Discrimination of GLUT4 vesicle trafficking from fusion using a temperature-sensitive Munc18c mutant

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To examine the temporal relationship between pre- and post-docking events, we generated a Munc18c temperature-sensitive mutant (Munc18c/TS) by substitution of arginine 240 with a lysine residue. At the permissive temperature (23°C), overexpression of both the wild type (Munc18c/WT) and the R240K mutant inhibited insulin-stimulated GLUT4/IRAP vesicle translocation. However, at the non-permissive temperature (37°C) only Munc18c/WT inhibited GLUT4/IRAP translocation whereas Munc18c/TS was without effect. Moreover, Munc18c/WT bound to syntaxin 4 at both 23 and 37°C whereas Munc18c/TS bound syntaxin 4 only at 23°C. This was due to a temperature-dependent conformational change in Munc18c/TS, as its ability to bind syntaxin 4 and effects on GLUT4 translocation were rapidly reversible while protein expression levels remained unchanged. Furthermore, insulin stimulation of Munc18c/TS-expressing cells at 23°C followed by temperature shift to 37°C resulted in an increased rate of GLUT4 translocation compared with cells stimulated at 37°C. To date, this is the first demonstration that the rate-limiting step for insulin-stimulated GLUT4 translocation is the trafficking of GLUT4 vesicles and not their fusion with the plasma membrane.

Keywords: adipocytes/glucose transporter/insulin/
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Introduction

The majority of insulin-stimulated glucose uptake in both striated muscle and adipose tissues is attributable to the presence of the insulin-responsive glucose transporter GLUT4 (Olson and Pessin, 1996). In the basal non-insulin stimulated state, the GLUT4 protein is localized to the perinuclear region and in both tubulovesicular elements and small intracellular vesicles scattered throughout the cell cytoplasm (Slot *et al.*, 1991a,b). Upon addition of insulin, a series of intracellular signaling events ensues leading to the movement (trafficking), association (docking) and eventual incorporation (fusion) of the GLUT4 storage vesicles with the plasma membrane (Czech, 1995; Kandror and Pilch, 1996; Rea and James, 1997; Pessin *et al.*, 1999). Similarly, the insulin-responsive aminopeptidase (IRAP) has a near identical subcellular distribution to GLUT4 and displays the same pattern of insulin-stimulated translocation (Kandror and Pilch, 1994, 1996;

Kandror *et al.*, 1994; Ross *et al.*, 1996; Malide *et al.*, 1997; Martin *et al.*, 1997). Although the physiological role of IRAP translocation remains unknown, the insulin-stimulated translocation of the GLUT4/IRAP-containing cargo vesicles results in a large increase in the number of functional glucose transporters on the cell surface and thereby accounts for the dramatic increase in glucose uptake.

This multi-step translocation process shares several important features with the exocytosis of synaptic vesicles during neurotransmitter release. For example, the interaction of the GLUT4 vesicle v-SNARE protein VAMP2 with the plasma membrane t-SNARE proteins syntaxin 4 and SNAP23 is necessary for insulin-stimulated GLUT4 translocation (Cheatham *et al.*, 1996; Volchuk *et al.*, 1996; Olson *et al.*, 1997; Tellam *et al.*, 1997; Martin *et al.*, 1998). In addition to these t- and v-SNAREs, there are adipocyte homologs of the n-Sec1 regulator of synaptic vesicle trafficking, Munc18b and Munc18c, of which only the Munc18c isoform binds to syntaxin 4 with high affinity (Tellam *et al.*, 1997; Thurmond *et al.*, 1998, 2000). Several studies have also demonstrated that increased expression of Munc18c but not Munc18b specifically inhibits insulin-stimulated GLUT4/IRAP translocation without affecting the trafficking of other recycling proteins (Tamori *et al.*, 1998; Thurmond *et al.*, 1998). More recently, it has been observed that the overexpression of Munc18c resulted in the occlusion of the plasma membrane vesicle binding sites and thereby prevented GLUT4 translocation at the step of plasma membrane docking (Thurmond *et al.*, 2000). These data are consistent with recent structural studies of the n-Sec1 (Munc18a) homolog, which appears to function in the transition of syntaxin 1A from the closed to open conformational state (Dulubova *et al.*, 1999; Misura *et al.*, 2000).

Unlike synaptic vesicle exocytosis, the rate-limiting step for insulin-stimulated GLUT4 translocation remains unknown. Since insulin-stimulated translocation of the GLUT4/IRAP vesicles can be subdivided into at least four overall processes (signaling, vesicle trafficking, plasma membrane docking and fusion), we proposed that the ability to regulate Munc18c function would provide a convenient tool to distinguish signaling and trafficking events from that of docking and fusion. To this end, we have generated a temperature-sensitive Munc18c mutant based upon its sequence similarity to yeast Sly1p. In this study, we demonstrate that the Munc18c mutant (Munc18c/TS) specifically inhibits GLUT4/IRAP-containing cargo vesicle translocation at the permissive temperature, which is rapidly reversible upon shifting to the non-permissive temperature. Furthermore, examination of the rate of GLUT4 translocation following temperature shift demonstrates that the rate-limiting step in insulin-stimulated GLUT4 translocation is the

Fig. 1. The rate of insulin-stimulated GLUT4 translocation is temperature dependent. Differentiated 3T3L1 adipocytes were incubated at either 23°C (a–f) or 37°C (g–l) and then either left untreated or stimulated with 100 nM insulin for 5, 15, 30, 60 or 90 min. Plasma membrane sheets were prepared as described in Materials and methods. The plasma membrane sheets were incubated with a GLUT4 antibody for 30 min at room temperature, followed by addition of anti-rabbit Texas Red. Rhodamine staining was visualized by confocal microscopy (60×).

trafficking of these vesicles to the plasma membrane and not docking or fusion.

Results

Insulin-stimulated GLUT4 translocation occurs at both 23 and 37°C in 3T3L1 adipocytes

At the physiological temperature of 37°C, GLUT4 vesicles have been shown to undergo an insulin-stimulated translocation to the plasma membrane of 3T3L1 adipocytes with a *t*_{1/2} of ~4 min. However, this translocation is prevented when the temperature is reduced to 19°C (Robinson *et al.*, 1992). Recently, we have observed that insulin-stimulated GLUT4 translocation can occur at 23°C but with a substantially slower rate, *t*_{1/2} ~30 min (Elmendorf *et al.*, 1999). To determine the time required for the full extent of insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes incubated at 23°C to equal that of cells incubated at 37°C, we isolated plasma membrane sheets and evaluated GLUT4 translocation using an endofacial GLUT4 antibody (Figure 1). In the basal state, very little GLUT4 immunofluorescence was detected in sheets prepared from cells incubated at either 23 or 37°C (Figure 1a and g). Upon insulin stimulation, a markedly reduced rate of GLUT4 protein immunofluorescence was observed in plasma membrane sheets isolated from cells incubated at 23°C compared with those incubated at 37°C (Figure 1a–e compared with g–k). Although the rate of GLUT4 translocation in cells incubated at 23°C was less than that of cells incubated at 37°C, similar levels were reached after 90 min of insulin stimulation (Figure 1f and l). Thus, incubation of cells at 23°C with insulin for 90 min resulted in comparable amounts of GLUT4 translocation to incubation at 37°C for 30 min.

Substitution of arginine 240 for lysine results in a temperature-sensitive Munc18c mutant protein

One useful approach to reversibly modulate a specific step in a biological process is with temperature-sensitive mutants. Recently, a temperature-sensitive allele in Sly1p, a yeast homolog of Munc18c, was isolated based

scSly1	R	G	V	L	I	I	L	D	R	N	I	D	F	270
scSec1	R	S	I	L	I	I	T	D	R	T	L	D	P	256
scVps45	T	P	V	L	L	I	L	D	R	N	T	D	P	238
ceUnc18	R	S	Q	L	I	I	I	D	R	G	Y	D	A	239
cbUnc18	R	S	Q	L	I	I	I	D	R	G	Y	D	A	237
ROP	R	S	Q	L	L	I	L	D	R	G	F	D	C	250
S-Sec1	R	S	Q	L	I	I	L	D	R	G	F	D	P	236
rSly1	R	P	L	L	V	L	V	D	R	N	I	D	L	273
mVps45	P	P	L	L	L	I	L	D	R	G	D	D	A	224
Munc18a/n-Sec1	R	S	Q	L	I	I	L	D	R	G	F	D	P	239
Munc18b	R	S	Q	L	L	I	M	D	R	A	A	D	P	239
Munc18c	Q	S	Q	L	L	I	I	D	R	G	F	D	P	244
Consensus	L	Ψ	I	/L	X	D	R	X	X	D				

Fig. 2. Amino acid sequence comparison between various Munc18c homologs in the region that confers temperature sensitivity to Sly1p. Amino acid residue numbers are listed to the right of each sequence and the conserved arginine residue is boxed. Abbreviations are as follows: scSly1, scSec1 and scVps45 from *S.cerevisiae*; ceUnc18 and cbUnc18 from *Caenorhabditis briggsae* and *C.elegans*, respectively; ROP, Ras2 opposite protein from *D.melanogaster*; s-Sec1, squid neuron Sec1; rSly1, rat Sly1p; mVps45, mouse Vps45p; Munc18a/n-Sec1 (rat neuronal Sec1), Munc18b and Munc18c, mammalian homologs of Unc18.

upon its ability to allow growth at 25°C but not at 37°C (Cao *et al.*, 1998). Sequence analysis revealed that this phenotype was the result of a single point mutation at arginine 266 that was mutated to lysine (R266K). Amino acid alignment shows that this arginine residue is highly conserved in Sly1p homologs from yeast to mammals (Figure 2). The 13 amino acid region flanking this conserved arginine displays between 38 and 62% amino acid identity with 77–92% sequence similarity amongst all the different isoforms and species. In addition, the core sequence surrounding this arginine residue is also highly conserved and can be defined by the consensus motif LΨI/LXDRXXD, where Ψ represents a hydrophobic and X any amino acid residue.

Although Sly1p functions in the trafficking of endoplasmic reticulum-derived vesicles to the Golgi, based upon the high degree of conservation we speculated that the same mutation in Munc18c might also result in a temperature-sensitive phenotype. We therefore co-trans-

Fig. 3. Expression of Munc18c/TS inhibits insulin-stimulated GLUT4 translocation at 23°C but not at 37°C. Differentiated 3T3L1 adipocytes were electroporated with 50 µg of pGLUT4-EGFP plus 200 µg of the empty vector (pcDNA3; **a**, **b**, **g** and **h**), the wild-type Munc18c (Munc18c/WT; **c**, **d**, **i** and **j**) or the temperature-sensitive R240K Munc18c (Munc18c/TS; **e**, **f**, **k** and **l**) and allowed to recover for 18 h. The cells were then incubated in serum-free medium for 2 h at either 23°C (**a–f**) or 37°C (**g–l**) followed by stimulation with 100 nM insulin for 90 min at 23°C (**b**, **d** and **f**) or 30 min at 37°C (**h**, **j** and **l**). The cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy (60×). These results are representative of at least 25 cells per experiment from three independent experimental sets of electroporated adipocytes.

fected 3T3L1 adipocytes with an enhanced green fluorescent protein (EGFP) epitope-tagged GLUT4 (GLUT4-EGFP) expression plasmid and either the pcDNA3 empty vector, the wild-type Munc18c (Munc18c/WT) or the R240K Munc18c (Munc18c/TS) mutant (Figure 3). As previously reported (Thurmond *et al.*, 1998, 2000), expression of GLUT4-EGFP in 3T3L1 adipocytes results in a similar distribution to the endogenous GLUT4, being primarily localized to the perinuclear region and small vesicles throughout the cytoplasm in cells maintained at 37°C (Figure 3g). As expected, 30 min of insulin stimulation resulted in a redistribution of the GLUT4-EGFP to the cell surface, resulting in the formation of an essentially continuous rim of fluorescence (Figure 3h). Similarly, the small vesicle and perinuclear distribution of GLUT4-EGFP was identical in cells incubated at 23°C, which also display an insulin-stimulated translocation to the plasma membrane (Figure 3a and b). Since the rate of translocation is significantly slower at 23°C compared with 37°C (Figure 1) the 23°C incubated cells were treated with insulin for 90 min. In any case, the expression of Munc18c/WT had no effect upon the basal distribution of GLUT4-EGFP but markedly inhibited the insulin-stimulated GLUT4-EGFP translocation at both 23 and 37°C (Figure 3, compare **c** with **d**, and **i** with **j**). Like Munc18c/WT, expression of Munc18c/TS had no effect on the basal GLUT4-EGFP localization at either temperature and prevented the insulin-stimulated translocation at 23°C (Figure 3e, **f** and **k**). Importantly, however, expression of

Munc18c/TS had no effect on the insulin stimulation of GLUT4-EGFP translocation at 37°C (Figure 3l). These data suggest that the R240K single point mutation in Munc18c resulted in the production of a temperature-sensitive Munc18c protein.

To confirm these results, we also examined the effect of Munc18c expression on another insulin-sensitive co-localized cargo protein, IRAP (Figure 4). Consistent with the effects of Munc18c/WT and Munc18c/TS on GLUT4-EGFP translocation, essentially identical results were obtained with the co-expression of the EGFP epitope-tagged IRAP protein, EGFP-IRAP. That is, expression of Munc18c/WT inhibited the insulin-stimulated translocation of EGFP-IRAP at both 23 and 37°C (Figure 4a–d and g–j). In contrast, the Munc18c/TS mutant was capable of inhibiting insulin-stimulated EGFP-IRAP translocation at 23 but not 37°C (Figure 4, compare **e** and **f** with **k** and **l**). Quantitation of the number of cells displaying a continuous cell surface fluorescence indicated that insulin induced the translocation of GLUT4-EGFP in 61 and 70% of the transfected cell population at 23 and 37°C, respectively (Figure 5A, bars 2 and 8). Expression of Munc18c/WT reduced the number of cells displaying GLUT4-EGFP translocation to 37 and 38% at 23 and 37°C, respectively (Figure 5A, bars 4 and 10). In contrast, expression of Munc18c/TS reduced the translocation of GLUT4-EGFP to 32% at 23°C, but there was no significant inhibition at 37°C with 64% of the cell population displaying GLUT4-EGFP translocation (Figure 5A, bars 6

Fig. 4. Expression of Munc18c/TS results in the inhibition of insulin-stimulated translocation of EGFP-IRAP at 23°C but not at 37°C. Differentiated 3T3L1 adipocytes were electroporated with 50 µg of pEGFP-IRAP plus 200 µg of the empty vector (pcDNA3; **a, b, g** and **h**), the wild-type Munc18c (Munc18c/WT; **c, d, i** and **j**) or the temperature-sensitive R240K Munc18c (Munc18c/TS; **e, f, k** and **l**) and allowed to recover for 18 h. The cells were then incubated in serum-free medium for 2 h at either 23°C (**a–f**) or 37°C (**g–l**) followed by stimulation with 100 nM insulin for 90 min at 23°C (**b, d** and **f**) or 30 min at 37°C (**h, j** and **l**). The cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy (60×). These results are representative of at least 25 cells per experiment from three independent experimental sets of electroporated adipocytes.

and 12). Similarly in cells expressing Munc18c/WT, insulin stimulated the translocation of EGFP-IRAP in 66% of the transfected cell population at both 23 and 37°C, which was reduced to 38 and 34%, respectively (Figure 5B, bars 2, 4, 8 and 10). However, expression of Munc18c/TS only inhibited EGFP-IRAP translocation in cells incubated at 23°C and not at 37°C (Figure 5B, bars 6 and 12).

In addition to the GLUT4/IRAP-containing vesicles, insulin can stimulate the plasma membrane translocation of other vesicular compartments containing proteins such as GLUT1, the transferrin receptor and the cation-independent mannose-6-phosphate receptor, CI/MPR (James *et al.*, 1994; Kandror and Pilch, 1996; Rea and James, 1997; Pessin *et al.*, 1999). Therefore, to assess the specificity of the Munc18c/TS mutant we also examined the insulin-dependent translocation of a CI/MPR-EGFP fusion protein (Figure 5C). In this case, insulin-stimulated the translocation of CI/MPR in ~30 and 40% of the transfected cell population at 23 and 37°C, respectively (Figure 5C, bars 2 and 8). This lower extent of translocation is consistent with previous studies demonstrating that the endogenous CI/MPR does not undergo the same degree of translocation as GLUT4 (Oka *et al.*, 1984; Wardzala *et al.*, 1984). More importantly, expression of neither Munc18c/WT nor Munc18c/TS had any significant effect on the extent of insulin-stimulated CI/MPR-EGFP translocation at either temperature (Figure 5C, bars 4, 6, 10 and 12). Together, these data demonstrate that Munc18c specifically regulates the translocation of the insulin-responsive GLUT4/IRAP storage vesicle compartments

but does not affect the general endosomal trafficking systems.

The temperature-sensitive function of Munc18c/TS is readily reversible

To determine if the negative effect of the Munc18c/TS protein on insulin-stimulated GLUT4 translocation was a reversible process, the 3T3L1 adipocytes were shifted from 23 to 37°C in the continuous presence of insulin (Figure 6). As expected, insulin stimulation for 90 min at 23°C resulted in the translocation of GLUT4-EGFP (Figure 6a and b). Similarly, cells maintained at 23°C that were then stimulated with insulin and immediately shifted to 37°C for 30 min also resulted in a similar extent of GLUT4-EGFP translocation (Figure 6e and f). However, although expression of Munc18c/TS prevented the insulin-stimulated translocation of GLUT4-EGFP in cells continuously maintained at 23°C, the cells that were temperature shifted displayed an equal extent of GLUT4-EGFP translocation to controls (Figure 6c, d, g and h compared with control panels a, b, e and f). In a complementary approach, we have also observed that maintenance of Munc18c/TS-expressing cells at 37°C, followed by a temperature shift to 23°C prevented insulin-stimulated GLUT4-EGFP translocation (data not shown). Thus, these data demonstrate that the regulatory properties of Munc18c/TS on GLUT4 translocation are fully reversible as a function of temperature within a 30 min time period.

Fig. 5. Expression of Munc18/TS specifically inhibits GLUT4/IRAP translocation but does not affect the insulin-stimulated recycling of the cation-independent mannose-6-phosphate receptor vesicle population. Differentiated 3T3L1 adipocytes were electroporated with 50 μ g of either (A) pGLUT4-EGFP, (B) pEGFP-IRAP or (C) pCI/MPR-EGFP plus 200 μ g of pcDNA3 (vector control), Munc18c/WT (18c/WT) or Munc18c/TS (18c/TS mutant) and allowed to recover for 18 h. The cells were incubated in serum-free medium for 2 h at either 23°C or 37°C followed by treatment without (odd-numbered bars) or with 100 nM insulin (even-numbered bars) for 90 min at 23°C or 30 min at 37°C. Cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy (60 $\times$). Each point represents the mean $\pm$ standard error of at least 25 cells per experiment from three independent sets of electroporated adipocytes.

The most likely explanation for the reversibility of Munc18c/TS function is a change in protein conformation.

However, it was formally possible that the Munc18c/TS mutant was either rapidly synthesized during the 90 min stimulation period with insulin at 23°C, or rapidly degraded at 37°C. To address the issue of protein synthesis, Munc18c/TS-expressing cells were maintained at 37°C for 2 h followed by a temperature shift to 23°C for 90 min with insulin plus cycloheximide (Figure 7A). In the absence of Munc18c/WT or Munc18c/TS protein, 59 and 51% of the transfected cells displayed GLUT4-EGFP cell surface fluorescence after a temperature shift from 37 to 23°C in the absence or presence of cycloheximide, respectively (Figure 7A, bars 2 and 8). This fluorescence was reduced to 26 and 20% by the co-expression of the Munc18c/WT protein (Figure 7A, bars 4 and 10). Co-expression of the Munc18c/TS protein also inhibited translocation (to 22%) both in the absence and presence of cycloheximide treatment (Figure 7A, bars 6 and 12). These data indicate that the inhibitory effect of the Munc18c/TS was reversible and was not due to the biosynthesis of new Munc18c/TS protein at the permissive temperature. Furthermore, to determine whether Munc18c/TS was specifically and rapidly degraded compared with Munc18c/WT, Flag immunoblots of whole cell detergent extracts were performed using cells expressing Flag-Munc18c/WT and Flag-Munc18c/TS maintained at either 23 or 37°C (Figure 7B). Although the steady-state expression levels of the Munc18c/TS mutant were slightly lower than those of Munc18c/WT, there were no significant changes in cells maintained at either 23 or 37°C (Figure 7B, lanes 1–4). In addition, incubation with insulin had no effect on protein expression (data not shown). Thus, changes in Munc18c/TS protein levels cannot account for the temperature sensitivity or reversibility of Munc18c/TS function on GLUT4 translocation.

Another property of Munc18c is its ability to interact specifically with the plasma membrane-bound syntaxin 4 protein (Tellam *et al.*, 1997; Tamori *et al.*, 1998; Thurmond *et al.*, 1998, 2000). We therefore compared the ability of Munc18c/TS to compete for Munc18c/WT binding at 23 and 37°C (Figure 8A). In this assay system, co-expression of syntaxin 4 with EGFP-Munc18c induces a strong plasma membrane fluorescence and competition of this interaction results in a re-distribution of EGFP-Munc18c to the cytosol (Thurmond *et al.*, 2000). In the absence of competitor protein, 72 and 77% of the transfected cells displayed cell surface fluorescence at 23 or 37°C, respectively (Figure 8A, bars 1 and 4). This fluorescence was reduced to 41 and 45% by the co-expression of the Munc18c/WT protein (Figure 8A, bars 2 and 5). In contrast, competition by the Munc18c/TS protein only occurred at 23°C with no significant effect at 37°C (Figure 8A, bars 3 and 6). These data indicate that the Munc18c/TS protein is capable of interacting competitively with Munc18c/WT for syntaxin 4 binding at the permissive temperature but not at the non-permissive temperature.

To examine the temperature-dependent interaction between Munc18c/TS and syntaxin 4 more directly, we next co-expressed Munc18c/WT or Munc18c/TS proteins with syntaxin 4 in CHO/IR cells (Figure 8B). These cells contain very low endogenous levels of Munc18c but express high levels of the transfected proteins (data not shown). Syntaxin 4 immunoblots of whole cell lysates

Fig. 6. Munc18c/TS-induced inhibition of insulin-stimulated GLUT4–EGFP translocation at 23°C is reversed by shifting cells to the non-permissive temperature (37°C). Differentiated 3T3L1 adipocytes were electroporated with 50 µg of pGLUT4–EGFP plus 200 µg of pcDNA3 (vector control) or Munc18c/TS (R240K mutant) and allowed to recover for 18 h. The cells were incubated in serum-free medium for 2 h at 23°C and either left untreated (a, c, e and g) or stimulated (b, d, f and h) with 100 nM insulin for 90 min and then either kept at 23°C (a–d) or shifted to 37°C (e–h) for an additional 30 min, respectively. Cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy (60×). In the presence of insulin, $63 \pm 3\%$ or $81 \pm 5\%$ of the cells expressing GLUT4–EGFP displayed a plasma membrane cell surface fluorescence when incubated at 23 or 37°C, respectively. This percentage was reduced to $20 \pm 5\%$ when Munc18c/TS was co-expressed and the cells incubated at 23°C. Temperature shifting of the cells expressing Munc18c/TS from 23 to 37°C resulted in $67 \pm 11\%$ of the cells displaying GLUT4–EGFP at the cell surface. These results are representative of the mean $\pm$ standard error of at least 25 cells per experiment from three independent sets of electroporated adipocytes.

isolated from cells incubated at 23 and 37°C demonstrated similar expression levels of the syntaxin 4 protein (data not shown). Immunoprecipitation of Flag-Munc18c/WT and Flag-Munc18c/TS proteins demonstrated the co-immunoprecipitation of the syntaxin 4 protein (Figure 8B, lanes 1–4). However, incubation of the Munc18c/TS-expressing cells at 37°C resulted in a decreased amount of syntaxin 4 protein that was immunoprecipitated with Flag-Munc18c/TS (Figure 8B, lane 4). This difference was not due to unequal expression (data not shown) or immunoprecipitation of Flag-Munc18c/TS at 23 and 37°C, as determined by Flag immunoblotting of the Flag immunoprecipitates (Figure 8B, lanes 3 and 4). Thus, these data demonstrate that binding interactions between syntaxin 4 and Munc18c/TS protein are temperature sensitive.

Signaling and/or GLUT4 vesicle trafficking is the rate-limiting step in insulin-stimulated GLUT4 translocation

We have recently demonstrated that increased Munc18c expression prevents GLUT4/IRAP vesicle translocation by inhibiting vesicle docking at the plasma membrane (Thurmond *et al.*, 2000). Having now established the reversibility of Munc18c/TS function, we recognized that this property could be utilized to determine whether the rate-limiting event in insulin-stimulated GLUT4 translocation occurs at a pre- or post-docking step (Figure 9). As controls, the GLUT4–EGFP-expressing cells were initially maintained at 23 or 37°C (Figure 9A). The cells maintained at 37°C were then treated with insulin and the time course of insulin-stimulated GLUT4–EGFP translocation was determined (Figure 9A, squares). In parallel,

the cells maintained at 23°C were simultaneously shifted to 37°C and treated with insulin followed by the determination of GLUT4–EGFP translocation (Figure 9A, circles). In both cases the extent and time dependence of insulin-stimulated GLUT4–EGFP translocation was essentially identical with a $t_{1/2}$ of ~ 4 min. These data demonstrate that pre-incubation at 23°C does not result in cell context changes that alter the subsequent kinetics of GLUT4 translocation at 37°C.

Consistent with our previous results, incubation of 3T3L1 adipocytes expressing Munc18c/WT at 23°C followed by shifting to 37°C in the presence of insulin resulted in a very low level of GLUT4–EGFP translocation (Figure 9B, diamonds). In contrast, cells expressing Munc18c/TS, when shifted from 23°C in the absence of insulin to 37°C in the presence of insulin, displayed a time-dependent translocation of GLUT4–EGFP (Figure 9B, squares). Under these conditions the $t_{1/2}$ for GLUT4 translocation was ~ 12 min, significantly longer than that observed in the absence of Munc18c/TS expression (Figure 9A). These data indicate that the temperature-dependent inactivation of Munc18c/TS occurred relatively slowly and thereby decreased the apparent overall rate of GLUT4 translocation. Nevertheless, when the same population of cells expressing Munc18c/TS was pre-incubated with insulin at 23°C and then shifted to 37°C, they underwent a significantly faster rate of GLUT4–EGFP translocation (Figure 9B, triangles). Thus, pre-activation of the insulin signaling pathways and stimulation of GLUT4 trafficking prior to the release of docking inhibition results in an enhanced rate of GLUT4 translocation. These data strongly indicate that the rate-limiting step for

Fig. 7. The reversibility of insulin-stimulated GLUT4 translocation by Munc18c/TS is not the result of increased protein expression at 23°C or increased protein degradation at 37°C. Differentiated 3T3L1 adipocytes were electroporated with 50 µg of pGLUT4-EGFP plus pcDNA3 (vector control, 200 µg), Munc18c/WT (18c/WT, 50 µg) or Munc18c/TS (18c/TS mutant, 200 µg) and allowed to recover for 18 h. The cells were incubated in serum-free medium for 2 h at 37°C followed by treatment without (odd-numbered bars) or with 100 nM insulin (even-numbered bars) for 90 min at 23°C or 30 min at 37°C in the presence of 10 µg/ml cycloheximide. (A) Cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy (60×). Each point represents the mean $\pm$ standard error of at least 25 cells per experiment from three independent sets of electroporated adipocytes. (B) In parallel, total detergent lysates were prepared from untreated cells incubated at either 23 or 37°C for 4 h and immunoblotted with the M5 Flag antibody (50 µg protein per lane).

insulin-stimulated GLUT4 translocation is either activation of insulin receptor downstream effectors or trafficking of the GLUT4 vesicles to the plasma membrane but not the docking or fusion of these compartments.

Discussion

The insulin-stimulated translocation of GLUT4/IRAP-containing vesicles is a complex multi-step process that is necessary for normal maintenance of glucose homeostasis (Kahn, 1992; Klip *et al.*, 1994; Olson and Pessin, 1996). Several specific molecular events and signaling molecules have been identified that can be categorized into a general four-step model (Figure 10). Initially, insulin binding to the insulin receptor activates the intrinsic protein kinase of the receptor β subunit, resulting in its autophosphorylation and tyrosine phosphorylation of several proximal docking proteins, most notably the IRS family of insulin receptor substrate proteins (White, 1998). In turn, this results in the

Fig. 8. The binding of Munc18c/TS to syntaxin 4 is disrupted in cells incubated at the non-permissive temperature. (A) Differentiated 3T3L1 adipocytes were electroporated with 50 µg pEGFP-Munc18c plus 200 µg of syntaxin 4 and either Munc18c/WT or Munc18c/TS competitor protein cDNAs in the pcDNA3 vector. The cells were allowed to recover for 18 h and incubated in serum-free medium for 4 h at 23 or 37°C. The cells were fixed with 4% paraformaldehyde and fluorescence visualized by confocal microscopy. The data are depicted as the percentage of cells exhibiting plasma membrane cell surface fluorescence. Each point represents the mean $\pm$ standard error of at least 25 cells per experiment from three independent sets of electroporated adipocytes. (B) CHO/IR cells were electroporated with 10 µg syntaxin 4 plus either 40 µg Flag-Munc18c/TS or 10 µg Flag-Munc18c/WT cDNAs and allowed to recover for 48 h. The cells were then incubated at 23°C (lanes 1 and 3) or 37°C (lanes 2 and 4) for 4 h. Whole cell detergent extracts (lysate) were prepared and immunoprecipitated (IP) with the M2 Flag antibody as described in Materials and methods. Lysates and the Flag immunoprecipitates were immunoblotted with an affinity-purified syntaxin 4 (lower bands) or M5 Flag antibody (upper bands). These results are representative of three sets of electroporated CHO/IR cells.

association, activation and targeting of the phosphatidylinositol kinase (PI 3-kinase) (Ruderman *et al.*, 1990; Kelly *et al.*, 1992; Chen *et al.*, 1993; Folli *et al.*, 1993). The active PI 3-kinase can then generate phosphatidylinositol-3,4,5-trisphosphate, which is necessary for the stimulation of both protein kinase B (PKB) and atypical protein kinase C isoforms through activation of the phosphoinositide-dependent protein kinases (PDK1 and PDK2) and/or through engagement of the PKB pleckstrin homology (PH) domain (Bandyopadhyay *et al.*, 1997; Kotani *et al.*, 1998; Vollenweider *et al.*, 1999). Although the precise role of these events in the stimulation of GLUT4 translocation remains highly controversial, this initial transduction

Fig. 9. Insulin receptor signaling or vesicle trafficking to the plasma membrane is the rate-limiting step in insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes. **(A)** Differentiated 3T3L1 adipocytes were electroporated with 50 μ g of pGLUT4-EGFP plus 200 μ g of the pcDNA3 empty vector and allowed to recover for 18 h. The cells were then incubated in serum-free medium for 2 h at either 23°C (solid circles) or 37°C (solid squares). The cells maintained at 37°C were then treated with insulin for the times indicated, while the cells maintained at 23°C were simultaneously shifted to 37°C and treated with insulin. **(B)** Differentiated 3T3L1 adipocytes were electroporated with 50 μ g of pGLUT4-EGFP plus 200 μ g of Munc18c/WT (solid diamonds) or Munc18c/TS (solid squares and triangles) and allowed to recover for 18 h. The cells were then incubated at 23°C either in the absence (solid squares and solid diamonds) or presence (solid triangles) of 100 nM insulin for 90 min. All cells were then shifted to 37°C and insulin added to those cells not pre-incubated with insulin at 23°C (solid squares and diamonds). Cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy (60 $\times$). These results are representative of the mean $\pm$ standard error of at least 25 cells per experiment from three independent sets of electroporated adipocytes.

pathway can be referred to as 'signaling'. Since the GLUT4/IRAP vesicles are physically separated from the plasma membrane, following insulin receptor signaling these intracellular storage compartments undergo vectorial movement towards the plasma membrane in a process we refer to as 'trafficking'. Upon close approach to the plasma membrane, these juxtaposed vesicles then bind to specific receptor proteins (t-SNAREs) on the plasma membrane and thus become 'docked'. Finally, the docked vesicles are functionally incorporated into the plasma membrane through the physical mixing of the two lipid bilayers by membrane 'fusion' events.

Although several essential players in the trafficking, docking and fusion events have been identified, the specific molecular steps and processes that these proteins mediate have remained elusive. For example, several studies have observed that increased expression of Munc18c, but not Munc18b specifically inhibits insulin-stimulated GLUT4/IRAP translocation without affecting the trafficking of other recycling proteins (Tamori *et al.*, 1998; Thurmond *et al.*, 1998). These data were interpreted as evidence for a specific repressor function of Munc18c and that insulin must therefore de-repress this activity to allow for GLUT4 translocation. However, we have recently observed that Munc18c overexpression results in the occlusion of the plasma membrane vesicle binding sites and thereby masks the true function of Munc18c as a required positive fusogenic protein (Thurmond *et al.*, 2000). This latter finding is consistent with the complete inhibition of target membrane fusion when the Munc18c homologs of *Drosophila melanogaster* (Rop), *Caenorhabditis elegans* (Unc) and *Saccharomyces cerevisiae* (Sec1) are genetically ablated or mutated (Novick and Schekman, 1979; Hosono *et al.*, 1992; Harrison *et al.*, 1994). Furthermore, a positive fusogenic role for another Munc18 homolog in yeast (Sly1p) has recently been demonstrated in the fusion of endoplasmic reticulum-derived vesicles with Golgi target membranes (Cao *et al.*, 1998). In this case, analysis of Sly1p function was made possible by the use of a temperature-sensitive conditional allele that was fully functional at 23°C but inactive at temperatures above 29°C. At the non-permissive temperature the vesicle membranes were fully capable of binding to the Golgi target membranes but were unable to undergo fusion. However, shifting to the permissive temperature allowed for fusion of these docked vesicles.

Given the extent of homology among this family of proteins both within and across species, we speculated that the identical mutation in Sly1p might confer a similar temperature sensitivity to Munc18c. In fact, our data directly demonstrate that substitution of arginine 240 for lysine in Munc18c generates a temperature-sensitive Munc18c that is fully functional at 23°C but inactive at 37°C. Fortunately, these temperatures lie within the range that can be used to examine insulin-stimulated GLUT4 translocation. However, even though the rate of insulin-stimulated GLUT4 translocation was slowed at 23°C compared with 37°C, the extent of translocation was not significantly different (Figure 1). A similar temperature dependence has also been observed for IRAP translocation (Elmendorf *et al.*, 1999). Importantly, the known proximal insulin receptor signaling events do not appear to display significant temperature dependence, at least between 23 and 37°C. For example, several laboratories have reported that insulin receptor autophosphorylation and IRS1 substrate phosphorylation occur normally at reduced temperature (Marshall and Olefsky, 1981; Carpentier, 1989; Heller-Harrison *et al.*, 1995). We have also found that the rate of insulin-stimulated PI 3-kinase activation, association with IRS1, *in vivo* phosphatidylinositol-3,4,5-trisphosphate production and rate of PKB activation are also largely unaffected by reduced temperature (Elmendorf *et al.*, 1999). Thus, the reduced rate of insulin-stimulated GLUT4 translocation at 23°C probably

Fig. 10. Schematic model depicting four general stages of insulin-stimulated GLUT4 translocation. Initiation of the translocation process begins with: (i) activation of the insulin receptor signal transduction pathway through the activation of the insulin receptor tyrosine kinase, phosphorylation of IRS proteins, targeting and activation of the PI3-kinase and subsequent activation of PKB and/or atypical PKC isoforms; (ii) vectorial movement of the GLUT4/IRAP-containing vesicles (GSV) to the plasma membrane binding sites containing the vesicle receptor protein complexes; (iii) interaction between the vesicle v-SNARE protein (VAMP2) and the plasma membrane t-SNARE proteins (syntaxin 4 and SNAP23) results in the docking or tethering of the GLUT4/IRAP vesicles to the plasma membrane; (iv) although not required for docking, Munc18c apparently facilitates the final plasma membrane fusion step through an as yet undefined mechanism.

does not arise due to a decrease in insulin signaling and therefore probably lies distal to these events.

We therefore hypothesized that expression of a reversible temperature-sensitive Munc18c mutant could be used to distinguish between the GLUT4/IRAP vesicle trafficking, docking and fusion. This is based upon the ability of overexpressed Munc18c/WT to prevent GLUT4/IRAP vesicle docking by occluding the syntaxin 4 binding sites, without having any effects on insulin receptor signaling or trafficking (Figure 10). Thus by stimulating cells at the permissive temperature (23°C), we were able to activate both the signaling and trafficking pathways but prevented GLUT4/IRAP vesicle docking. Release of this blockade by temperature shift to the non-permissive temperature (37°C) would then allow for vesicle progression to the dock and fused state. If docking and/or fusion were the rate-limiting step, then release of the temperature block would have resulted in an identical rate of translocation in cells pre-incubated with insulin at 23°C compared with those stimulated with insulin at 37°C. However, under these conditions the pre-incubation with insulin resulted in an accelerated translocation process, indicating that either

the trafficking or the signaling steps were rate limiting. Based upon the apparent relatively normal rates of proximal signal transduction at 23°C compared with 37°C, we conclude that the rate-limiting step for insulin-stimulated GLUT4/IRAP-containing vesicle translocation is trafficking to the plasma membrane.

In summary, we report here the synthesis of a temperature-sensitive form of Munc18c protein that specifically inhibits GLUT4/IRAP storage vesicle translocation at 23°C but not at 37°C. This mutation appears to cause a conformational change in the protein such that at the non-permissive temperature it can no longer bind to syntaxin 4. Insulin pre-incubation of adipocytes expressing the Munc18c/TS mutant at 23°C results in an accelerated rate of GLUT4 translocation compared with cells stimulated at 37°C, suggesting that vesicle trafficking is the rate-limiting step in GLUT4/IRAP translocation. The availability of a reversible temperature-sensitive mammalian Munc18c protein will now allow us to probe further into the molecular details of the SNARE complex directly involved in the GLUT4/IRAP vesicle fusion process.

Materials and methods

Materials

The polyclonal GLUT4, syntaxin 4 and Munc18c antibodies were obtained as described previously (Olson *et al.*, 1997). Rabbit polyclonal IRAP antibody was kindly provided by Dr Steven Waters (Metabolex, Inc., Hayward, CA). Monoclonal antibodies to the Flag epitope (M2 and M5) and cycloheximide were purchased from Sigma (St Louis, MO). Vectashield was obtained from Vector Laboratories (Burlingame, CA). Mini-prep DNA and DNA agarose extraction kits were purchased from Qiagen (Santa Clarita, CA). Other chemicals were reagent grade or the best quality commercially available.

Plasmids

The EGFP-Flag-Munc18c, GLUT4-EGFP and EGFP-IRAP constructs were prepared as described previously (Thurmond *et al.*, 1998). The name of the EGFP construct denotes the position of the EGFP as either amino (EGFP-IRAP) or carboxyl (GLUT4-EGFP) terminal fusions, respectively. The full-length syntaxin 4 cDNA was obtained from Dr Richard Scheller (Stanford University) and PCR primers directed against the 5' and 3' UTRs were used to amplify the DNA for subcloning into the *EcoRI* and *XhoI* sites of pcDNA3 (Invitrogen). The pcDNA3.1-Flag-Munc18c construct described previously (Thurmond *et al.*, 1998) was modified to include an N-terminal Kozak sequence and start codon upstream of the Flag epitope tag to ensure a high level of expression. Synthetic complementary oligonucleotides of the following sequence were inserted into the *EcoRI* and *SacII* restriction sites to create the in-frame Kozak/start sequence/Flag tag: 5'-AATTCATGGATTATAAGATGATGATGATAAAGCCGC-3'. The resulting cDNA was inserted into the *EcoRI* and *XhoI* sites of pGEX4T-1 so that the 106 bp region between the *StuI* and *NdeI* sites in the Munc18c/WT cDNA could be replaced with the same region containing the R240K temperature-sensitive (TS) point mutation. The mutated region was made by annealing synthetic complementary oligonucleotides. The mutated pGEX4T-1-Munc18c cDNA was excised and inserted into the pcDNA3.1 vector, creating pcDNA3-Munc18c/TS. The pcDNA3-Munc18c/WT and pcDNA3-Munc18c/TS plasmids were sequenced to verify identical construction and the presence of the R240K point mutation. The full-length cDNA encoding the cation-independent mannose-6-phosphate/IGF-II receptor was obtained from Dr Richard Roth (Stanford University, Stanford, CA) in the pECE vector. To create the CI/MPR-EGFP construct, the entire cDNA (8.4 kb) was excised by partial digestion with *EcoRI* and inserted upstream of the EGFP cDNA using the *EcoRI* site of pEGFP-N3 (Clontech). The CI/MPR stop codon and 3' UTR were removed by digestion with *NdeI* and *SalI*, which also removed the transmembrane domain. The transmembrane domain DNA fragment was then synthesized by PCR for direct reinsertion into the *NdeI-SalI* sites, and all junctions of the construct were sequenced to verify continuity and frame.

Cell culture and transient transfection

3T3L1 preadipocytes were purchased from the American Type Tissue Culture repository, differentiated into adipocytes and transfected by electroporation as described previously (Thurmond *et al.*, 1998). As indicated in the figure legends, co-transfected experiments were performed using 50 µg of EGFP-tagged plasmid DNA plus 50–200 µg of additional plasmid DNA for analysis of EGFP fluorescence (see figure legends). Following electroporation, the cells were allowed to adhere to cover slips in 35-cm tissue culture dishes for 18 h and were then serum starved for 2 h prior to temperature studies and stimulation with 100 nM insulin. Temperature studies were carried out in Boekel incubators supplied with 8% CO₂ in a 4°C cold room. Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR) were obtained as previously described (Waters *et al.*, 1995). CHO/IR cells were incubated in Minimal Eagle's Medium (MEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Fully confluent CHO/IR cells were transiently transfected by electroporation (0.34 kV and 960 µF) with 10–40 µg plasmid DNA per cuvette as described previously (Yamauchi and Pessin, 1994). Following electroporation, the cells were allowed to adhere to 10-cm tissue culture dishes for 40–48 h at 37°C and then shifted to either the permissive or non-permissive temperature for 4 h.

Plasma membrane sheet assay and confocal microscopy

Control and insulin-stimulated adipocytes were washed with ice-cold phosphate-buffered saline (PBS) and plasma membrane sheets were prepared as described previously (Robinson *et al.*, 1992). Cells were

treated with 0.55 mg/ml poly-L-lysine followed by three washes with hypotonic buffer (23 mM KCl, 10 mM HEPES pH 7.5, 1.7 mM MgCl₂, 1 mM EGTA). The cells were placed in sonication buffer (3× hypotonic buffer plus 100 µM PMSF and 1 mM dithiothreitol) and sonicated with a Fisher probe membrane disrupter. Plasma membrane sheets were washed three times with sonication buffer and fixed in 4% paraformaldehyde for 20 min on ice. The fixed plasma membrane sheets were quenched for 15 min at room temperature in 100 mM glycine. The isolated plasma membrane sheets were washed and blocked with 5% donkey serum for 1 h at 37°C followed by incubation with GLUT4 antibody (1:100 dilution of antisera) for 30 min at room temperature. Plasma membrane sheets were then washed three times with PBS, incubated with Texas Red-conjugated donkey anti-rabbit secondary antibody for 30 min at room temperature, washed three times with PBS, overlaid with a drop of Vectashield and visualized by confocal fluorescence microscopy using a Bio-Rad MRC 600 laser confocal microscope.

Co-immunoprecipitation and immunoblotting

Whole cell detergent extracts were prepared by solubilization in an NP-40 lysis buffer (25 mM Tris pH 7.4, 1% NP-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium vanadate, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml leupeptin) for 10 min at 4°C. Insoluble material was separated from the soluble extract by microcentrifugation for 10 min at 4°C. Whole cell lysate (2–3 mg) was combined with 4.5 µg of Flag (M2) antibody for 2 h at 4°C followed by a second incubation with protein G-Sepharose for 1.5 h. The resultant immunoprecipitates were subjected to electrophoresis on 10% SDS-PAGE followed by transfer to PVDF and immunoblotting as described previously (Thurmond *et al.*, 1998).

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