Insulin-triggered repositioning of munc18c on syntaxin-4 in GLUT4 signalling

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One of the most important actions of insulin is the stimulation of the uptake of glucose into fat and muscle cells. Crucial to this response is the translocation of GLUT4 (glucose transporter-4) to the plasma membrane. The insulin-stimulated GLUT4 vesicle docking at the plasma membrane requires an interaction between VAMP-2 (vesicle-associated membrane protein-2) on the GLUT4 vesicle and syntaxin-4 in the plasma membrane. In the basal state, munc18c is thought to preclude GLUT4 vesicle docking by inhibiting this interaction. Here, we have used FCS (fluorescence correlation spectroscopy) in single living cells to show that munc18c binds to syntaxin-4 in both the basal and insulin-stimulated states. We show that munc18c contains two binding sites for syntaxin-4, one of which is disrupted by insulin, while the other is activated by insulin. Insulin-triggered repositioning of munc18c on syntaxin-4 in this way in turn allows syntaxin-4 to adopt its ‘open’ conformation and bind VAMP-2, resulting in the docking of the GLUT4 vesicle at the cell surface. The results also demonstrate the utility of using FCS in intact single living cells to elucidate cell signalling events.

Key words: fluorescence correlation spectroscopy (FCS), glucose transporter-4 (GLUT4), glucose uptake, insulin, munc18c, vesicle-associated membrane protein (VAMP).

INTRODUCTION

The most important action of insulin is the regulation of glucose homoeostasis. Activation of PKCζ (protein kinase Cζ) and PKB (protein kinase B; also called Akt) leads to the translocation of GLUT4 (glucose transporter-4) from an intracellular vesicle to the plasma membrane. The GLUT4 vesicle contains the v-SNARE [vesicle SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor)] VAMP-2 (vesicle-associated membrane protein 2) and membrane binding requires interaction with the t-SNARE (target membrane SNARE) syntaxin-4. In the basal state, the v- and t-SNARE interaction is precluded by accessory proteins such as munc18c. In unstimulated cells, the association between munc18c and syntaxin-4 is stronger than that between VAMP-2 and syntaxin-4. This blocks the GLUT4 vesicle from docking to the plasma membrane. Upon insulin stimulation, the situation reverses, the association between munc18c and syntaxin-4 weakens considerably and is out-competed by the syntaxin-4 VAMP-2 interaction and thus the GLUT4 vesicle docks at the cell surface. However, the details of this mechanism, whereby insulin weakens the syntaxin-4–munc18c interaction, are poorly understood [1–4]. The present study utilized FCS (fluorescence correlation spectroscopy) to investigate interactions between munc18c and syntaxin-4 in intact living cells. FCS analysis indicated that munc18c did not dissociate from syntaxin-4 in response to insulin. Rather, insulin triggered the repositioning of munc18c on syntaxin-4 via an additional binding site, thus allowing VAMP-2–syntaxin-4 interaction and GLUT4 vesicle docking at the plasma membrane.

EXPERIMENTAL

Plasmid constructs

The full-length syntaxin-4 clone was supplied in a pCMVSPORT 6 vector (Invitrogen). The full-length syntaxin-4 sequence was amplified by PCR using Pfu DNA polymerase and the sequence-specific forward primer 5′-ATGCCGCAGAGCCAC-3′ and reverse primer 5′-TGTTATTGCAACCTATT-3′. The DNA product from this reaction was then used as a template for further amplification by PCR using Pfu DNA polymerase and the forward primer (containing the EcoRI restriction site) 5′-GCGTGAATTCTAGCGGCAGACAGG-3′ and reverse primer (containing the KpnI restriction site) 5′-GTGATATGCCAACTACATGCTGG-3′. Other constructs were described by Hodgkinson et al. [5,6]. Digestion of the PCR products by EcoRI and KpnI then allowed ligation in frame with the EYFP (enhanced yellow fluorescent protein) epitope of the pEYFP-N2 vector (BD Biosciences).

Transient transfection

CHO (Chinese-hamster ovary; CHO-K1) cells were purchased from A.T.C.C. (A.T.C.C. number CCL-61; Manassas, VA, U.S.A.) and maintained at 37°C and 5% CO₂ on 25 cm² tissue culture dishes in HAM-12 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) glucose. CHO cells were transfected using Polyfect transfection reagent (Qiagen) as per the manufacturer’s instructions and serum-starved 24 h prior to FCS measurements. 3T3-L1 adipocytes were obtained and cultured as described previously [7,8]. For electroporation, 3T3-L1 adipocytes were detached from culture dishes by incubation with 0.25% trypsin (0.5 mg/ml), and 5 × 10⁶ cells were mixed with 5 μg of plasmid in the solution provided for the cell line Nucleofector kit V (Amaxa, Cologne, Germany). The plasmid was then introduced into the 3T3-L1 adipocytes by electroporation with the use of a Nucleofector (Amaxa) instrument according to the T-20 program. For immunoblotting, cells were extracted into 200 μl of lysis buffer [62.5 mM Tris, pH 7.4, and 1% (w/v) SDS].
FCS and confocal microscopy

FCS and confocal microscopy were carried out with a Leica × 63 water-immersion objective and Leica SP2 confocal laser-scanning microscope (Leica). Samples were excited with a 514 nm Ar/Kr laser at low-average laser power. Cells were first washed twice with 1 ml of prewarmed Krebs–Ringer buffer (10 mM Heps, pH 7.4, 136 mM NaCl, 4.7 mM KCl and 1.25 mM MgSO4). Cells were then incubated with 2 ml of the same buffer. A prebleaching illumination period of 160 s was used prior to data collection. Correlation curves of 600 s were collected from cells and traces were analysed with the Leica FCS software. Experiments were performed at room temperature to reduce the mobility of the cells. When required, cells were stimulated with 100 nM insulin in Krebs–Ringer buffer for 20 min. Data were collected from six individual cells both before and after insulin stimulation. Values are expressed as means ± S.E.M. The presence of the overexpressed proteins was confirmed by detection of the fluorescence by microscopy and subsequent Western blotting of the cell extract (SySy; results not shown).

GST (glutathione transferase) pull-down protocol

BL21 cells were used to express the GST-tagged syntaxin-4 by using a standard protocol supplied by Amersham Biosciences. BL21 cells overexpressing GST-tagged syntaxin-4 were lysed using Bugbuster (Novagen) and clarified extracts were incubated with glutathione beads (Amersham Biosciences). After several washes with PBS, the glutathione bead syntaxin-4 complex was exposed to cell extracts prepared from CHO cells overexpressing Xpress-tagged munc18c constructs. After 4 h of constant agitation at 4 °C, complexes were washed three times with PBS and then resuspended in a small volume of PBS.

Immunoprecipitation

Cells were extracted into lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl and 1 % (v/v) Sigma Protease and Phosphatase Inhibitor cocktails] to give a 500 µl final volume (500 µg). Co-immunoprecipitation was carried out by incubating 500 µg of cell lysate with a monoclonal Xpress antibody (5 µl; Invitrogen) and Protein G–Protein A beads (50:50; 20 µl; Sigma). After 5 h continuous gentle agitation at 4 °C, the beads were collected by pulse spin and then washed three times with lysis buffer, after which they were resuspended in PBS.

Immunoblotting

Samples were resolved by SDS/PAGE [7,8] and transferred to a nitrocellulose filter. The membranes were probed with various primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Primary antibodies [GST (NEB), munc18c (BD Biosciences), Living Colours antibody (SySy), syntaxin-4 (SySy), Xpress (Invitrogen) and 80K-H (BD Biosciences)] were used according to the manufacturer’s instructions. Blots were developed using the ECL® system according to the manufacturer’s instructions (Amersham Biosciences).

RESULTS

The FCS results from the present study were fitted using a three-dimensional Gaussian triplet calculation (Leica software) and the $\tau_{D1}$ value was calculated in microseconds. $\tau_{D1}$ represents the average time the EYFP-tagged construct spends in the observation volume and thus can be called a diffusion coefficient. A larger diffusion coefficient corresponds to a longer time spent in the observation volume and hence relates to a slower-moving molecule. Knowledge of the average speed of the protein of interest in vivo provides valuable insights into its behaviour.

To investigate insulin-triggered interactions between munc18c, syntaxin-4 and other proteins in vivo within intact cells, one or two of the proteins, or non-binding constructs, were expressed. In all experiments, cells were serum-starved 24 h prior to data collection and were bleached for 120 s prior to data collection in order to provide optimal signal-to-noise ratio for FCS (this typically being 10–100 fluorescent molecules in the focal volume). Levels of the constructs were determined by immunoblotting. Analysis of the immunoblots showed that expression levels were similar for all the constructs used in the present study (results not shown).

Table 1: Action of insulin on the diffusion coefficients of munc18c and syntaxin-4 in CHO cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>No Insulin</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFL</td>
<td>526 ± 41</td>
<td>592 ± 78</td>
</tr>
<tr>
<td>MFL Syntaxin-4</td>
<td>795 ± 69*</td>
<td>804 ± 61*</td>
</tr>
<tr>
<td>M295</td>
<td>576 ± 64</td>
<td>489 ± 36</td>
</tr>
<tr>
<td>M295 Syntaxin-4</td>
<td>619 ± 34</td>
<td>821 ± 32*</td>
</tr>
<tr>
<td>Syntaxin-4</td>
<td>459 ± 54</td>
<td>491 ± 71</td>
</tr>
<tr>
<td>Syntaxin-4 MFL</td>
<td>720 ± 59*</td>
<td>789 ± 88*</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with absence of the interactor. MFL, munc18c full-length; M295, munc18c 295.

Table 2: Action of insulin on the diffusion coefficients of munc18c and syntaxin-4 in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Construct</th>
<th>No Insulin</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFL</td>
<td>480 ± 25</td>
<td>507 ± 17</td>
</tr>
<tr>
<td>MFL Syntaxin-4</td>
<td>770 ± 71*</td>
<td>772 ± 27*</td>
</tr>
<tr>
<td>M295</td>
<td>501 ± 69</td>
<td>561 ± 58</td>
</tr>
<tr>
<td>M295 Syntaxin-4</td>
<td>493 ± 62</td>
<td>748 ± 57*</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with absence of the interactor. MFL, munc18c full-length; M295, munc18c 295.

The indicated EYFP-tagged constructs were expressed in the presence or absence of the indicated interactor. Cells were serum-starved for 24 h and stimulated with or without 10−7 M insulin for 20 min. FCS data were collected and diffusion coefficients were measured (microseconds). Results are the means ± S.E.M. for three separate experiments each using six separate cells.

Inhibitor cocktails to give a 500 µl final volume (500 µg). Co-immunoprecipitation was carried out by incubating 500 µg of cell lysate with a monoclonal Xpress antibody (5 µl; Invitrogen) and Protein G–Protein A beads (50:50; 20 µl; Sigma). After 5 h continuous gentle agitation at 4 °C, complexes were washed three times with PBS and then resuspended in PBS.

Insulin did not affect the $\tau_{D1}$ value of the EYFP-tagged full-length munc18c construct when expressed alone (Tables 1 and 2). When the EYFP-tagged munc18c construct was co-expressed with syntaxin-4, the diffusion coefficient of EYFP-tagged full-length munc18c was markedly increased compared with the single transfection both before and after insulin stimulation (Tables 1 and 2). The effects of co-expressing syntaxin-4 were highly statistically significant, with P values of <0.01, and indicate that munc18c binds syntaxin-4 both in the presence and absence of insulin. Example FCS traces are shown in Figure 1.
Insulin stimulation did not shift the curve. The original FCS traces are shown.

When full-length munc18c was employed, the diffusion coefficient of the EYFP-tagged munc18c 295 construct; this corresponds to a reduction in the speed of the munc18c construct (Tables 3 and 4). As with the EYFP-tagged munc18c 295, which lacks the N-terminal 295 amino acids. GST pull-down assays in our laboratory showed that this construct is unable to bind to syntaxin-4 when expressed in the non-insulin-responsive Cos-1 cell line (results not shown). This is in accordance with other published data [9] that showed that the N-terminal 139 amino acids of munc18c bind to syntaxin-4. The diffusion coefficient for the EYFP-tagged munc18c 295 was not significantly affected by insulin stimulation (Tables 1 and 2). The co-expression of syntaxin-4 with EYFP-tagged munc18c 295 had no statistically significant effect on the diffusion coefficient of the EYFP-tagged munc18c 295 construct in the basal state. In contrast, insulin stimulation caused a major increase in the diffusion coefficient of the EYFP-tagged munc18c 295 construct (Tables 1 and 2; Figure 1). This effect was highly significant with a P value of <0.01 and indicated that munc18c 295 only binds syntaxin-4 in the insulin-stimulated state. These results suggest that munc18c has two binding sites for syntaxin-4. One of them lies between residues 1 and 295, as has been shown previously, and the other lies between residues 295 and 592 and binds syntaxin-4 in the presence of insulin.

To further confirm that insulin was working in the cells and to help validate the FCS approach, the interaction of munc18c with 80K-H was analysed. 80K-H is believed to be an upstream regulator of munc18c that is crucial for delivering an insulin signal to the GLUT4 translocation machinery [5,6]. Co-expression of EYFP-tagged full-length munc18c with 80K-H resulted in insulin eliciting a major increase in the mobility of the full-length EYFP-tagged munc18c of approx. 2-fold (P < 0.01; Tables 3 and 4). To test whether this effect was dependent on the ability of 80K-H to bind munc18c, a truncation construct of munc18c, namely EYFP-tagged munc18c 338, was employed. This construct is unable to bind 80K-H and inhibits insulin-stimulated GLUT4 translocation to a greater extent than full-length munc18c. As with the EYFP-tagged full-length munc18c, insulin did not affect the diffusion coefficient of EYFP-tagged munc18c 338 when expressed alone. Importantly, when EYFP-tagged munc18c 338 and 80K-H were co-expressed, insulin was no longer able to trigger a change in the speed of the munc18c construct (Tables 3 and 4). As a control, a less truncated version of munc18c was employed,

Table 3 Insulin increases the mobility of munc18c in an 80K-H-dependent manner in CHO cells

<table>
<thead>
<tr>
<th>Effect of insulin on mobility</th>
<th>EYFP</th>
<th>80K-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>80K-H</td>
<td>No 80K-H</td>
<td></td>
</tr>
<tr>
<td>MFL</td>
<td>0.83 ± 0.07</td>
<td>1.75 ± 0.18*</td>
</tr>
<tr>
<td>M295</td>
<td>1.1 ± 0.01</td>
<td>1.96 ± 0.27*</td>
</tr>
<tr>
<td>M338</td>
<td>0.83 ± 0.06</td>
<td>0.98 ± 0.06</td>
</tr>
</tbody>
</table>

markedly increased both before and after insulin stimulation (Table 1). These increases are virtually identical with those for the EYFP-tagged full-length munc18c construct in the EYFP-tagged full-length munc18c and syntaxin-4 transfections. Again, insulin had no effect on the diffusion coefficients.

To further confirm the binding of munc18c to syntaxin-4, we employed a truncation construct of munc18c, namely munc18c 295, which lacks the N-terminal 295 amino acids. GST pull-down assays in our laboratory showed that this construct is unable to bind to syntaxin-4 when expressed in the non-insulin-responsive Cos-1 cell line (results not shown). This is in accordance with other published data [9] that showed that the N-terminal 139 amino acids of munc18c bind to syntaxin-4. The diffusion coefficient for the EYFP-tagged munc18c 295 was not significantly affected by insulin stimulation (Tables 1 and 2). The co-expression of syntaxin-4 with EYFP-tagged munc18c 295 had no statistically significant effect on the diffusion coefficient of the EYFP-tagged munc18c 295 construct in the basal state. In contrast, insulin stimulation caused a major increase in the diffusion coefficient of the EYFP-tagged munc18c 295 construct (Tables 1 and 2; Figure 1). This effect was highly significant with a P value of <0.01 and indicated that munc18c 295 only binds syntaxin-4 in the insulin-stimulated state. These results suggest that munc18c has two binding sites for syntaxin-4. One of them lies between residues 1 and 295, as has been shown previously, and the other lies between residues 295 and 592 and binds syntaxin-4 in the presence of insulin.

To further confirm that insulin was working in the cells and to help validate the FCS approach, the interaction of munc18c with 80K-H was analysed. 80K-H is believed to be an upstream regulator of munc18c that is crucial for delivering an insulin signal to the GLUT4 translocation machinery [5,6]. Co-expression of EYFP-tagged full-length munc18c with 80K-H resulted in insulin eliciting a major increase in the mobility of the full-length EYFP-tagged munc18c of approx. 2-fold (P < 0.01; Tables 3 and 4). To test whether this effect was dependent on the ability of 80K-H to bind munc18c, a truncation construct of munc18c, namely EYFP-tagged munc18c 338, was employed. This construct is unable to bind 80K-H and inhibits insulin-stimulated GLUT4 translocation to a greater extent than full-length munc18c. As with the EYFP-tagged full-length munc18c, insulin did not affect the diffusion coefficient of EYFP-tagged munc18c 338 when expressed alone. Importantly, when EYFP-tagged munc18c 338 and 80K-H were co-expressed, insulin was no longer able to trigger a change in the speed of the munc18c construct (Tables 3 and 4). As a control, a less truncated version of munc18c was employed.

To confirm this, we next determined the diffusion coefficient of EYFP-tagged syntaxin-4. Insulin stimulation had no effect on the diffusion coefficient of EYFP-tagged syntaxin-4 (Table 1). However, when full-length munc18c was co-expressed, the diffusion coefficients for EYFP-tagged syntaxin-4 were again

**Figure 1** Example FCS traces showing that syntaxin-4 reduces the diffusion coefficient of munc18c 295, but not full-length munc18c, in an insulin-dependent manner

Syntaxin-4 was expressed with the EYFP-tagged full-length munc18c construct (A, B) or the EYFP-tagged munc18c 295 construct (C, D), which lacks the well-characterized syntaxin-4-binding site. Cells were serum-starved for 24 h, stimulated with (B, D) or without (A, C) 10−7 M insulin for 20 min and FCS data were collected. Note the shift to the right of the curve in (D) compared with that in (C), demonstrating an insulin-stimulated increase in the diffusion coefficient of the EYFP-tagged munc18c 295 construct; this corresponds to a reduction in the speed of the EYFP-tagged munc18c 295 construct. When full-length munc18c was employed, insulin stimulation did not shift the curve. The original FCS traces are shown.
namely EYFP-tagged munc18c 295. This construct still has the ability to bind 80K-H and, like the full-length munc18c, to inhibit GLUT4 translocation when overexpressed. Insulin did not affect the mobility of the EYFP-tagged munc18c 295 construct when expressed alone. However, when EYFP-tagged munc18c 295 and 80K-H were co-expressed, insulin stimulation caused an approx. 2-fold increase in the speed of EYFP-tagged munc18c 295.

To further test the nature of the interactions between munc18c and syntaxin-4, co-immunoprecipitation experiments were performed. For this, Xpress-tagged munc18c 295 or full-length munc18c constructs were expressed in CHO cells and the cells were incubated with and without insulin. Only with the insulin-stimulated cells did syntaxin-4 co-immunoprecipitate with the Xpress-tagged 295 construct, whereas with both unstimulated and insulin-stimulated cells, syntaxin-4 co-immunoprecipitated with the Xpress-tagged full-length munc18c construct (Figure 2). As expected, 80-KH co-immunoprecipitated with munc18c 295 and syntaxin-4 from insulin-stimulated but not unstimulated cells (Figure 3). Crucially, these co-immunoprecipitation data support, by a different method, the FCS data.

The above results establish that full-length munc18c can bind syntaxin-4 both in the presence and absence of insulin, whereas munc18c 295 can only bind syntaxin-4 in the presence of insulin. Munc18c 295 lacks the well-established binding site for syntaxin-4 located in the N-terminus of the protein. The results above thus indicate that munc18c contains a second syntaxin-4-binding site. To help define the nature of the second syntaxin-binding site, further co-immunoprecipitation experiments were performed by more extensively truncating munc18c. For this, various Xpress-tagged munc18c truncation constructs were expressed in CHO cells and the cells were incubated with insulin. Cell extracts were then incubated with GST-tagged syntaxin-4 glutathione beads. As expected, the Xpress-tagged munc18c 295 construct was pulled down by the GST-tagged syntaxin-4 glutathione beads. In contrast, the Xpress-tagged munc18c 308, 326 and 338 constructs were not pulled down by the GST-tagged syntaxin-4 glutathione beads (Figure 4). This indicates that key residues involved in forming the second binding site are located between amino acids 295 and 308 of munc18c.

**DISCUSSION**

Insulin regulates glucose homoeostasis predominantly by causing a rapid increase in glucose uptake in muscle and adipose cells via the translocation of the GLUT4 vesicle to the cell surface. GLUT4 vesicle trafficking is similar to that seen in synaptic vesicles. The GLUT4 vesicle contains the v-SNARE VAMP-2, which binds to its cognate t-SNARE receptor syntaxin-4 in the plasma membrane [10–12]. Insulin regulates the VAMP-2–syntaxin-4 binding by virtue of accessory proteins, the most important of which is munc18c.

There have, however, been few studies conducted to elucidate the exact role of munc18c in GLUT4 vesicle exocytosis. munc18c has been shown to inhibit GLUT4 vesicle exocytosis and yet to be also essential for this process. In vitro assays have shown that in the basal state munc18c binds to syntaxin-4 with higher affinity than...
VAMP-2. This prevents VAMP-2 from binding to syntaxin-4 and hence inhibits GLUT4 vesicle docking at the plasma membrane [11,13,14]. These same in vitro assays also showed that when insulin stimulation decreases the binding strength of munc18c towards syntaxin-4, the VAMP-2 interaction with syntaxin-4 becomes relatively stronger. This allows the GLUT4 vesicle to bind to the plasma membrane. Due to the in vitro nature of these assays, the mechanistic detail in vivo remained unclassified. Several scenarios would fit these data in vivo. Either munc18c would dissociate from syntaxin-4 in response to insulin or weaker binding association would not cause munc18c dissociation but rather allow syntaxin-4 to undergo a conformational change allowing syntaxin-4 to bind VAMP-2.

In our study, FCS has been utilized to investigate munc18c biology in intact living cells. Co-expression of EYFP-tagged full-length munc18c with syntaxin-4 resulted in a significant increase in the diffusion coefficient of the EYFP-tagged full-length munc18c in the absence of insulin stimulation. This slow down in the mobility of munc18c is expected as munc18c is known to bind syntaxin-4. The insulin-independent binding between munc18c and syntaxin-4 was confirmed using EYFP-tagged syntaxin-4 in the presence and absence of co-expression of munc18c. If insulin caused munc18c to physically dissociate from syntaxin-4, then insulin stimulation would be expected to increase the mobility of the EYFP-tagged full-length munc18c. However, the diffusion coefficient of EYFP-tagged munc18c did not change in response to insulin. The same result was seen when the diffusion coefficient of EYFP-tagged syntaxin-4 was measured in the presence of munc18c. These in vivo results clearly show that munc18c is not released from syntaxin-4 in response to insulin. Munc18c belongs to the nSec1 family of proteins. Yeast Sec1 proteins have been the most heavily studied. Several mechanisms appear to be used for interaction between Sec1 proteins and syntaxins. Sec1 only binds to the yeast syntaxin Sso1p when the complete SNARE complex is formed [15]. The structure of the neuronal SM protein (Sec/Munc18 protein), nSec1, bound to the closed conformation of syntaxin 1a has been interpreted as an intermediate required to convert syntaxin into the ‘open’ conformation, which then assembles with the other SNAREs to form the fusogenic SNARE complex [16]. An indirect syntaxin-binding mode describes the interaction of the yeast vacuolar SM protein, Vps33p, which is part of a large protein complex that binds to the cognate syntaxin, Vam3p [17]. Indeed, null mutations in the genes for the SM proteins cause dramatic reductions in vesicle exocytosis, suggesting that these proteins are essential for normal SNARE function [18]. The munc18c–syntaxin-4 complex has been shown to be specifically required for insulin-stimulated GLUT4 vesicle fusion, and munc18c has been described as a positive fusogenic protein [19]. Our FCS data agree with these findings. If munc18c is not actually released by syntaxin-4, some change in munc18c must allow syntaxin-4 to bind VAMP-2.

To test whether munc18c has a binding site for syntaxin-4 distinct from the well-known site in the N-terminal portion of the protein, the EYFP-tagged munc18c 295 construct, which lacks the site, was employed. The mobility of the EYFP-tagged munc18c 295 construct was similar in the presence and absence of co-expressed syntaxin-4 in the absence of insulin. This indicates that, unlike full-length munc18c, the munc18c 295 construct does not associate with syntaxin-4 in the basal state and that the N-terminal region is responsible for binding syntaxin-4 in the basal state. Upon insulin stimulation of cells co-expressing the EYFP-tagged munc18c 295 construct and syntaxin-4, there was a marked increase in the diffusion coefficient of the EYFP-tagged munc18c 295 construct. This indicates that the munc18c 295 construct was able to associate with syntaxin-4 in the insulin-stimulated state and that munc18c contains a second binding site for syntaxin-4 that is outside the N-terminal region. Thus insulin may trigger a conformational change in munc18c that results in it repositioning on syntaxin-4 and in turn allowing syntaxin-4 to adopt its ‘open’ conformation and bind VAMP-2. Very recent data from D’Andrea-Merrins et al. [20] support our view that munc18c has more than one binding site on syntaxin-4.

To analyse how the insulin signal may be delivered to munc18c to induce such a conformational change, experiments were conducted with 80K-H. Recent evidence suggests that 80K-H links insulin signalling to the machinery controlling GLUT4 exocytosis through an insulin-triggered association with munc18c [5,6]. For this, EYFP-tagged full-length munc18c was expressed in the presence and absence of 80K-H. While insulin did not affect the mobility of EYFP-tagged full-length munc18c when expressed alone, in the presence of 80K-H, insulin induced a large increase in the mobility of the EYFP-tagged full-length munc18c. Use of various constructs of munc18c showed that this increase in speed of munc18c was critically dependent on the presence of an intact 80K-H-binding site on munc18c. These results indicate that 80K-H interacts with munc18c in intact living cells. How then does insulin trigger 80K-H to increase the speed of munc18c if, as shown above, munc18c remains associated with syntaxin-4? One way that the mobility of munc18c can increase is if there is a decrease in the tethering of the syntaxin-4 complex with other known interactors, such as synip or tomosyn or other scaffolding proteins. In this model, insulin would break or reduce these interactions. This model is shown in Figure 5. However, we did not observe an increase in mobility of syntaxin-4 when cells co-expressing munc18c and syntaxin-4 were stimulated with insulin. This is probably because the availability of the other interactors was limiting and because most of the overexpressed syntaxin-4 was not tethered in the first place. Tyrosine phosphorylation of munc18c has been shown to promote VAMP-2 binding to syntaxin-4 and this provides a further mechanism of delivering an insulin signal to munc18c [21].
exposed by insulin. Thus insulin may elicit a repositioning of munc18c on syntaxin-4, which in turn allows syntaxin-4 to adopt its ‘open’ conformation and bind VAMP-2.

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REFERENCES


Figure 5  Hypothetical model showing how insulin triggers the repositioning of munc18c on syntaxin-4

Upstream signalling molecules including PKCζ and 80K-H relay the insulin signal to munc18c. Activated munc18c then repositions itself on syntaxin-4, thereby triggering syntaxin-4 to adopt the ‘open’ formation enabling it to bind VAMP-2 of the GLUT4 vesicle. Breakage of interactions between syntaxin-4 and other known interactors, such as synip, tomosyn, SNAP-23 or other scaffolders, may account for the observed increase in munc18c mobility.

The above conclusions, based on differences in the diffusion coefficients between full-length munc18c and munc18c 295 (Tables 1 and 2), suggest that in unstimulated cells the latter does not interact with syntaxin-4, whereas association is enhanced with syntaxin-4 in insulin-stimulated cells. This is a critical result that raises the concept that reorganizing the molecular interaction between munc18c and syntaxin-4 serves as a gating mechanism that permits interaction between SNARE proteins. To underpin this finding it was important to substantiate these interactions by another approach. For this Xpress-tagged munc18c 295 or full-length munc18c, constructs were expressed in CHO cells. If the proposition holds, then only in insulin-stimulated cells should syntaxin-4 co-immunoprecipitate with munc18c 295. Western blots in Figure 2 show that this was indeed the case. Additionally, if the proposition is correct, full-length munc18c should not undergo any change in association with syntaxin-4 upon insulin treatment. Again, this was found to be the case (Figure 2). These results corroborate the repositioning hypothesis.

In conclusion, we have identified that insulin does not trigger the release of munc18c from syntaxin-4 in intact single living cells. Rather munc18c appears to contain two binding sites for syntaxin-4, one of which is disrupted by insulin and the other

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