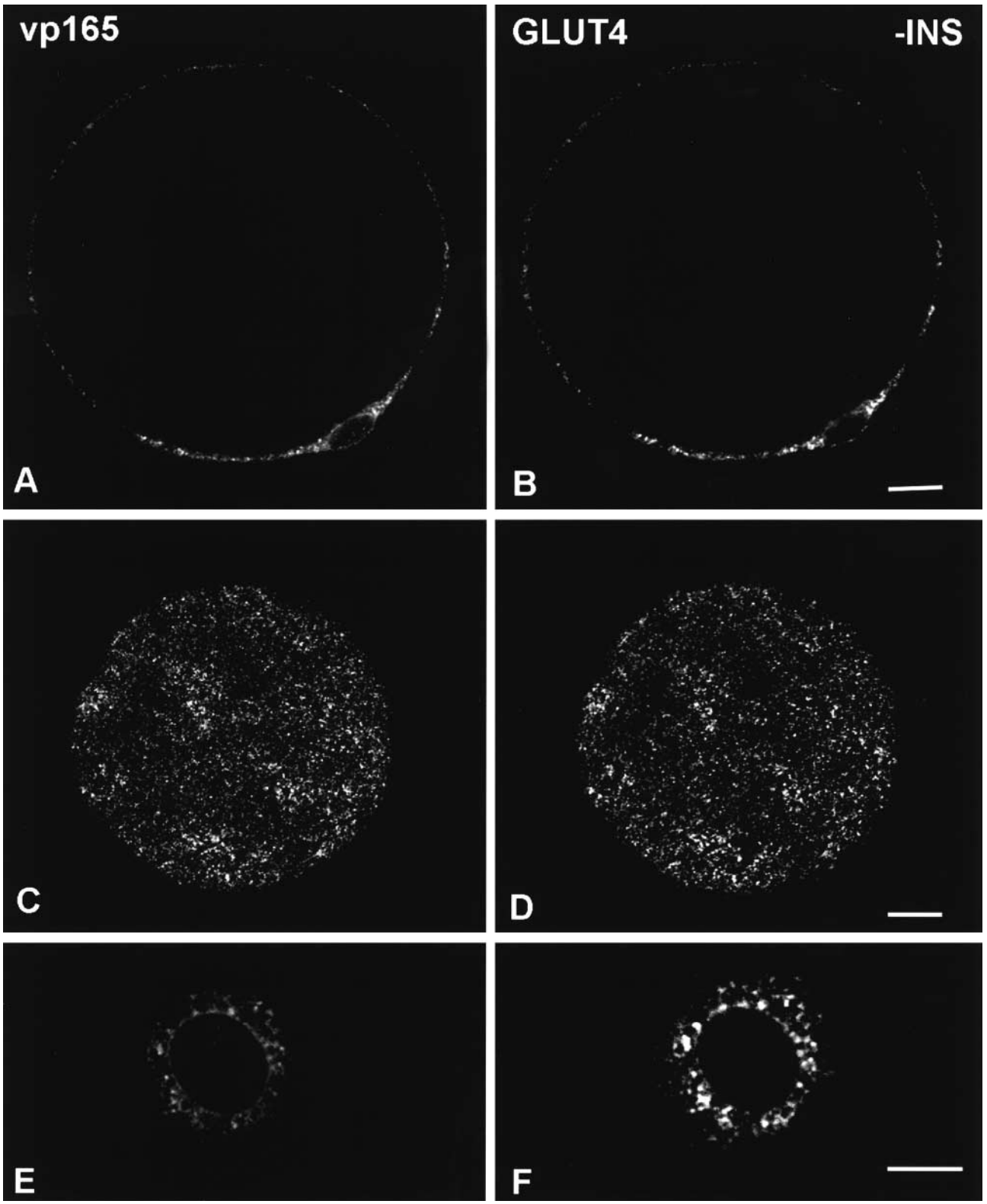

Fig. 3. Co-localization of vp165 and GLUT4 in basal rat adipose cells. Images from the same confocal optical sections double labeled for vp165 (A,C,E) and GLUT4 (B,D,F) are presented side by side. For most confocal sections through the middle (A,B) and closer to the surface (C,D) of rat adipose cells, very similar intracellular punctate staining is observed for vp165 and GLUT4. In contrast, in the perinuclear region, vp165 exhibit only weak punctate immunofluorescence partially co-stained by GLUT4; the latter is present also in 'large dots' distinct from vp165 (E–F). Bars: 10 μm.

LDM (58% and 76%, respectively). The distributions of GLUT4 and vp165 in subcellular fractions of cells incubated first with insulin for 15 min followed by the addition of WT or incubated simultaneously with WT and insulin are un-

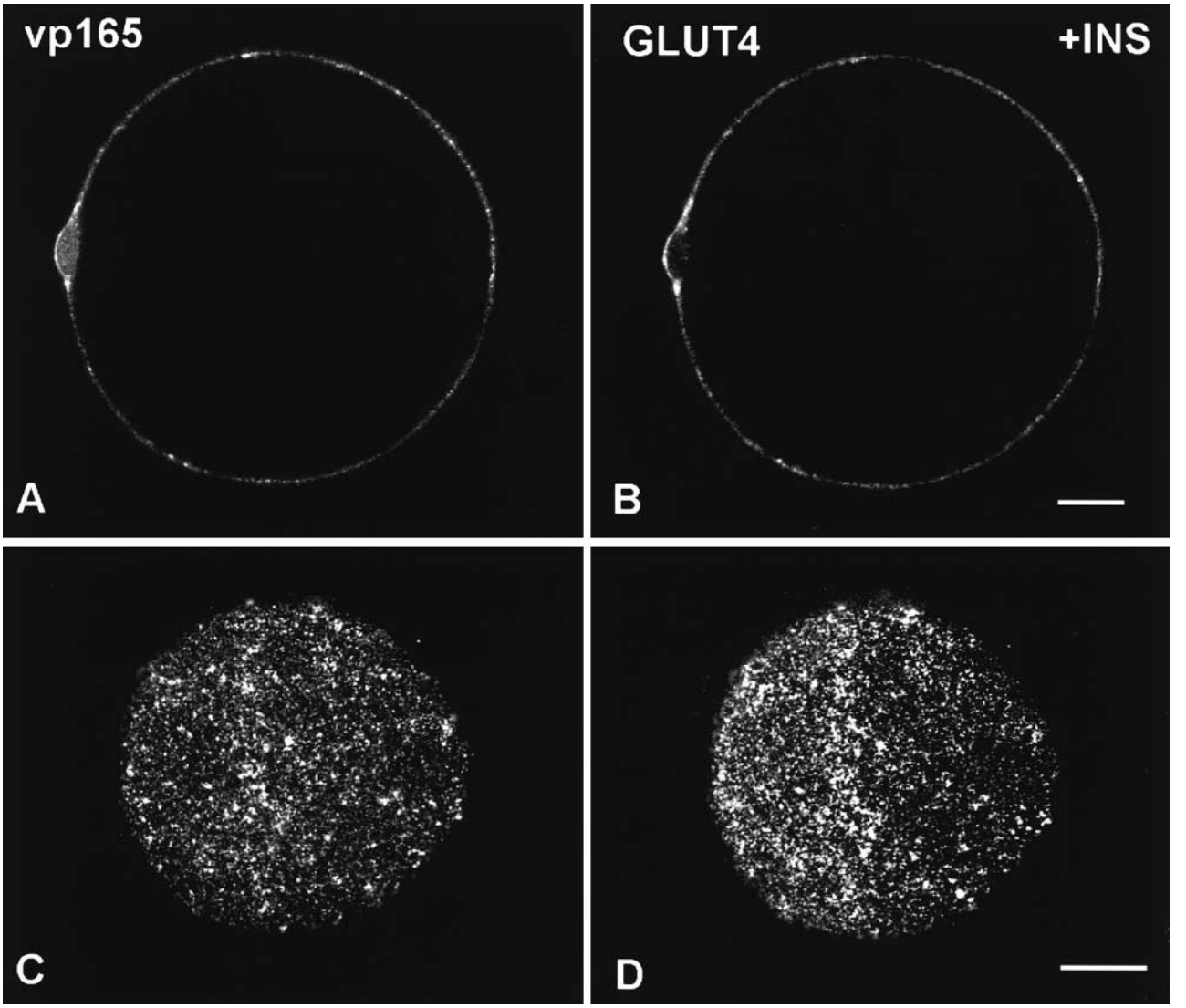

Fig. 4. Comparable translocation of vp165 and GLUT4 to the cell surface in insulin-treated rat adipose cells. Images from the same confocal optical sections double labeled for vp165 (A,C) and GLUT4 (B,D) are presented side by side. In confocal cross-sections (A,B), the cell surface staining of vp165 and GLUT4 can be seen outlining the periphery of the cell; in grazing views (C,D), the vp165 and GLUT4 staining can be seen obscuring the visualization of the lipid droplet. Note the high degree of correspondence of vp165 and GLUT4 immunofluorescence. Bars: 10 μm.

changed compared to their respective basal distributions. Thus WT reverses the effects of insulin when added after insulin and blocks the effects of insulin when added to cells together with insulin. These results are consistent with the insulin-stimulated increases of vp165 and GLUT4 in PM of rat adipose cells reported previously [15,17]. In addition, we demonstrate here comparable inhibitory effects of WT on the insulin-stimulated translocation of vp165 and GLUT4 in rat adipose cells. Finally, our data using WT treatment following insulin stimulation reveal a remarkable parallelism between the subcellular distributions of vp165 and GLUT4 along the return pathway from the plasma membrane to the intracellular compartment as well.

Because the observed parallelism of vp165 and GLUT4 in subcellular membrane fractions does not necessarily prove their co-localization in the same vesicle, we next used confocal microscopy to directly visualize their subcellular distributions. Adipose cells were incubated under the experimental conditions described above and then double labeled for vp165 and GLUT4. When cells are incubated in the absence of insulin (basal cells), we observe very similar patterns and almost complete overlap of immunofluorescence for vp165 and GLUT4 (Fig. 3). Confocal optical sections (0.2 μm in thickness) through the middle, closer to the surface, and through the perinuclear area of a rat adipose cell are presented. The staining is almost entirely intracellular and found in the perinuclear region and in fine punctate spots distributed throughout the cytoplasm (Fig. 3A,B). Only weak staining is observed at the cell surface (Fig. 3C,D). A high degree of co-localization is demonstrated in most confocal cross-sections (Fig. 3A,B) and grazing sections (Fig. 3C,D). In contrast, in the perinuclear region overlap is not complete; close examination reveals some differences in the distributions and immunofluorescence patterns of the proteins. In this region GLUT4 exhibit a fine punctate staining with partial overlap with vp165 and a 'large dot' staining for the most part distinct from vp165 (Fig.

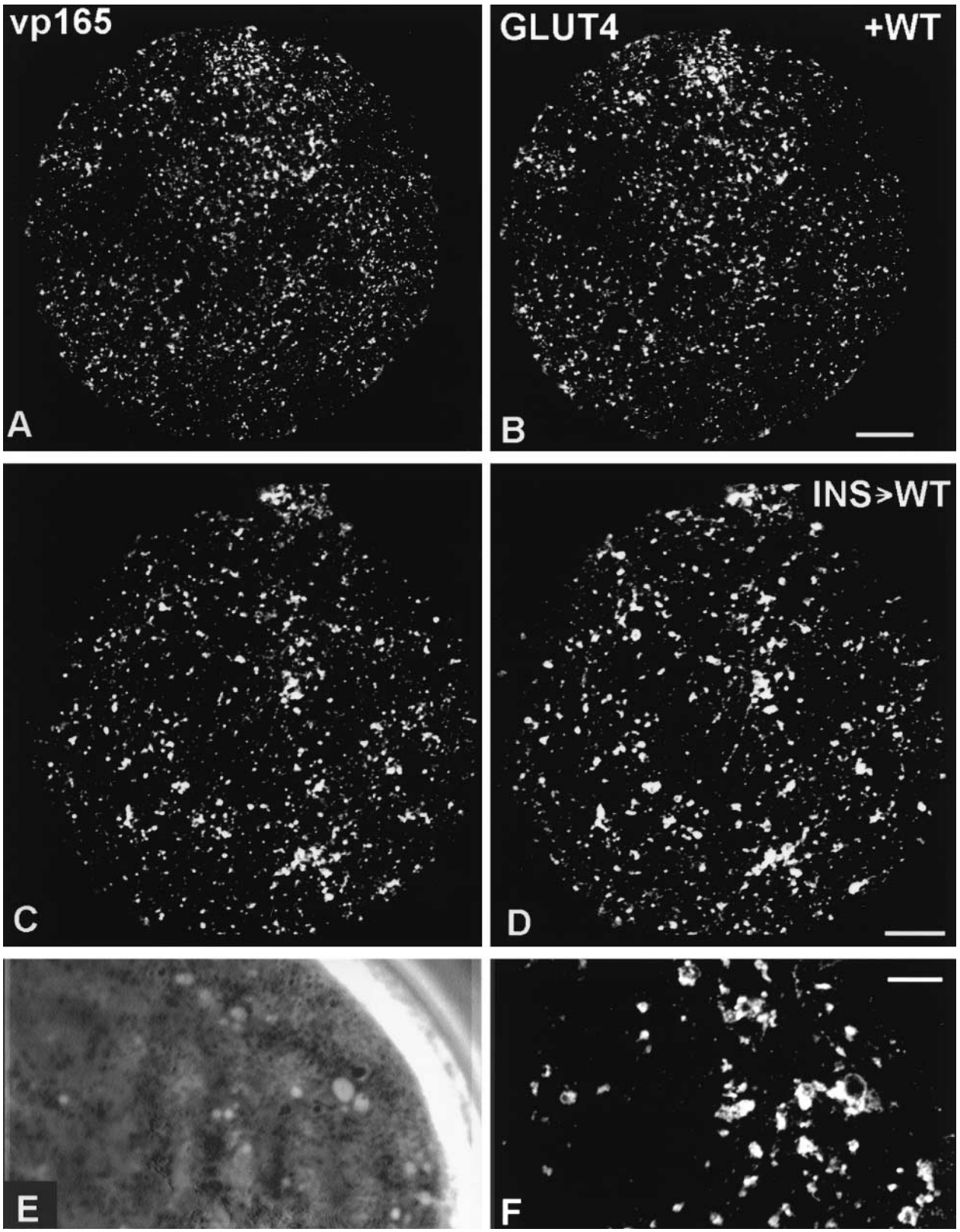

Fig. 5. Co-localization of vp165 and GLUT4 in WT-treated rat adipose cells. Images from the same confocal optical sections double labeled for vp165 (A,C) and GLUT4 (B,D) are presented side by side. In grazing sections of WT-treated cells (A,B), vp165 and GLUT4 exhibit a remarkably similar intracellular punctate pattern indistinguishable from that seen with basal cells (Fig. 3). In grazing sections of cells incubated with insulin followed by the addition of WT in the continuous presence of insulin (C,D), vp165 and GLUT4 immunofluorescence coincides for the most part in vacuoles and vesicle-like structures. WT-induced phase-lucent vacuoles are illustrated in a phase micrograph (E) and a corresponding GLUT4 immunofluorescence image (F). Bars: 10 μm (A,B,C,D); 5 μm (E,F).

3E,F). A semi-quantitative estimation of the degree of the overlap suggests that approximately 85% of the total GLUT4-containing vesicles present in a cell contains vp165 as well.

Several control experiments were performed to confirm the specificity of these results (not shown). Staining of the adipose cells with normal non-immune rabbit or mouse sera instead of the primary antibodies was not detectable.

After a 45-min treatment with insulin, very similar redistributions of vp165 and GLUT4 immunofluorescence to the cell surface are observed (Fig. 4A–D). Highly comparable co-staining is shown in most of the cross-sections (Fig. 4A,B) and grazing sections (Fig. 4C,D) examined.

The effects of WT observed in the present study on the subcellular distributions of vp165 and GLUT4 are summarized in Fig. 5. Wortmannin does not significantly affect the basal patterns of vp165 and GLUT4 staining (Fig. 5A,B), but prevents the insulin-stimulated appearance of immunodetectable vp165 and GLUT4 on the cell surface (data not shown). Under both these conditions, vp165 and GLUT4 immunofluorescence exhibits an intracellular punctate pattern indistinguishable from the basal staining (Fig. 5A,B). It is well documented in other cell types that WT induces morphological perturbations in the endosomal system in the absence of insulin [32–36]. Furthermore, in a CHO cell line overexpressing transferrin receptors, Martys et al. [34] have shown that WT treatment induces a redistribution of transferrin receptors to the morphologically altered compartment along with a slowed rate of receptor recycling. In contrast, our data show that the recycling of GLUT4 and vp165 under basal conditions is not changed by WT. Assuming that the recycling of transferrin receptors under basal conditions in rat adipose cells is the same as that in CHO cells, it is clearly different from those of vp165 and GLUT4. Of particular interest is our observation that although morphological alterations are present rat adipose cells in the form of large phase-lucent vacuoles (Fig. 5E), these vacuoles are devoid of GLUT4 and vp165. These data suggest that in the absence of insulin both proteins are outside the endosomal system, thus further supporting the concept of a specialized basal GLUT4 compartment.

By incubating cells with insulin for 15 min to induce translocation to the plasma membrane and then adding WT for the next 30 min to block further translocation despite the continuous presence of insulin, we were able to follow by immunofluorescence the internalization and return of vp165 and GLUT4 to the intracellular compartments (Fig. 5C–F). Consistent with our previous observations, WT induces the formation of phase-lucent vacuoles (Fig. 5E) which stain for GLUT4 (Fig. 5F). Likewise, vp165 show almost complete co-localization with GLUT4 in these vacuoles and throughout the remaining vesicle-like structures (Fig. 5C,D). However, the effects of WT on the distributions of vp165 and GLUT4 observed in insulin-treated adipose cells may not be specific only for these proteins. These changes probably reflect the trafficking of vp165 and GLUT4 through multiple WT-sensitive steps along the endocytic pathway. Several recent reports have documented that WT directly affects both receptor-mediated and fluid-phase endocytosis [32–36]. Our data are in agreement with these studies and add more support to the concept that both vp165 and GLUT4 are internalized and recycled through the endocytic pathway together with other membrane proteins.

In conclusion, at the level of resolution of confocal microscopy, the pattern of vp165 distribution closely resembles that of GLUT4 in the basal intracellular compartment indicating that both proteins reside in the same vesicle. Further, our data strongly indicate that vp165 and GLUT4 travel as part of an identical vesicle pool translocated to the plasma membrane in response to insulin. Interestingly, the use of WT provides direct evidence for the localization of both proteins in similar endocytic vesicles after re-internalization from the plasma membrane. These results are consistent with vp165 and GLUT4 residing in and recycling through the same subcellular compartments. Because the role of the aminopeptidase vp165 remains largely unknown, the significance of the presence of vp165 within GLUT4-containing vesicles is as yet unclear. Moreover, the expression of vp165 is not restricted to cells expressing GLUT4 [16]. Whether vp165 plays a role in the biogenesis of GLUT4 vesicles or in their fusion with the plasma membrane remains to be established.

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