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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.

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.3

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
insulin-responsive GLUT4 translocation.\textsuperscript{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),\textsuperscript{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,\textsuperscript{6,7} such that depletion of mVps45 also destabilizes VAMP; (ii) direct interaction of VAMP2 or 4 with Sx16 (the levels of which also fall upon mVps45 depletion);\textsuperscript{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 Qa-SNARE known to interact with VAMP2 in adipocytes underlies decreased VAMP2 levels.\textsuperscript{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.\textsuperscript{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.\textsuperscript{6-10} Hence, we
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 over-expressing 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 Qa-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. Antibodies against the different VAMP isoforms were all rabbit polyclonal species from Synaptic Systems, Germany; GST-VAMP2 and 3 were as described. 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. 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.

**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 x LSB.

**Pull downs with mVps45**

Yeast lysates expressing mVps45 were grown to mid-log phase, pelleted, resuspended in 1/100th volume of binding...
buffer (40 mM HEPES pH 7.4 KOH, 150 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5% (v/v) Triton X 100) then lysed by vortexing with 425-600 μl 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 for 20 min. 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 x LSB.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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