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Cellular munc18c levels can modulate glucose transport rate and GLUT4 translocation in 3T3L1 cells

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Abstract Munc18c has been shown to bind syntaxin 4 and to play a role in GLUT4 translocation and glucose transport, although this role is as yet poorly defined. In the present study, the effects of modulating the available level of munc18c on glucose transport and GLUT4 translocation were examined. Over-expression of munc18c in 3T3L1 adipocytes inhibited insulin-stimulated glucose transport by approximately 50%. Basal glucose transport rates were also decreased by approximately 25%. In contrast, microinjection of a munc18c polyclonal antibody stimulated GLUT4 translocation by approximately 60% over basal levels without affecting insulin-stimulated GLUT4 levels. Microinjection of a control antibody had no effect. These data are consistent with the likelihood that antibody microinjection sequesters munc18c enabling translocation/fusion of GLUT4 vesicles. Mutagenesis of a potential proline-directed kinase phosphorylation site in munc18c, T569, that in previous studies of its neuronal counterpart munc18a caused its dissociation from its complex with syntaxin 1a, had no effect on munc18c's association with syntaxin 4 or its inhibition of glucose transport, indicative that phosphorylation of this residue is not important for insulin regulation of glucose transport. The over-expression and microinjection sequestration data support an inhibitory role for munc18c on translocation/fusion of GLUT4 vesicles. They further show that altering the level of available munc18c in 3T3L1 cells can modulate glucose transport rates, indicating its potential as a target for therapeutics in diabetes.

Key words: Munc18c; Sec1; Syntaxin 4; SNARE protein; Glucose transport; GLUT4

1. Introduction

Insulin stimulation of glucose transport in insulin-sensitive tissues results predominantly from the translocation of GLUT4 transporters from a pool within tubovesicular membrane structures in the cell, to the cell surface, through a process that shares some similarities with regulated exocytosis (see [1,2] for review). In particular, proteins involved in docking and fusion of exocytic vesicles with the cell surface are present in insulin-sensitive cells and have been implicated in insulin-dependent GLUT4 trafficking [2–4]. These proteins include vesicle associated membrane protein 2 (VAMP2), syntaxin 4 and synaptosome associated protein of 23 kDa (SNAP23), whose counterparts in neural cells comprise the fusogenic machinery for regulated trafficking of neurotransmitter to nerve terminals. Vesicles containing vesicle soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (vesicle SNAREs; VAMP2 in GLUT4 translocation) on their surface interact with their cognate target SNAREs (syntaxin 4 and SNAP23 in GLUT4 translocation) on the plasma membrane in a highly specific manner. This specificity was initially believed to be controlled by cellular compartmentalisation and specificity of the different SNARE proteins involved. However, it was recently demonstrated that non-cognate SNARE proteins could form complexes in vitro and the properties of these complexes were similar regardless of whether the SNARE proteins were closely or distantly related [5]. This implies that specificity and membrane fusion must be controlled by additional events. These events could be mediated through a variety of regulatory proteins including munc18c [5–12], synip [13] and/or GTP binding proteins such as Rab4 [14].

The munc18 family are syntaxin binding proteins and are the mammalian homologues of the yeast Sec1 and *Caenorhabditis elegans* Unc18 proteins, but their role remains largely undefined. They apparently have a critical role in vesicle docking, fusion and/or exocytosis, since null or temperature-sensitive mutations in yeast and/or *Drosophila* homologues markedly inhibit vesicle exocytosis [15–17]. Equally, an inhibitory effect on vesicle fusion is evident from studies that show that over-expression of munc homologues inhibits exocytosis [9,10,18]. Understanding the nature of munc interactions with its binding partners and the processes it regulates will clearly provide further insight into understanding vesicle fusion and/or exocytosis events.

To date three mammalian munc18 homologues have been identified; munc18a, b and c [11,19–21]. Munc18a and b interact with syntaxin 1, 2 and 3. Munc18c interacts with syntaxin 4, the syntaxin homologue shown to be of importance for fusion of GLUT4 vesicles with the plasma membrane, and to a lesser extent syntaxin 2. In neuronal cells, the binding of munc18 to syntaxin prevents syntaxin forming a SNARE complex with VAMP and SNAP25. In 3T3L1 adipocytes, consistent with these data, over-expression of munc18c inhib-

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Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle associated membrane protein; SNAP23, synaptosome associated protein of 23 kDa; BSA, bovine serum albumin; GST, glutathione S-transferase

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its insulin-stimulated glucose transport and GLUT4 translocation [8,10].

Studies with the neuronal munc homologue, munc18a, demonstrated greatly reduced syntaxin binding capacity when phosphorylated by protein kinase C [22], although phosphorylation could not be demonstrated when munc18a was already bound to syntaxin. More recently, cyclin-dependent kinase 5 was found to directly associate with munc18a, phosphorylate T574 and induce disassembly of munc18a from syntaxin: munc18a heterodimer complexes [23]. This phosphorylation thus provides a potential site for regulation of fusion. Interestingly, this site (HILTPQK), and surrounding residues, is conserved in all mammalian homologues of munc18, including munc18c (HILTPRK), indicating that regulation of phosphorylation of this site could be of importance in mediating available levels of munc for regulation of activities such as glucose transport.

In the present study, the nature of munc18c involvement in GLUT4 translocation and glucose transport is investigated by examining the effect of modulating the available level of munc18c. We show that lowering endogenous munc18c levels by microinjection of munc18c antibody stimulated GLUT4 translocation to the plasma membrane. We confirm that over-expression of munc18c inhibits insulin-stimulated glucose transport. We further show that mutagenesis of T569 in munc18c (equivalent to T574 in munc18a) to alanine, serine or glutamic acid fails to affect munc inhibition of glucose transport, indicating that regulation of available munc18c binding activity is unlikely to be manifested through phosphorylation of this site. These data taken together thereby demonstrate that the level of cellular munc18c can modulate glucose transport rate and are of importance for consideration of potential therapeutics for increasing glucose transport rate in the treatment of type 2 diabetes.

2. Materials and methods

2.1. Antibodies

Immunopurified munc18b and 18c rabbit polyclonal antibodies were gifts from Professor David James (University of Queensland, St. Lucia, Qld, Australia). 9E10 anti-c-myc monoclonal antibody was used to detect c-myc-epitope tagged munc18c expression. Glutathione-S-transferase (GST)–syntaxin 4 was detected with a GST polyclonal antibody from Amrad Biotech, Australia.

2.2. Constructs and plasmid preparation

Full-length mouse munc18c (residues 1–592), GenBank accession number U19521; a gift from Professor David James, University of Queensland, St. Lucia, Qld, Australia) was subcloned into pCR Script™ (Stratagene) and a c-myc tag inserted into the N-terminus. Mutagenised constructs (T569A, T569S and T569E) were prepared using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutagenised constructs were subcloned back into munc18cWT (wild type) as *Pst*I/*Bam*HI fragments and the resulting constructs checked by restriction analysis and sequencing. WT and mutant munc18c constructs were subcloned into pLXIN (Clontech) for retroviral infection of 3T3L1 fibroblasts. Each construct was excised out of pCR Script as a blunt-ended *Xho*I/*Bam*HI fragment and subcloned into the *Hpa*I/*Bam*HI sites of pLXIN maintaining the 5′ c-myc tag in munc18c. Correct insertion of the munc18c sequence was checked by restriction enzyme analysis.

2.3. Cell culture

3T3L1 fibroblasts obtained from the American Type Culture Collection (Rockville, MD, USA) were maintained and passaged as preconfluent cultures in Dulbecco's modified Eagle's medium (Sigma) with 5% foetal calf serum (CSL, Australia). Cells were differentiated into adipocytes for study as previously described [4].

2.4. Retroviral infection and transfection

Munc18c constructs were retrovirally infected into 3T3L1 fibroblasts after generation of virus in BOSC23 cells using the replication-incompetent retroviral vector, pLXIN (Clontech). Cells were maintained under G418 selection and used as mixed populations. Infection efficiency ($>70\%$) was monitored with parallel infections using virus from BOSC23 cells transfected with pLEIN (Clontech, pLXIN vector encoding enhanced green fluorescent protein). For experiments, 3T3L1 cells were used in the differentiated state.

2.5. GST-syntaxin 4 purification

A GST fusion protein consisting of the cytoplasmic portion of syntaxin 4, GST–syntaxin 4^{2-273}, was produced in *Escherichia coli* and purified as described previously [6,24].

2.6. Cell lysis, immunoprecipitation and in vitro binding studies

Cells in 55 cm^2 dishes were washed twice with phosphate buffered saline (PBS), pH 7.4, and then lysed by the addition of 800 μl/dish of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer, pH 7.2, containing 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 200 U/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 2 mM phenylmethylsulphonyl fluoride essentially as detailed previously [25]. Dishes were incubated 5 min on ice, the lysate collected, incubated a further 5 min on ice and then clarified by centrifugation at $12\,500\times g$ for 2 min. munc18cWT or mutants in the supernatants were immunoprecipitated by incubation overnight at 4°C with 2 μg/ml purified 9E10 anti-c-myc monoclonal antibody and 20 μl of protein A–Sepharose (Zymed, CA, USA) slurry. The Sepharose beads were pelleted by centrifugation, washed three times in 0.5 ml 20 mM HEPES, pH 7.2, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol (HNTG buffer), and munc18c preparations used for interaction assay with GST–syntaxin 4^{2-273} or for SDS–PAGE and Western transfer. For interaction assay, the immunoprecipitate from one 55 cm^2 dish was incubated for 90 min at 4°C with 10 μg GST–syntaxin 4^{2-273}. The immunoprecipitate was then washed four times with PBS and syntaxin 4 binding to munc18c detected, after SDS–PAGE and Western transfer of the immunoprecipitates, by probing blots with anti-GST polyclonal antibody with enhanced chemiluminescence detection with SuperSignal® (Pierce, IL, USA). In one experimental series, total cell lysates (equivalent of 0.025 of a dish of cells) were analysed by SDS–PAGE, Western transfer and blotting with munc18c polyclonal antibody.

2.7. Microinjection and GLUT4 translocation assay

Our procedure for microinjection of 3T3L1 adipocytes and quantitation of insulin-stimulated GLUT4 translocation by confocal microscopy has been described elsewhere [4,11]. Briefly, cells grown to confluence and differentiated on coverslips were transferred for 45 min to Krebs–Ringer bicarbonate HEPES buffer, pH 7.4, containing 2 mM pyruvate, 0.5% bovine serum albumin (BSA) and 2.5 mM glucose. Antibody in buffer containing 5 mM sodium phosphate (pH 7.2), 100 mM KCl or buffer alone was microinjected over a 45 min period using a Zeiss automated injection system (Carl Zeiss, Germany) coupled to an Eppendorf (Germany) microinjector. Cells were transferred into fresh medium and allowed to recover for 60–90 min following injection, prior to stimulation with 100 nM insulin for 15 min and analysis of GLUT4 translocation using the plasma membrane lawn assay. Plasma membrane GLUT4 levels in microinjected cells were compared to that in non-injected cells in the immediate vicinity on the same coverslip as described previously [26,11]. Briefly, after cell treatment, 3T3L1 cells grown on coverslips were washed sonicated and the membrane fragments immunolabelled with polyclonal rabbit anti-GLUT4 antibody (R1159, 1/100) [27] and either Alexa 488 goat anti-rabbit (Molecular Probes) or CY3-labelled goat anti-rabbit antibody (Amersham, UK). Coverslips were visualised and imaged using a BioRad (USA) Lasersharp MRC-500 confocal laser scanning immunofluorescence microscope or Optiscan F900e Confocal microscope. Data were analysed using either BioRad COMOS confocal imaging software or ImageJ. Efforts were made to maintain confocal microscope gain settings over the period of experiments although alterations in these as well as differences in 3T3L1 populations contribute to between-experiment variability.

2.8. Glucose transport assay

Glucose transport was measured as 2-deoxy-[U-^{14}C]glucose uptake

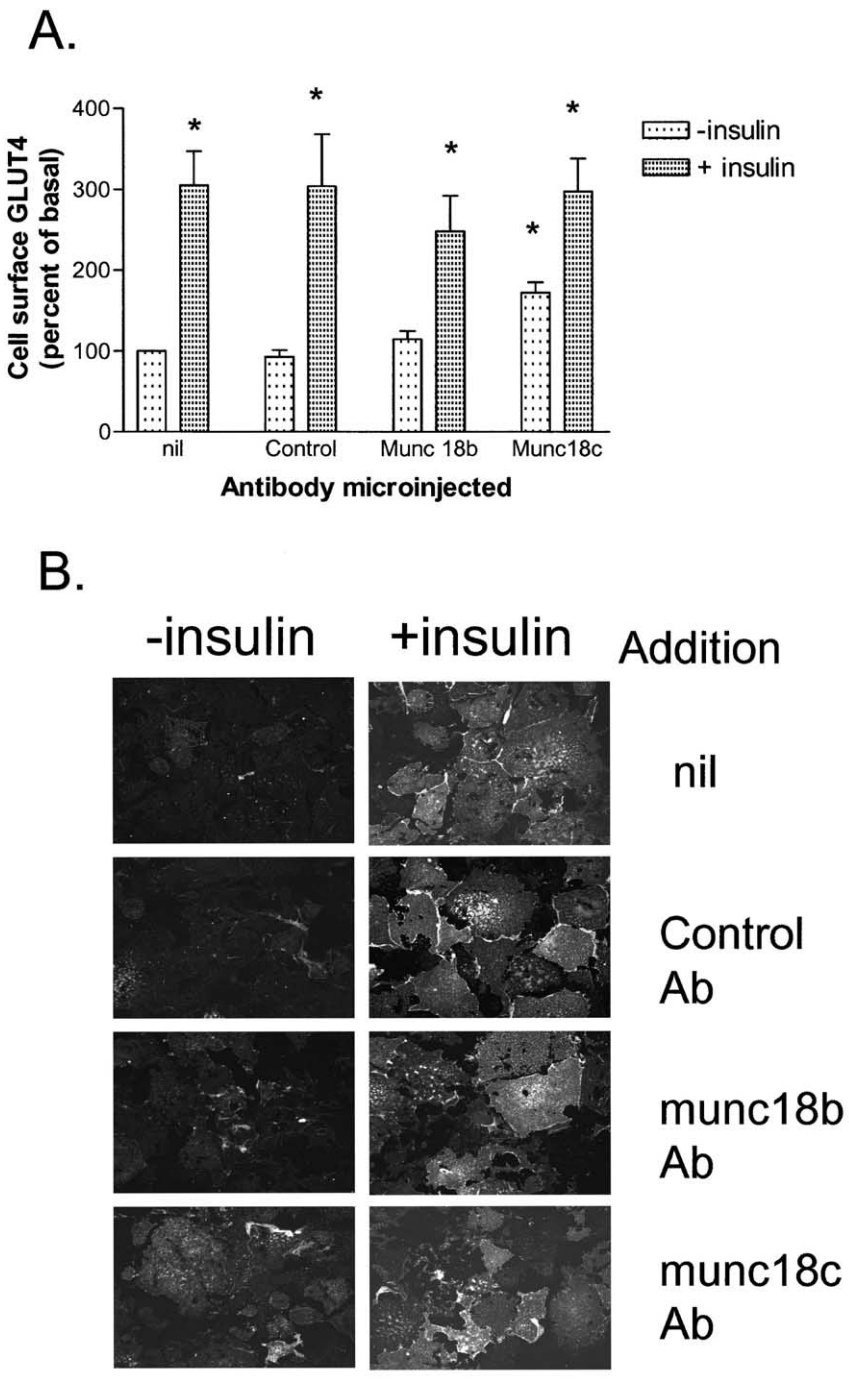

Fig. 1. Microinjection of munc18c antibody stimulates GLUT4 translocation. 3T3L1 adipocytes were preincubated in Krebs–Ringer buffer pH 7.4 for 45 min and then microinjected with buffer, anti-munc18c, anti-munc18b or control antibody as described in Section 2. The cells were transferred to fresh buffer and allowed to recover for 90 min prior to incubation for 15 min in the presence or absence of 100 nM insulin. A: GLUT4 translocation was determined by the plasma lawn assay. Results are expressed as 'percent of basal cell surface GLUT4'. They represent the mean ± S.E.M. of 6–11 experiments in which four or more separate field determinations were analysed within any single experiment. $*P < 0.05$ vs. minus insulin control (repeated measures, analysis of variance). Insulin-stimulated plasma membrane levels of GLUT4 in microinjected cells did not differ significantly from insulin-stimulated cells that had not been microinjected. B: Representative lawns for each condition.

as previously described [28]. Cells at least 48 h post-differentiation in 24-well plates were washed twice in Krebs–Ringer bicarbonate HEPES buffer, 1% BSA, 2 mM pyruvate, containing half the normal Ca (1.15 mM $CaCl_2$), pH 7.4, and then allowed to equilibrate in the incubator for 90 min. Insulin was added over the concentration range 0.7–70 nM for 30 min and 5 mM 2-deoxy-[U-^{14}C]glucose uptake measured over the final 10 min.

3. Results

3.1. Effect of microinjection of anti-munc18c antibodies on GLUT4 translocation to the plasma membrane of 3T3L1 adipocytes

We previously showed, using an antibody microinjection

approach, that syntaxin 4 plays a role in translocation of GLUT4 to the plasma membrane of 3T3L1 adipocytes [11]. In those studies, microinjection of a syntaxin 4 antibody blocked insulin-dependent GLUT4 translocation in this cell type by about 70% over control. A similar approach was used in the present study to assess the effects of disruption of munc18c function on GLUT4 translocation in 3T3L1 adipocytes (Fig. 1). A polyclonal munc18c antibody, described previously [11], was microinjected into 3T3L1 adipocytes prior to stimulation with insulin. The plasma membrane lawn assay was then used to quantitate cell surface GLUT4 levels. We had previously established that microinjection per se did not affect GLUT4 translocation in these cells. Injection of the munc18c antibody increased plasma membrane GLUT4 levels by approximately 60% over control in the absence of insulin ($P < 0.002$), whereas an unrelated control antibody had no effect (Fig. 1). The same effect was seen using a different polyclonal munc18c antibody raised against the C-terminal portion of munc (data not shown). Moreover, the effect observed was specific for munc18c, since an antibody directed against another munc homologue, munc18b, failed to increase the level of GLUT4 at the plasma membrane. The effect of munc18c was not additive to that of insulin since munc18c antibody microinjection failed to stimulate GLUT4 translocation over that caused by insulin addition alone. Thus, interference of munc18c function influences GLUT4 appearance at the plasma membrane.

3.2. *Expression of munc18c and T569 munc18c mutants in 3T3L1 cells*

Since sequestration of munc18c with munc18c antibodies stimulated GLUT4 translocation, it was of interest to assess the effects of over-expression of munc18c in 3T3L1 adipocytes. Additionally, the role of T569 in regulating munc18c binding activity was assessed since phosphorylation of the conserved equivalent residue in munc18a, T574, was found

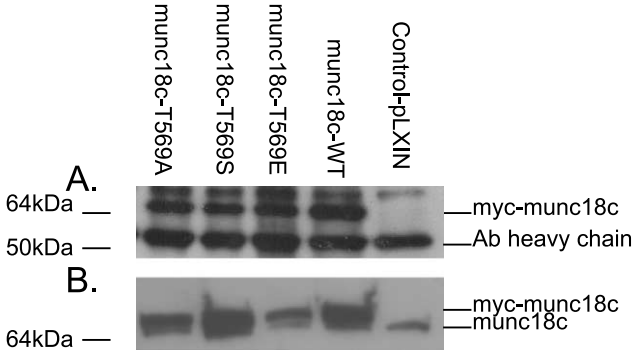

Fig. 2. Western blot analysis of 3T3L1 cells infected with retrovirus encoding munc18cWT and T569 mutations. Cell lysates were prepared from 3T3L1 cells infected with virus encoding munc18cWT, munc18cT569A, munc18cT569S or munc18cT569E. Empty vector (pLXIN) was used as a negative control. A: Munc18c in each lysate was immunoprecipitated overnight at 4°C with 2.5 μg 9E10 anti-myc antibody and 20 μl protein A–Sepharose and analysed by SDS–PAGE, Western transfer and blotting with c-myc antibody, 9E10. The lower band at ~50 kDa represents the heavy chain of the immunoprecipitating antibody. The upper band, c-myc-munc18c, is indicated. B: Total cell lysate from each cell line was analysed by SDS–PAGE, Western transfer and blotting with munc18c polyclonal antibody. The upper band, c-myc-munc18c, is indicated, as is endogenous munc18c running as a fainter band slightly below the myc-tagged protein.

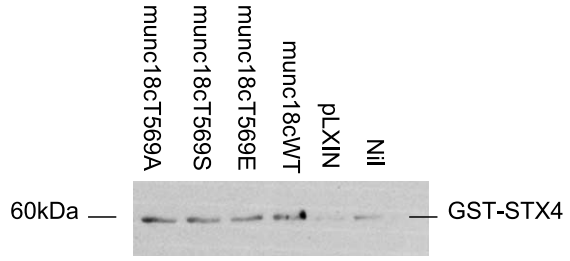

Fig. 3. Munc18cWT and T569 mutants bind to GST–syntaxin 4^{2-273} in vitro. c-Myc-tagged munc18cWT and mutants were extracted from 3T3L1 cells expressing each construct by immunoprecipitation with 9E10 anti-myc antibody. Control analyses included immunoprecipitates from 3T3L1 cells infected with virus packaged from empty vector transfected BOSC cells (second last lane, pLXIN), and 9E10 bound protein A–Sepharose alone (last track, Nil). Each immunoprecipitate was incubated with 10 μg GST–syntaxin 4^{2-273} for 90 min at 4°C. The immunoprecipitates were then washed four times with PBS and syntaxin 4 binding to munc18c detected, after SDS–PAGE and Western transfer of the immunoprecipitates, by probing blots with anti-GST polyclonal antibody.

to cause its dissociation from syntaxin in chromafin cells [23]. We had previously not been able to demonstrate significant phosphorylation of munc18c in 3T3L1 cells (unpublished) but this does not preclude transient phosphorylation as a regulatory mechanism in munc18c activity. We therefore generated 3T3L1 cells over-expressing munc18c, munc18c-T569A, T569S, or T569E, by retroviral infection and selection with neomycin. These cell lines were assessed as pooled populations. Immunofluorescence studies with 9E10 anti-c-myc epitope monoclonal antibody indicated that each mutant had a similar distribution pattern to that of munc18cWT, predominantly cytosolic with additional foci of staining at the periphery (data not shown) similar to the subcellular localisation of wild type munc18c we reported previously [11]. Immunoprecipitation and Western blot analysis with 9E10 antibody confirmed the expression of munc18c and each of the munc18c mutants at the expected molecular weight of approximately 65 kDa (Fig. 2A). The 9E10 antibody heavy chain was also detected at ~50 kDa. Co-precipitation of endogenous syntaxin 4 with munc18c was detected by immunoblotting these immunoprecipitates with a syntaxin 4 antibody, but only weakly (data not shown). Western blots of total cell lysates were probed with munc18c polyclonal antibody (Fig. 2B). Both c-myc epitope–munc18c (upper band) and endogenous munc18c (lighter lower band) were detected in cell lysates from each of the cell lines except for the empty vector transfected cell line that expressed only endogenous munc18c as expected. The c-myc epitope–munc18c ran at a slightly higher molecular weight than endogenous munc18c and was expressed at levels five- to 10-fold higher than endogenous munc18c (Fig. 2B).

To assess the binding of syntaxin 4 to each of the munc18c mutants, munc18c proteins isolated from 3T3L1 adipocytes by immunoprecipitation with 9E10 were incubated with a recombinant GST-fusion protein encompassing the cytoplasmic portion of syntaxin 4 (GST–syntaxin 4^{2-273}). GST–syntaxin 4^{2-273} association with the immunoprecipitated c-myc-munc18c was analysed after extensive washing by SDS–PAGE, transfer to nitrocellulose and immunoblotting with a GST antibody (Amrad Biotech, Australia) (Fig. 3). GST–syntaxin 4^{2-273} bound each of the constructs approximately equivalently. No GST–syntaxin 4^{2-273} binding was detected

to the extract from the vector control or to protein A–Sepharose alone. There was also no binding of GST to munc18c (data not shown) as demonstrated in our earlier studies [6]. Interestingly, pre-incubation of the c-myc-munc18c cell lysates with polyclonal munc18c antibody prior to immunoprecipitation and GST–syntaxin 4^{2-273} binding failed to markedly affect its binding to c-myc epitope–munc18c (data not shown), suggesting the binding epitopes lie in regions outside the syntaxin binding region of munc18c.

3.3. Effects of over-expression of munc18c mutants on glucose transport and GLUT4 translocation

The effect of over-expression of each of the munc18c mutants on glucose transport in 3T3L1 cells was examined over a range of insulin concentrations (Fig. 4). Insulin caused an approximately four-fold stimulation of glucose transport in both 3T3L1 cells and 3T3L1 cells infected with virus from BOSC23 cells transfected with the empty vector. Insulin-stimulated glucose transport in cells transfected with c-myc-tagged munc18c was only half that of non-infected or vector alone infected cells at all concentrations of insulin tested. Basal glucose transport rate was also lower than empty vector infected or non-infected cells. Insulin stimulation of glucose transport and basal glucose transport rates for each of the mutant munc18c-expressing cell lines were similar to that of wild type munc18c-expressing cell line, consistent with the lack of effect of these mutations on the binding to syntaxin seen in the initial studies.

The effects of insulin on glucose transport are manifested primarily through the translocation of GLUT4 to the cell surface. The effects of over-expression of each of the munc mutants in 3T3L1 adipocytes on GLUT4 translocation were therefore determined at maximal insulin concentration (70 nM) (Fig. 5). Insulin stimulated GLUT4 translocation more than four-fold in 3T3L1 adipocytes from cells infected with virus from BOSC cells transfected with pLXIN vector alone. Cells expressing c-myc epitope–munc18c, and each of

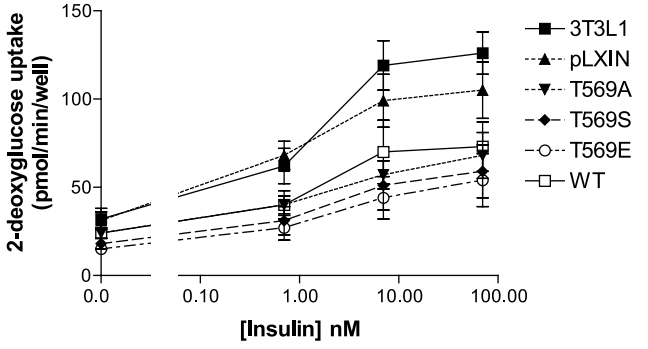

Fig. 4. Effect of insulin on glucose transport in 3T3L1 adipocytes expressing munc18cWT and T569 mutations. 3T3L1 cells expressing munc18cWT or each of the munc18cT569 mutations were incubated in the presence or absence of the indicated concentrations of insulin for 20 min prior to the measurement of 2-deoxy[U-^{14}C]glucose uptake over the following 10 min. Results are expressed as the mean ± S.E.M. from six to seven independent experiments in which three independent cell incubations were performed within each experiment for each point. In each case, insulin significantly stimulated glucose transport at the lowest concentration tested, 0.07 nM, $P < 0.05$. Cells over-expressing munc18cWT or the T569 mutations showed a significantly impaired response to insulin compared to 3T3L1 cells at all concentrations, $P < 0.05$. Empty vector pLXIN infected cells were not different from non-infected 3T3L1 cells.

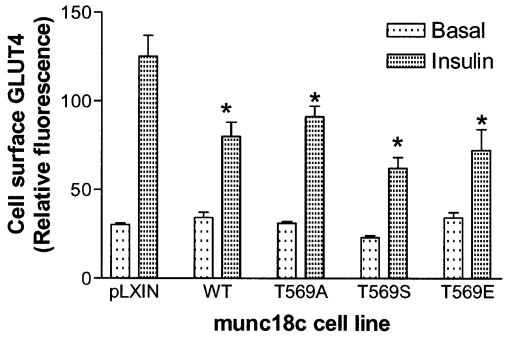

Fig. 5. Effect of insulin on GLUT4 translocation in 3T3L1 adipocytes expressing munc18cWT and T569 mutations. 3T3L1 cells expressing munc18cWT or each of the munc18cT569 mutations were incubated in the presence or absence of 70 nM insulin for 20 min prior to the measurement of plasma membrane GLUT4 levels as described in Section 2. Results are mean ± S.E.M. of five or more fields within the same experiment. *$P < 0.05$ vs. insulin-stimulated pLXIN empty vector infected cells. Similar results were obtained in two separate experiments.

the munc18c mutants, had diminished responses to insulin of 35–65% in the assay shown, consistent with the effects of insulin on glucose transport shown in Fig. 4.

4. Discussion

In this study we show that modulating the level of munc18c in 3T3L1 adipocytes can alter glucose transport rate and GLUT4 translocation. Over-expression of munc18c inhibited glucose transport, confirming previous reports [8,10,29]. Microinjection of munc18c antibody into 3T3L1 cells, which we infer depletes available munc18c, stimulated GLUT4 translocation. The modulation of glucose transport and GLUT4 translocation by manipulating munc18c protein levels supports a potential regulatory role for munc18c protein levels in mediating fusion/translocation of GLUT4 vesicles to the cell surface. This activity of munc does not appear to be mediated through phosphorylation of T569 since munc18c mutants lacking the phosphorylation site, munc18cT569A, or a mutant designed to mimic phosphorylation with respect to size and charge, munc18cT569E, failed to affect syntaxin 4 binding to munc18c. Consistent with these data, over-expression of each of these mutants inhibited insulin-stimulated glucose transport and GLUT4 translocation to a similar extent as over-expression of wild type munc18c.

The lack of effect of the munc18c mutations on syntaxin 4 binding and on munc inhibition of glucose transport is perhaps surprising given previous findings [23,30], demonstrating that munc18a phosphorylation at the equivalent site displaced syntaxin 1a binding and the fact that sequences of munc isoforms are highly conserved. However, a number of observations make it unlikely that munc18c binds syntaxin 4 through an interaction encompassing T569. First, the crystal structure of the munc18a/syntaxin 1 complex shows no contact in this region [31]. Structural similarity between munc18c and munc18a would be expected since the alignment of munc18c with munc18a is similar throughout (51% identity) and particularly in the region of T569 [12]. Second, we previously showed that residues 1–139 of munc18c are sufficient for binding to syntaxin 4 [6], consistent with the structure reported for syntaxin 1 binding to munc18a. The major sites in munc18a

for interaction with syntaxin 1 were found to be located in domain 1 (residues 4–134) (similar to our findings for munc18c) and some residues in domain 3 [31]. In this structure, syntaxin 1a adopts a closed conformation with its N-terminus interacting with its C-terminus. Removal of munc may allow syntaxin to adopt an open conformation for binding to SNAP25 and VAMP2, thus enabling membrane fusion. However, free syntaxin has been shown to adopt a closed conformation in solution similar to its munc-bound form [32]. The T569 site is buried within domain 2, away from this syntaxin-binding domain. Thus, if important, T569 could represent a site of interaction for other effectors such as Rab proteins. Supporting a role for this region is the finding that a C-terminal 20 amino acid munc18a peptide stimulated insulin secretion from HIT cells [33]. These authors suggested that the peptide potentially disrupted a Rab protein interaction. Our studies do not rule out a potential effect of phosphorylation of T569 to affect the binding of a munc effector such as a Rab to potentiate fusion since the inhibitory effects of munc overexpression would be expected to dominate. However, the data show that modification of this residue is unlikely to directly affect syntaxin binding.

The stimulation of GLUT4 appearance at the plasma membrane by munc18c antibody microinjection is consistent with the possibility that the antibody caused dissociation of the munc18c:syntaxin 4 complex, freeing syntaxin to interact with SNAP23 and VAMP2 to enable fusion. A previous study in HIT cells demonstrated that administration of a munc18a antibody stimulated insulin release [33], consistent with our studies and this interpretation for munc18c. These findings are important since they indicate that manipulating munc18c availability can both positively and negatively affect glucose transport. An alternative possibility is that the antibody binding altered the conformation of munc18c making it competent for fusion. This possibility is unlikely, given that one antibody used was directed toward the C-terminal amino acids and therefore a small epitope. Further, reports of conformational change induced by antibodies in homeotypic protein interactions are rare.

In addition to the inhibitory effect of munc on SNARE complex formation suggested above, munc has also been ascribed an essential chaperone role for syntaxin and a further potentiating role in fusion. Deletion of munc homologues in yeast or mammalian cells resulted in a block of vesicle transport [34,35]. Deletion of the yeast munc homologue, Vps45p, was furthermore shown to cause rapid proteosomal degradation of its target SNARE, Tlg2p [35]. Active participation of munc homologues in the fusion process has also been suggested [8,35,36]. Thurmond et al. [8] showed that introduction into 3T3L1 cells of a peptide corresponding to the predicted exteriorised loop between domains 2 and 3 of munc made cells competent to translocate GLUT4 following an insulin stimulus without enabling fusion. In this case, GLUT4 aligned in storage vesicles just below the plasma membrane. Similarly, Dresbach et al. [36] showed that microinjection of C-terminal portion of squid sec1 into squid axons led to the accumulation of docked synaptic vesicles but prevention of fusion.

The antibody results reported here for GLUT4, and for insulin secretion previously [33], appear incompatible with the suggested active role for munc on fusion. How might these seemingly opposing or unrelated effects be manifested? It is likely that munc is required for optimal fusion but it is also clear that fusion can proceed in the absence of munc from in vitro data using purified SNARE proteins in micelles [5]. The antibody data presented here and previously [33] are consistent with the possibility that removal of munc from predocked vesicles can enable fusion. It is possible that munc peptides described above interact with an effector such as a Rab that normally alters the conformation of munc, making it competent for fusion, perhaps priming syntaxin for fusion. Without this interaction munc may remain bound to syntaxin and fusion-incompetent, resulting in vesicles accumulating below the cell surface. It is possible that antibody microinjection sequesters munc, enabling fusion of vesicles in proximity to the plasma membrane to proceed. Consistent with this, electron microscopy has demonstrated that a significant proportion of GLUT4 vesicles lie in close proximity to the cell surface [37]. It is clear that only when the nature of the additional munc interacting proteins are defined, including how they impact syntaxin binding, will the complex role of munc in fusion be understood.

In conclusion, our data show that manipulation of the level of munc18c in cells modulates glucose transport. Over-expression of munc18c inhibits glucose transport in 3T3L1 cells whereas reduced expression, by sequestering munc18c with an antibody, stimulates glucose transport. Whether phosphorylation plays a role in the regulation of munc activity remains to be addressed but our studies do not support a direct role for proline-directed kinase phosphorylation of the T569 site affecting the interaction of munc18c with syntaxin 4. Our studies clearly support a regulatory role for munc18c in glucose transport and indicate available protein level may be involved in this regulation.

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References

[1] Jahn, R. and Sudhof, T.C. (1999) Annu. Rev. Biochem. 68, 863–911.
[2] Bryant, N.J., Govers, R. and James, D.E. (2002) Nat. Rev. Mol. Cell Biol. 3, 267–277.
[3] Pessin, J.E., Thurmond, D.C., Elmendorf, J.S., Coker, K.J. and Okada, S. (1999) J. Biol. Chem. 274, 2593–2596.
[4] Macaulay, S.L., Hewish, D.R., Gough, K.H., Stoichevska, V., MacPherson, S.F., Jagadish, M. and Ward, C.W. (1997) Biochem. J. 324, 217–224.
[5] Fasshauer, D., Antonin, W., Margittai, M., Pabst, S. and Jahn, R. (1999) J. Biol. Chem. 274, 15440–15446.
[6] Grusovin, J., Stoichevska, V., Gough, K.H., Nunan, K., Ward, C.W. and Macaulay, S.L. (2000) Biochem. J. 350, 741–746.
[7] Thurmond, D.C. and Pessin, J.E. (2000) EMBO J. 19, 3565–3575.
[8] Thurmond, D.C., Kanzaki, M., Khan, A.H. and Pessin, J.E. (2000) Mol. Cell Biol. 20, 379–388.
[9] Thurmond, D.C., Ceresa, B.P., Okada, S., Elmendorf, J.S., Coker, K. and Pessin, J.E. (1998) J. Biol. Chem. 273, 33876–33883.
[10] Tamori, Y., Kawanishi, M., Niki, T., Shinoda, H., Araki, S., Okazawa, H. and Kasuga, M. (1998) J. Biol. Chem. 273, 19740–19746.
[11] Tellam, J.T., Macaulay, S.L., McIntosh, S., Hewish, D.R., Ward, C.W. and James, D.E. (1997) J. Biol. Chem. 272, 6179–6186.
[12] Tellam, J.T., McIntosh, S. and James, D.E. (1995) J. Biol. Chem. 270, 5857–5863.
[13] Min, J., Okada, S., Kanzaki, M., Elmendorf, J.S., Coker, K.J., Ceresa, B.P., Syu, L.J., Noda, Y., Saltiel, A.R. and Pessin, J.E. (1999) Mol. Cell 3, 751–760.

[14] Li, L., Omata, W., Kojima, I. and Shibata, H. (2002) J. Biol. Chem. 276, 5265–5273.
[15] Wu, M.N., Littleton, J.T., Bhat, M.A., Prokop, A. and Bellen, H.J. (1998) EMBO J. 17, 127–139.
[16] Harrison, S.D., Broadie, K., van de Goor, J. and Rubin, G.M. (1994) Neuron 13, 555–566.
[17] Hosono, R., Hekimi, S., Kamiya, Y., Sassa, T., Murakami, S., Nishiwaki, K., Miwa, J., Taketo, A. and Kodaira, K.I. (1992) J. Neurochem. 58, 1517–1525.
[18] Misura, K.M., May, A.P. and Weis, W.I. (2000) Curr. Opin. Struct. Biol. 10, 662–671.
[19] Tellam, J.T., James, D.E., Stevens, T.H. and Piper, R.C. (1997) J. Biol. Chem. 272, 6187–6193.
[20] Hata, Y. and Sudhof, T.C. (1995) J. Biol. Chem. 270, 13022–13028.
[21] Pevsner, J., Hsu, S.C. and Scheller, R.H. (1994) Proc. Natl. Acad. Sci. USA 91, 1445–1449.
[22] Fujita, Y., Sasaki, T., Fukui, K., Kotani, H., Kimura, T., Hata, Y., Sudhof, T.C., Scheller, R.H. and Takai, Y. (1996) J. Biol. Chem. 271, 7265–7268.
[23] Fletcher, A.I., Shuang, R., Giovannucci, D.R., Zhang, L., Bittner, M.A. and Stuenkel, E.L. (1999) J. Biol. Chem. 274, 4027–4035.
[24] Jagadish, M.N., Fernandez, C.S., Hewish, D.R., Macaulay, S.L., Gough, K.H., Grusovin, J., Verkuylen, A., Cosgrove, L., Alafaci, A., Frenkel, M.J. and Ward, C.W. (1996) Biochem. J. 317, 945–954.
[25] Lammers, R., Gray, A., Schlessinger, J. and Ullrich, A. (1989) EMBO J. 8, 1369–1375.
[26] Marsh, B.J., Alm, R.A., McIntosh, S.R. and James, D.E. (1995) J. Cell Biol. 130, 1081–1091.
[27] Kelada, A.S., Macaulay, S.L. and Proietto, J. (1992) J. Biol. Chem. 267, 7021–7025.
[28] Olefsky, J.M. (1978) Biochem. J. 172, 137–145.
[29] Khan, A.H., Thurmond, D.C., Yang, C., Ceresa, B.P., Sigmund, C.D. and Pessin, J.E. (2001) J. Biol. Chem. 276, 4063–4069.
[30] Shuang, R., Zhang, L., Fletcher, A., Groblewski, G.E., Pevsner, J. and Stuenkel, E.L. (1998) J. Biol. Chem. 273, 4957–4966.
[31] Misura, K.M., Scheller, R.H. and Weis, W.I. (2000) Nature 404, 355–362.
[32] Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T.C. and Rizo, J. (1999) EMBO J. 18, 4372–4382.
[33] Zhang, W., Efanov, A., Yang, S.N., Fried, G., Kolare, S., Brown, H., Zaitsev, S., Berggren, P.O. and Meister, B. (2000) J. Biol. Chem. 275, 41521–41527.
[34] Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H.J. and Sudhof, T.C. (2000) Science 287, 864–869.
[35] Bryant, N.J. and James, D.E. (2001) EMBO J. 20, 3380–3388.
[36] Dresbach, T., Burns, M.E., O'Connor, V., DeBello, W.M., Betz, H. and Augustine, G.J. (1998) J. Neurosci. 18, 2923–2932.
[37] Slot, J.W., Geuze, H.J., Gigengack, S., Lienhard, G.E. and James, D.E. (1991) J. Cell Biol. 113, 123–135.