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mVps45 knockdown selectively modulates VAMP expression in 3T3-L1 adipocytes

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Insulin stimulates the delivery of glucose transporter-4 (GLUT4)-containing vesicles to the surface of adipocytes. Depletion of the Sec1/Munc18 protein mVps45 significantly abrogates insulin-stimulated glucose transport and GLUT4 translocation. Here we show that depletion of mVps45 selectively reduced expression of VAMPs 2 and 4, but not other VAMP isoforms. Although we did not observe direct interaction of mVps45 with any VAMP isoform; we found that the cognate binding partner of mVps45, Syntaxin 16 associates with VAMPs 2, 4, 7 and 8 *in vitro*. Co-immunoprecipitation experiments in 3T3-L1 adipocytes revealed an interaction between Syntaxin 16 and only VAMP4. We suggest GLUT4 trafficking is controlled by the coordinated expression of mVps45/Syntaxin 16/VAMP4, and that depletion of mVps45 regulates VAMP2 levels indirectly, perhaps via reduced trafficking into specialized subcellular compartments.

Keywords: adipocyte, biochemistry, cell biology, glucose transport, hormones, insulin, intracellular membranes, membrane protein trafficking, membrane trafficking, SNARE proteins, VAMP

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Introduction

Insulin stimulates glucose transport in adipose and muscle tissue by inducing the movement of specialized intracellular vesicles enriched in glucose transporters (GSVs) to the cell surface, where they dock and fuse, resulting in increased levels of functional GLUT4 glucose transporters at the cell surface.[1,2] Understanding GSV formation and function is important, as individuals with insulin resistance and/or type-2 diabetes exhibit blunted rates of insulin-stimulated glucose transport that may arise as a result of defective formation or trafficking of the GSVs. [3]

Insulin-regulated GLUT4 translocation is an example of regulated membrane traffic, and like all such events utilizes the SNARE machinery to mediate both fusion of the GSVs with the plasma membrane and the sorting of GLUT4 into the GSV compartment.[3] Recent studies from our group identified Syntaxin 16 (Sx16) as an important t-SNARE for the sorting of GLUT4 into GSVs.[4] Sx16 is regulated by its cognate Sec1/Munc18 protein, mVps45; depletion of mVps45 results in decreased GLUT4 levels and abrogated sorting of GLUT4 into GSVs.[5] Depletion of mVps45 also resulted in a decrease in cellular VAMP4 levels, consistent with mVps45 regulating a SNARE complex comprised of Sx16 and VAMP4; by contrast, no effect on VAMP3 levels was observed.[5]

Here we show that mVps45 knockdown reduced levels of VAMP2 and VAMP4 selectively, with no effect on VAMP3, 5, 7 or 8. Using recombinant proteins, we observed interactions between Sx16 and multiple VAMP isoforms, including VAMP2 and VAMP4, *in vitro*; however, Sx16 was found to selectively co-immunoprecipitate only VAMP4. We suggest that mVps45, Syntaxin 16 and VAMP4 are co-ordinately regulated, and function to control GLUT4 sorting; perturbation of this pathway may result in decreased levels of VAMP2 as a consequence of altered GLUT4 sorting.

Results and Discussion

We have shown that shRNA-mediated depletion of mVps45 reduces Sx16 and GLUT4 protein levels and blunts

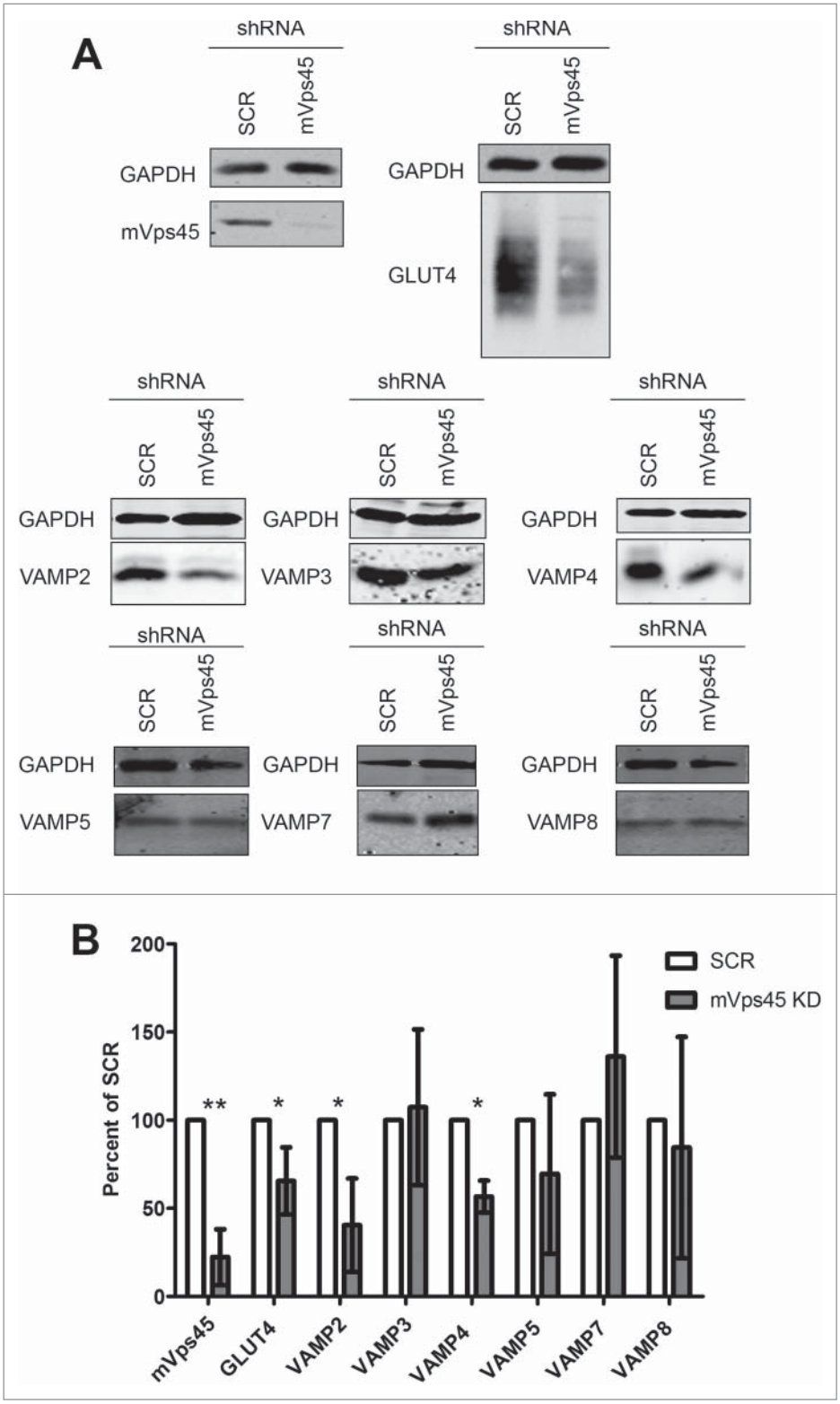

Figure 1. mVps45 Knock-down reduces the levels of VAMPs 2 and 4 in 3T3 L1 adipocytes. Knockdown of mVps45 was achieved as described. Shown are immunoblots of total cell membranes from a typical experiment following treatment with scrambled shRNA (SCR) or shRNA targeting mVps45 (mVps45) using the antibodies indicated (Panel **A**); 25 μg of protein was loaded per lane. Panel (**B**) shows the levels of the indicated protein in mVps45 knockdown cells expressed as a percentage of that in SCR-control cells (mean ± SD of 3 separate experiments, * = p,0.05, ** = $p < 0.01$ vs scrambled control).

insulin-responsive GLUT4 translocation.[5] We also observed decreased levels of VAMP4, a v-SNARE proposed to act in concert with Sx16, suggesting that mVps45 may act to stabilize said complex. We examined the consequences of mVps45 knockdown in adipocytes on all the VAMP isoforms (**Fig. 1**). We observed no consistent change in levels of VAMP3, 5, 7 or 8 in these experiments. Strikingly, we found that in addition to the previously reported decrease in VAMP4 levels (41.3% decrease, $p = 0.04$; **Fig. 1**),[5] VAMP2 levels were also significantly decreased (73.3% reduction, $p = 0.02$; see **Fig. 1**). These selective decreases in VAMP2 and 4 expression may reflect (i) binding of mVps45 directly to the VAMP, as has been reported for another Sec1/Munc18 protein,[6,7] such that depletion of mVps45 also de-stabilizes VAMP; (ii) direct interaction of VAMP2 or 4 with Sx16 (the levels of which also fall upon mVps45 depletion);[5] or (iii) an indirect effect, perhaps by a reduction in GSV numbers as a result of impaired trafficking via Sx16. Note that levels of Sx4 did not change upon mVps45 knockdown, making it unlikely that reduced levels of the Q_a-SNARE known to interact with VAMP2 in adipocytes underlies decreased VAMP2 levels.[5]

We first tested the ability of recombinant purified GST-tagged VAMP proteins to capture mVps45 expressed in yeast (**Fig. 2**). Equal amounts of GST-VAMP were loaded onto glutathione beads and incubated with yeast lysate over-expressing human mVps45 containing an HA-epitope tag. As shown, we were unable to detect an interaction between mVps45 and any of the VAMP isoforms. This may reflect either that no direct interaction in the case of these homologues, or that the interaction is weaker than that previously shown for VAMP2/Munc18c in our hands.[6] By contrast, Sx16 efficiently captured recombinant HA-mVps45 from the same lysate, confirming that the mVps45 was correctly folded.

Studies from other groups have suggested that there may be plasticity among SNARE interactions.[8-10] Hence, we

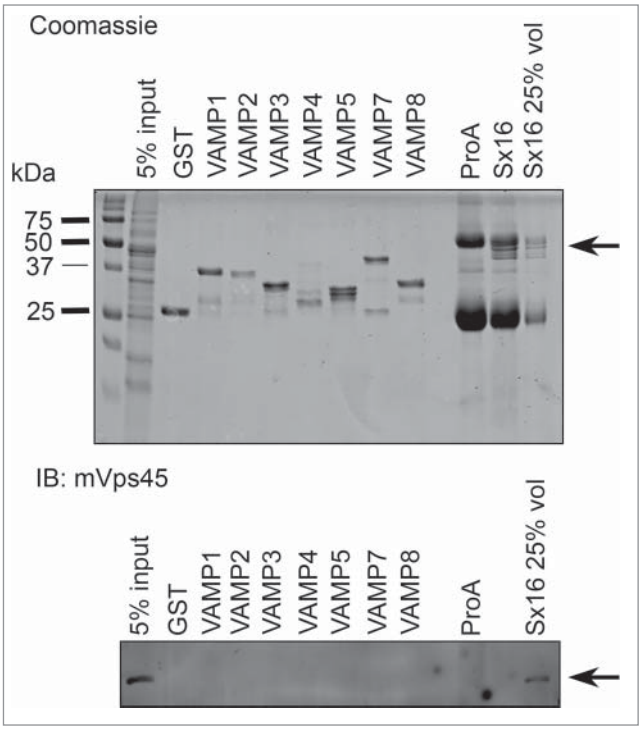

Figure 2. mVps45 does not directly interact with VAMP proteins. GST alone or GST-VAMP proteins were immobilized on Glutathione-Sepharose beads, protein-A alone or protein-A tagged syntaxin 16 was bound to IgG beads. Beads and associated proteins were incubated with equal volumes of yeast lysate expressing HA-tagged mVps45 overnight at 4°C. Beads were recovered by centrifugation, washed and bound protein eluted in 2 × LSB. 75% of the eluate was subjected to SDS-PAGE and stained with Coomassie brilliant blue to examine the amounts of each protein bound to beads (**A**). 25% of the eluate was immunoblotted with anti-HA tag to determine the presence of mVps45; due to the high levels of interaction seen between mVps45 and syntaxin 16, in this specific case the fraction of the elute loaded was reduced a further 25% (**B**). Shown are data from a typical experiment that was repeated 3 times using 3 separate batches of yeast lysate, molecular marker sizes are shown in kDa. In (**A**) arrow indicates syntaxin 16; in (**B**) arrow indicates mVps45.

reasoned that another explanation for decreased VAMP2 levels upon mVps45 knockdown may be a consequence of interaction between VAMP2 and Sx16. To test this, we used recombinant Sx16 in a series of pull-downs from bacterial lysates overexpressing GST-tagged VAMPs. We observed interaction between Sx16 and VAMP2, 4, 7 and 8 (but not VAMP3 and VAMP5) (**Fig. 3A**). Whether these data reflect interactions *in vivo* is unclear. To address this, we quantitatively immunoprecipitated Sx16 from 3T3-L1 adipocytes treated with or without insulin and probed the immunoprecipitated material for VAMP isoforms (**Fig. 3B** shows the data from cells not exposed to insulin; insulin treatment did not change the pattern of interactions [data not shown]). Our data revealed a consistent association between Sx16 and VAMP4, but we were unable to observe co-immunoprecipitation of VAMP2, VAMP3, VAMP7 or VAMP8. (Interaction between Sx4 and VAMP2 was observed in the same lysates; data not shown).

In sum, depletion of mVps45 in 3T3-L1 adipocytes results in significant reduction in cellular levels of VAMP2 and VAMP4 (but not VAMP3, 5, 7 or 8). This is unlikely to be explained by a direct interaction between mVps45 and either VAMP. mVps45 depletion also depletes the corresponding Q_a-SNARE Sx16, and we report here that this SNARE directly binds VAMPs 2, 4, 7 and 8 *in vitro*. These promiscuous interactions were not, however, recapitulated *in vivo*, as immunoprecipitation of Sx16 co-precipitated only VAMP4. We therefore suggest that mVps45 controls VAMP4 levels by regulating Sx16 levels; as Sx16 levels decline upon mVps45 knockdown, we propose that coordinate regulation of the cognate VAMP4 also results. Finally, we postulate that VAMP2 levels decline in mVps45-knockdown cells as a consequence of reduced trafficking of GLUT4 into GSVs, reflecting an important role in localization in the control of intracellular SNARE levels.

Materials and Methods

Knockdown of mVps45 in 3T3-L1 adipocytes was performed using lentivirus as described; control cells infected with scrambled shRNA were used in all experiments.[5] Antibodies against the different VAMP isoforms were all rabbit polyclonal species from Synaptic Systems, Germany. GST-VAMP2 and 3 were as described.[11] GST-VAMP4, −5 and −7 and −8 were from Andrew Peden (Sheffield). Sx16-protein A and Sx4-protein A constructs and the methodology for the pull-down experiments were as described.[6,12,13] SDS-PAGE and immunoblotting were performed as described.[14,15] Immunoblot signals were quantified using the Licor system; changes in expression of VAMP isoform signals were normalized to those obtained from anti-GAPDH immunoblots on the same gel. Data are presented as a change relative to levels of expression in cells infected with scrambled shRNA virus.[5]

Immunoprecipitation

3T3-L1 adipocytes were treated with 1 mM NEM and then lysed in immunoprecipitation buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 150 mM NaCl, 2 mM β-glycerophosphate, 1 mM DTT, 1% (v/v) Triton X100 and protease inhibitors) and centrifuged at 12,500 xg for 20 min at 4°C. One.5 mg of lysate was precleared using Protein-A beads then incubated with 5 μl anti-syntaxin 16 or random rabbit serum for 2 h on ice. Protein A beads were added for a further 2 h and then separated from unbound material by brief centrifugation. Unbound material was retained for analysis and bound material washed 3 times and eluted using 2 × LSB.

Pull downs with mVps45

Yeast lysates expressing mVps45 were grown to mid-log phase, pelleted, resuspended in 1/100th volume of binding

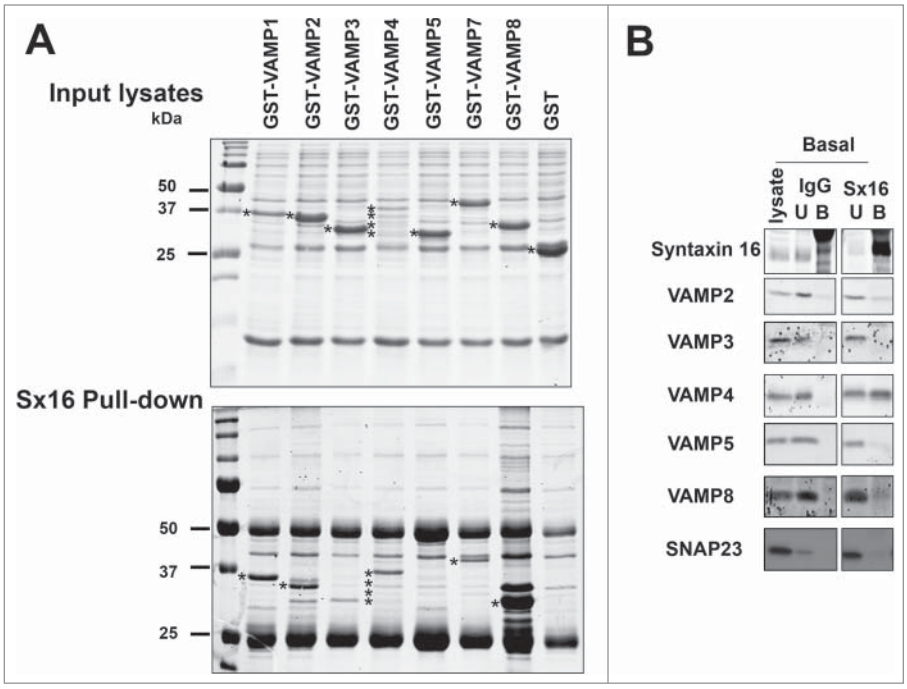

Figure 3. Interactions between Syntaxin 16 and VAMPs *in vitro* and *in vivo*. Panel **A**: GST-VAMP proteins were expressed in bacteria and lysates prepared. Shown in the upper panel are Coomassie Blue stained gels of these lysates, with * identifying the recombinant GST-VAMP species. Protein-A tagged syntaxin 16 was bound to IgG beads and used to capture VAMPs from such lysates. After 2h incubation at 4°C, the beads were recovered, washed and resuspended in 2×LSB. Equal volumes were analyzed on SDS-PAGE ((**A**) lower panel). The bands at 50 and 25 kDa are the heavy and light chains from the IgG beads; * indicates the captured recombinant GST-VAMP. Data from a typical experiment is shown. Panel **B**: Sx16 was immunoprecipitated from a lysate prepared from 3T3-L1 adipocytes incubated with 1 mM NEM and ±1 μM insulin for 30 min. Proteins were immunoprecipitated from 1.5 mg lysate with 5μl anti syntaxin 16 (Sx16) or random rabbit IgG (IgG). Immunoprecipitated proteins were recovered by centrifugation, washed and eluted in 2×LSB. Samples (material bound to beads – B, unbound material – U) were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Shown are data from a typical experiment, molecular marker sizes are shown in kDa.

buffer (40 mM HEPES pH7.4 KOH, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5% (v/v)Triton X 100) then lysed by vortexing with 425-600 μ glass beads. 200 μl lysate was incubated with GST-VAMPs, GST alone, syntaxin16-PrA or PrA alone bound to the appropriate beads in a volume of 1 ml at 4°C with rotation. Lysate was separated from bound material by brief centrifugation, washed and resuspended in 2 × LSB.

Pull downs with Sx16 from bacterial lysates

BL21(DE3) *E. coli* expressing GST or GST-VAMP proteins were induced by 1 mM IPTG and grown overnight at 22°C. The following day, bacteria were harvested, resuspended in PBS, lysed in microfluidiser and incubated with 1 mg/ml lysozyme for 30 min on ice. The lysate was then cleared by centrifugation at 10,000 xg at 4°C for 30 min. In the pull-down experiments, 60 μl of 1:1 IgG bead-PBS slurry containing Sx16-protein A was incubated with 1 ml of cleared VAMP lysate at 4°C for 2 h with rotation. The beads were collected, washed then bound material eluted in 2 × LSB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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