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Demonstration of differential quantitative requirements for NSF among multiple vesicle fusion pathways of GLUT4 using a dominant-negative ATPase-deficient NSF

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Abstract

In this study, we investigated the relative participation of *N*-ethylmaleimide-sensitive factor (NSF) in vivo in a complex multistep vesicle trafficking system, the translocation response of GLUT4 to insulin in rat adipose cells. Transfections of rat adipose cells demonstrate that over-expression of wild-type NSF has no effect on total, or basal and insulin-stimulated cell-surface expression of HA-tagged GLUT4. In contrast, a dominant-negative NSF (NSF-D1EQ) can be expressed at a low enough level that it has little effect on total HA-GLUT4, but does reduce both basal and insulin-stimulated cell-surface HA-GLUT4 by ~50% without affecting the GLUT4 fold-translocation response to insulin. However, high expression levels of NSF-D1EQ decrease total HA-GLUT4. The inhibitory effect of NSF-D1EQ on cell-surface HA-GLUT4 is reversed when endocytosis is inhibited by co-expression of a dominant-negative dynamin (dynamin-K44A). Moreover, NSF-D1EQ does not affect cell-surface levels of constitutively recycling GLUT1 and TfR, suggesting a predominant effect of low-level NSF-D1EQ on the trafficking of GLUT4 from the endocytic recycling compared to the intracellular GLUT4-specific compartment. Thus, our data demonstrate that the multiple fusion steps in GLUT4 trafficking have differential quantitative requirements for NSF activity. This indicates that the rates of plasma and intracellular membrane fusion reactions vary, leading to differential needs for the turnover of the SNARE proteins.
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The interaction between SNARE (soluble NSF attachment protein receptors) proteins from the vesicle (v-SNAREs) and target (t-SNAREs) membranes is an essential part of vesicle-membrane fusion [1]. NSF is an hexameric ATPase containing an N-terminal domain and two homologous ATP-binding domains, and it is required for numerous intracellular vesicle fusion processes [2]. NSF binds to the SNARE complex in the presence of α-SNAP and ATP under conditions. It is proposed that NSF uses the energy from ATP hydrolysis to disassemble SNARE complexes after membrane fusion so that the individual SNARE proteins can be recycled [2].

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Insulin stimulates glucose transport in adipose and muscle cells through the translocation of the GLUT4 glucose transporter isoform [3,4]. In the basal state, the majority of GLUT4 resides in an intracellular GLUT4-specific compartment (GSC). Insulin stimulation induces a rapid redistribution of GLUT4 from the intracellular pool to the plasma membrane. Studies on the subcellular trafficking of GLUT4 demonstrate that, in the insulin-stimulated state, GLUT4 continuously cycles through multiple intracellular compartments including early endosomes involved in GLUT4 internalization and GSC from which GLUT4 undergoes rapid exocytosis to the plasma membrane. The recruitment of an intrinsic membrane protein such as GLUT4 to the plasma membrane from an intracellular pool of vesicles is conceptually similar to neurosecretion as well as other regulated secretory processes.

In adipose cells, we and others have previously identified VAMP-2 and VAMP-3/cellubrevin in GSC, and two plasma membrane SNAREs, syntaxins 2 and 4 [5,6]. The following observations from previous studies demonstrate roles for SNARE proteins in GLUT4 translocation [5,7–10]. First, VAMPs are co-localized with GLUT4 and insulin stimulates the trafficking of VAMPs to the plasma membrane along with GLUT4 [5]. Second, both inactivation of VAMP-2 and VAMP-3/cellubrevin with tetanus toxin and inhibition of syntaxin 4 block insulin-stimulated GLUT4 translocation [9]. Finally, interference with the formation of 20S SNARE complexes by over-expressing SNAP23-Δ 8, which binds to syntaxin 4 but not to VAMP-2, inhibits insulin-induced translocation of GLUT4 [10]. These data strongly suggest that SNARE complex proteins are involved in insulin-stimulated GLUT4 translocation. However, the role of NSF in the SNARE complex-regulated GLUT4 translocation is assumed, but largely uncharacterized. Mastick and Falick [11] have investigated the potential role of NSF in GLUT4 trafficking by examining the association of NSF and SNAPs with GLUT4 vesicles in rat adipose cells. In their study, NSF and SNAPs are highly enriched in GLUT4 vesicles and low-density microsomes in the absence of insulin. Very little NSF and SNAPs are associated with the plasma membrane fraction. Insulin does not stimulate the translocation of NSF and SNAPs to the plasma membrane with GLUT4. These data suggest differential quantitative requirements for NSF activity among the various membrane fusion reactions comprising the complex multistep GLUT4 vesicle trafficking system, but the details are unknown.

To investigate the relative participation of NSF in the multiple fusion reactions comprising the trafficking of GLUT4, we expressed wild-type and mutant NSF in rat adipose cells. NSF-D1EQ is a dominant-negative ATP hydrolysis-deficient mutant, which binds SNARE complexes, but cannot disassemble them [12]. We have observed a differential sensitivity of the various fusion events of GLUT4 vesicle trafficking to disruption by the NSF mutant. Low-level expression of the NSF-D1EQ mutant predominantly influences intracellular membrane fusion events involved in GLUT4 cycling from the endocytic recycling compartment (ERC) to the GSC, but not biosynthesis and plasma membrane fusion.

Materials and methods

Plasmid constructs. NSF wild-type and NSF-D1EQ were generated in the pCIS2 mammalian expression vector. Construction of the HA-tagged GLUT4, HA-GLUT4-GFP, HA-GLUT1, and dominant-negative K44A dynamin1 (Dyn-K44A) in pCIS2 has been described previously [13–16]. The vpTfR construct was a generous gift from Dr. T.E. McGraw (Weill Medical College of Cornell University). For transfection experiments, the plasmids were purified using a maxiprep kit (Qiagen).

Cell culture and transfection of rat adipose cells. Preparation of isolated rat epididymal adipose cells from male rats (CD strain, Charles River Breeding Laboratories) was performed as described previously [13]. Isolated cells were washed twice with Dulbecco's modified Eagle's medium containing 25 mM glucose, 25 mM Hepes, 4 mM L-glutamine, 200 nM *N*-6-(2-phenylisopropyl)-adenosine, and 75 μg/ml gentamicin, and resuspended in a cytocrit of 40% (5–6×10^6 cells/ml). Two hundred microliters of the cell suspension was added to 200 μl of Dulbecco's modified Eagle's medium containing 100 μg of carrier DNA (sheared herring sperm DNA, Boehringer Mannheim) and expression plasmids as indicated. The total concentration of plasmid DNA in each cuvette was adjusted to 2 μg/ml for HA-GLUT4 and 18 μg/ml for dominant-negative K44A dynamin1. In time course experiments, HA-GLUT4 and NSF-D1EQ were co-transfected, and the total concentration of plasmid DNA in each cuvette was adjusted to 2 μg/ml for HA-GLUT4 and various concentrations of 0.4, 2, and 4 μg/ml for NSF-D1EQ. In experiments where HA-GLUT1 or TfR was co-transfected with NSF-D1EQ, the total concentration of plasmid DNA in each cuvette was adjusted to 2 μg/ml for HA-GLUT1 and TfR, and 0.4 μg/ml for NSF-D1EQ. Electroporation was carried out in 0.4-cm gap-width cuvettes (Bio-Rad) using a T810 square wave pulse generator (BTX). After applying three pulses (12 ms, 200 V), the cells were washed once in Dulbecco's modified Eagle's medium, pooled in groups of 4–10 cuvettes, and cultured at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium containing 3.5% bovine serum albumin.

Cell-surface antibody-binding assay. Rat adipose cells were harvested 20–24 h post-transfection and washed in Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, 200 nM adenosine (KRBH buffer) containing 5% bovine serum albumin. Samples corresponding to the cells from one cuvette were distributed into 1.5-ml microcentrifuge tubes. After stimulation with 67 nM (1×10^4 μU/ml) insulin for 30 min at 37 °C, subcellular trafficking of GLUT4 was stopped by the addition of 2 mM KCN. All of the following steps were performed at room temperature. A monoclonal anti-HA antibody (HA.11, Berkeley Antibody Co.) or anti-TfR antibody (Santa Cruz) was added at a dilution of 1:1000 or 1:10, respectively, and the cells were incubated for 1 h. Excess antibody was removed by washing the cells three times with KRBH, 5% bovine serum albumin. Then 0.1 μCi of ^{125}I sheep anti-mouse antibody (Amersham Pharmacia Biotech) was added to each reaction, and the cells were incubated for 1 h. Finally, the cells were spun through dinonylphthalate oil to remove the unbound antibody, and the cell-surface-associated radioactivity was counted in a γ-counter. The resulting counts were normalized to the lipid weight of the samples [17]. Unless stated otherwise, the values obtained for

pCIS-transfected cells were subtracted from all other values to correct for non-specific antibody binding. Antibody-binding assays were performed in duplicate or triplicate.

Membrane isolation and immunoblotting to detect expression of HA-GLUT4. Rat adipose cells were co-transfected with HA-GLUT4 (4 μg/ml) and NSF-D1EQ at the various concentrations of 0.4, 2, and 4 μg/ml. Transfected cells were harvested after 20 h of incubation. Crude total membrane fractions were prepared essentially as described previously [18]. Proteins in the crude total membranes were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Immunoblot analysis of HA-GLUT4 was performed with the monoclonal anti-HA antibody described above (1:1000 dilution) and 0.2 μCi/ml ^{125}I-protein A. The HA-GLUT4 signals were quantified using Image Gauge V3.12, Science lab 98 (Fuji Photo Film).

Confocal microscopy. For morphological analysis, electroporation was carried out using HA-GLUT4-GFP (8 μg/ml, 0.5 ml per cuvette) together with NSF-D1EQ (0–0.5 μg/ml). For the experiment of chronic insulin treatment, cells were cultured with or without 67 nM insulin for 20–24 h at 37 °C, 5% CO_2. For acute insulin treatment, cells were treated with or without 67 nM insulin for 30 min at 37 °C after culturing in the absence of insulin for 20–24 h. Cells were then fixed in a suspension of 4% paraformaldehyde (Electron Microscopy Sciences) in 0.15 M PBS, pH 7.4, for 20 min at room temperature, rinsed with PBS, and then mounted using Vectashield mounting medium (Vector Laboratories). GFP fluorescence was observed with an Optiphot 2 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Bio-Rad MRC1024 confocal laser scanning imaging system (CSLM, Bio-Rad Laboratories). Specimens were observed using a planapochromat 60×/1.4 NA oil immersion objective. To excite GFP, the 488 nm line of a mixed argon/krypton laser was used at 100% laser energy. All images were taken with Kalman averaging of four frames. For each cell, optical sections for a whole cell were recorded in 0.5-μm steps along the *Z*-axis and projected images were produced using Confocal Assistant 4.02 software.

Results

Effect of a dominant-negative ATPase-deficient NSF (NSF-D1EQ) on cell-surface translocation of HA-GLUT4

To determine the role of NSF in GLUT4 translocation, HA-GLUT4 and wild-type or mutant NSF were co-transfected into rat adipose cells, and cell-surface levels of HA-GLUT4 were detected by anti-HA antibody binding. The effect of NSF expression on the total expression of HA-GLUT4 was assessed by immunoblotting for the HA-epitope. Over-expression of wild-type NSF has no effect on cell-surface levels of HA-GLUT4 in the basal and insulin-stimulated states, nor on total HA-GLUT4 protein expression (data not shown). In contrast, as illustrated in Fig. 1, expression of the NSF-D1EQ mutant decreases both basal and insulin-stimulated cell-surface levels of HA-GLUT4 and total HA-GLUT4 in a dose-dependent manner. However, as compared with the total expression of HA-GLUT4, low-level expression of NSF-D1EQ mutant (0.25 μl/ml) reduces cell-surface HA-GLUT4 by about 50% in both the basal and insulin-stimulated states (Fig. 1A), with little reduction in total HA-GLUT4 expression (Fig. 1B). The reduction in total HA-GLUT4 is observed only when higher levels of the NSF-D1EQ mutant are expressed (Fig. 1B). Moreover, the fold GLUT4 translocation response to insulin is not changed by expression of the NSF-D1EQ mutant (Fig. 1A).

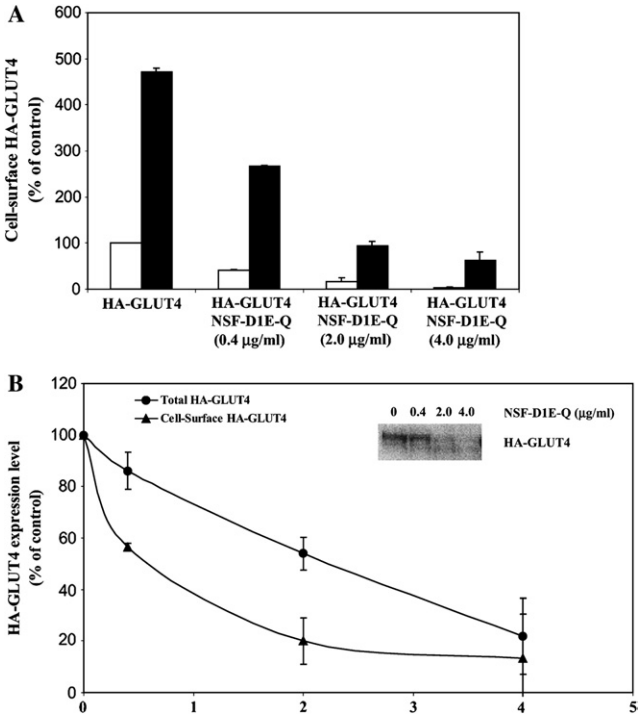

Fig. 1. A mutant NSF (NSF-D1EQ) differentially inhibits cell-surface and total HA-GLUT4 expression in a concentration-dependent manner. Rat adipose cells were co-transfected with 2 μg/ml HA-GLUT4 and various concentrations of NSF-D1EQ, and cultured for 20 h. After harvesting the cells, cell-surface levels of HA-GLUT4 were detected in the basal (open bars) and insulin-stimulated states (closed bars) using an HA antibody-binding assay (A). The cell-surface-associated radioactivity was normalized to the basal control. Total expression levels of HA-GLUT4 were determined by immunoblotting membranes with an anti-HA antibody. Cell-surface HA-GLUT4 was then compared to total expression levels of HA-GLUT4 (B). Results are means ± SEM of the mean values obtained from at least duplicate determinations in 3 independent experiments.

A dominant-negative dynamin GTPase (Dyn-K44A) reverses the inhibitory effect of NSF-D1EQ on cell-surface HA-GLUT4

The above data suggest that low-level expression of the NSF-D1EQ mutant may interfere with the intracellular trafficking of GLUT4. To further study the particular GLUT4 trafficking steps in which NSF might be involved, we used Dyn-K44A to examine whether NSF-D1EQ affects cell-surface accumulation of HA-GLUT4 when the endocytosis of GLUT4 is blocked. NSF-D1EQ, Dyn-K44A, and HA-GLUT4 were co-transfected into adipose cells, and cell-surface HA-GLUT4 was detected by anti-HA antibody-binding assay. Fig. 2 shows that Dyn-K44A-induced basal cell-surface HA-GLUT4 is not different between adipose

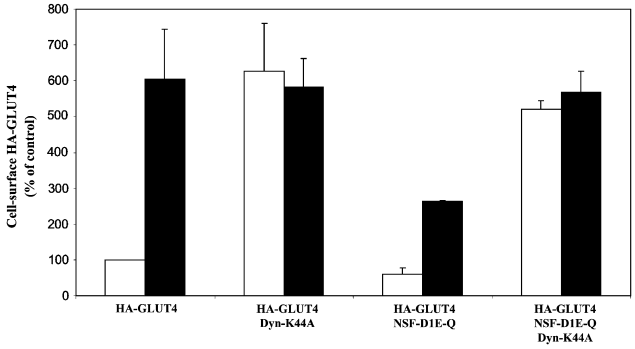

Fig. 2. A dominant-negative dynamin GTPase (Dyn-K44A) reverses the NSF-D1EQ inhibitory effect on cell-surface HA-GLUT4. Rat adipose cells were co-transfected with the indicated combinations of HA-GLUT4, 2 μg/ml; NSF-D1EQ, 0.4 μg/ml; and Dyn-K44A, 18 μg/ml. Cells were then cultured for 20 h. After harvesting the cells, cell-surface levels of HA-GLUT4 were detected in the basal (open bars) and insulin-stimulated states (closed bars) using an HA antibody-binding assay. The cell-surface-associated radioactivity was normalized to the basal control. Results are means ± SEM of the mean values obtained from at least duplicate determinations in three independent experiments.

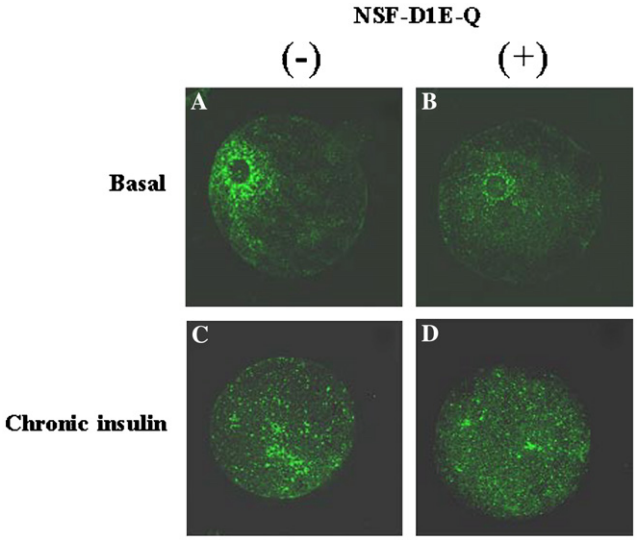

Fig. 3. Localization analysis of HA-GLUT4-GFP in rat adipose cells transfected with or without a dominant-negative NSF-D1EQ in the presence or absence of insulin. HA-GLUT4-GFP was co-transfected without (A) or with (B) NSF-D1EQ, 0.4 μg/ml, and the cells were cultured in the absence of insulin for 20 h. Co-transfected cells without (C) or with NSF-D1EQ (D) were treated with insulin during 20 h culture.

cells with and without low-level expression of NSF-D1EQ. These data indicate that GLUT4 is able to traffic to and fuse with the plasma membrane in the presence of low-level expression of NSF-D1EQ. Like control cells, cells transfected with NSF-D1EQ do not show a further increase in cell-surface HA-GLUT4 in response to insulin in the presence of Dyn-K44A.

Effect of low-level expression of NSF-D1EQ on the subcellular localization of HA-GLUT4-GFP

In order to further examine the effect of the NSF-D1EQ mutant on the subcellular localization of GLUT4, adipose cells were co-transfected with NSF-D1EQ and HA-GLUT4-GFP, and cultured in the presence or absence of insulin for 20 h. The subcellular localization of HA-GLUT4-GFP was then examined by confocal microscopy. Fig. 3 shows that after 20 h of culture in the absence of insulin, HA-GLUT4-GFP displays a concentrated localization in the perinuclear region of control cells (A). In cells co-transfected with HA-GLUT4-GFP and NSF-D1EQ, the accumulation of HA-GLUT4-GFP in the perinuclear region is significantly decreased, and the HA-GLUT4-GFP is more dispersed throughout the cytosol Fig. 3B. The presence of insulin during the entire period of 20-h culture also almost completely prevents the aggregation of HA-GLUT4-GFP in the perinuclear region, such that the subcellular distribution of HA-GLUT4-GFP is not different without and with co-transfected NSF-D1EQ Figs. 3C and D. These data indicate that the reduction of cell-surface GLUT4 by low-level expression of a dominant-negative NSF may be associated with a reduction in the recycling of internalized GLUT4 back to the insulin-responsive intracellular compartment.

Comparison of the NSF-D1EQ effects on cell-surface HA-GLUT4, TfR, and HA-GLUT1 in short-term culture

To further test the hypothesis that the recycling process of internalized-GLUT4 to the intracellular GSC is more dependent on NSF activity, we compared the NSF-D1EQ effect on HA-GLUT4 with those on TfR and HA-GLUT1, membrane proteins that constitutively recycle. Transfected cells were harvested after 4 h of culture and incubated for 30 min in the absence and presence of insulin. The levels of cell-surface HA-GLUT4, TfR, and HA-GLUT1 were then determined in intact cells by antibody-binding assays against the HA-epitope tag and the extracellular domain of the TfR. As illustrated in Fig. 4, similar to 20 h of culture, low-level expression of NSF-D1EQ for 4 h inhibits cell-surface expression of HA-GLUT4 in both basal and insulin-stimulated cells. As observed previously, similar cell-surface levels of TfR and HA-GLUT1 are seen in the basal and insulin-stimulated states. Furthermore, low-level expression of NSF-D1EQ does not affect the cell-surface expression of TfR and HA-GLUT1, again in either the presence or absence of insulin. Finally, we cultured HA-GLUT4-transfected cells in the presence of insulin for 4 h to provoke constitutive recycling. We found that chronic insulin itself reduces cell-surface expression of HA-GLUT4, but that low-level co-expression of

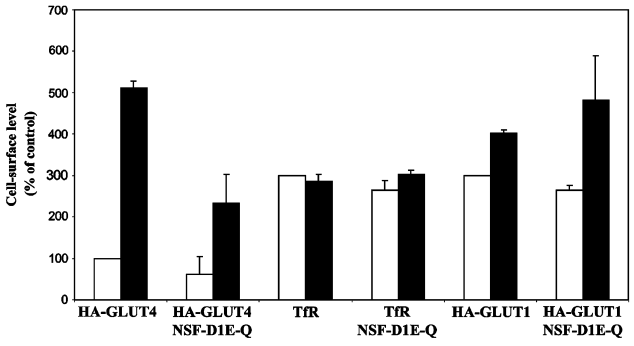

Fig. 4. A mutant NSF (NSF-D1EQ) differentially affects cell-surface expression of HA-GLUT4, TfR, and HA-GLUT1. Rat adipose cells were co-transfected with the indicated combinations of HA-GLUT4, 2.0 μg/ml; TfR, 2.0 μg/ml; HA-GLUT1, 2.0 μg/ml; NSF-D1EQ, 0.4 μg/ml. Cells were then cultured for 4 h. After harvesting the cells, cell-surface levels of HA-GLUT4, TfR, and HA-GLUT1 were detected in the basal (open bars) and insulin-stimulated states (closed bars) using antibody-binding assays. The cell-surface-associated radioactivity was normalized to the basal control. Results are means ± SEM of the mean values obtained from at least duplicate determinations in two independent experiments.

NSF-D1EQ does not have any additional inhibitory effect (data not shown).

Discussion

NSF-mediated SNARE disassembly has been implicated in many cellular fusion reactions, of both constitutive and regulated pathways. The precise role of NSF in the docking/fusion/recycling steps of membrane fusion has been studied in a number of eukaryotic cells [19]. In this study, we investigated the relative participation of NSF in vivo in a complex multistep vesicle trafficking system, the translocation response of GLUT4 to insulin in rat adipose cells. Using NSF and dynamin mutants, we have shown that the ATPase activity of NSF is required for both cell-surface localization of GLUT4 and the biosynthesis of GLUT4, but that the latter is less sensitive to a loss of NSF activity than is GLUT4 trafficking. Furthermore, a comparison of NSF's effects on the trafficking of TfR, GLUT1, and GLUT4 demonstrates that NSF activity is not critical for exocytosis and the initial steps of endocytic recycling of GLUT4. Rather, the NSF-D1EQ-induced decrease in cell-surface GLUT4 appears to impair endocytic recycling of GLUT4 from ERC to GSC.

The formation of an hexamer of NSF is crucial for the activation of NSF and introduction of a single inactive subunit of NSF can poison an hexamer [20]. The mutant NSF-D1EQ shows concentration-dependent effects on both the biosynthesis and trafficking of GLUT4. The loss of total HA-GLUT4 as the concentration of co-transfected NSF-D1EQ is increased (Fig. 2B) appears to be indicative of a defect in the biosynthetic processing of GLUT4 to and through the Golgi that leads to accumulation in the ER and subsequent degradation. However, since mutant NSF presumably also blocks lysosome delivery, the degradation of GLUT4 would have to occur through another pathway. For instance, it has been recently reported that GLUT4 can be degraded through the proteasome pathway and that this process is regulated by sentrin-conjugating enzyme mUbc9 [21].

Nevertheless, low-level expression of NSF-D1EQ significantly diminishes the cell-surface levels of HA-GLUT4 without affecting either total HA-GLUT4 protein expression or insulin-stimulated translocation of HA-GLUT4 to the plasma membrane. These observations suggest that the former is more sensitive to NSF ATPase activity than the latter. This would be consistent with the morphological data seen in Fig. 3 demonstrating that NSF-D1EQ prevents the aggregation of HA-GLUT4-GFP in the perinuclear region of the cell, maintaining the normal diffuse appearance, with overnight culture. Such differential sensitivity in the different trafficking steps could possibly depend upon the rate and the total number of NSF-required membrane fusion reactions. For instance, the sorting of biosynthesized GLUT4 from ER to Golgi and the basal rate of exocytosis of GLUT4 from GSC to the plasma membrane may be less sensitive to NSF-D1EQ inhibition due to a slow rate of trafficking, i.e., a low rate of membrane fusion, whereas post-Golgi trafficking of GLUT4, including endocytosis and intracellular sorting, is more sensitive to the inhibitory activity of NSF-D1EQ because a rapid recycling is required.

In general, endocytic recycling pathways consist of several steps including clathrin-coated vesicle formation at the plasma membrane, formation of sorting endosomes, and transport of molecules to their correct destinations [22]. In adipose cells, the endocytic itinerary of GLUT4 after ERC has been proposed to be transport to GSC [22]. McGraw's group has demonstrated that the intracellular cycling of GLUT4 between the GSC and the endosomes takes much less time than that to fuse with the plasma membrane in 3T3-L1 adipocytes [23], indicating that intracellular cycling of GLUT4 is relatively rapid. In addition, studies on the subcellular localization of NSF and quantification of SNARE proteins in the intracellular compartments suggest that the total number of intracellular fusion reactions is larger than the total number of plasma membrane fusion reactions. For example, one study has shown that the NSF and SNAPs are highly enriched in GLUT4 vesicles and low-density microsomes (LDM) under steady state conditions, and that this association is not changed by insulin stimulation [11]. Another study has demonstrated that the endosomal v-SNARE cellubrevin is expressed at 10-fold higher levels than syntaxin 4 or

VAMP-2, the latter believed to be involved in plasma membrane fusion events [24]. In these two cases, it is reasonable to consider that rapid or extensive intracellular fusion reactions of GLUT4 require more NSF.

Dynamin is an important GTPase required for the pinching off of the clathrin-coated vesicle and therefore involves the initial step of the endocytic recycling [25]. In previous studies, we have observed in adipose cells that dynamin regulates the rate of GLUT4 endocytosis [15]. In the present study, Dyn-K44A was used to test whether NSF activity is necessary for the fusion of GLUT4 to the plasma membrane and the first step of endocytic recycling. Our data show that Dyn-K44A leads to an increase in basal cell-surface GLUT4 such that insulin stimulation produces little increase. This result argues that the bulk of the GLUT4 is "trapped" on the cell-surface, suggesting that GLUT4 is trafficked to the plasma membrane even when the NSF-D1EQ is expressed, but perhaps at a slower rate. Thus, when Dyn-K44A blocks the return of GLUT4 from the plasma membrane, plasma membrane accumulation is seen even with the lower level of trafficking in the presence of NSF-D1EQ expression. The effect of Dyn-K44A overcomes the effect of the mutant NSF. This observation supports the conclusion stated above regarding relative trafficking rates and fits with the work of Ganetzky's group looking at NSF and dynamin in *Drosophila* synaptosomes [19]. In the Ganetzky report, use of a dynamin mutant to slow endocytosis caused a reversal of the accumulation of SNARE complexes seen with the NSF mutants.

To further address the possibility that the requirement for NSF is specific for different membrane trafficking and recycling processes, we compared the effects of NSF-D1EQ on GLUT4 to those on GLUT1 and TfR. GLUT4, GLUT1, and TfR share the same initial endocytic pathways from the plasma membrane to the ERC. From the endosome system, they traffic to distinct compartments, for instance, GLUT1 and TfR return back to cell surface while GLUT4 go to GSC [22,26]. Co-expression of NSF-D1EQ does not affect TfR or GLUT1 cell-surface expression. These data suggest that the trafficking of GLUT4 is more sensitive to overall cellular trafficking rates, perhaps requiring rapid or extensive transit that is not required for GLUT1 and TfR. This explanation fits with the observation reported by Yang et al. [27] that TfR recycling is not dependent on VAMP-3, suggesting that fewer SNARE proteins and a lower NSF requirement are involved in this process. It could mean that the requirements for GLUT4 transit are limiting and must be recycled by active NSF. In this case, the secretory machinery needed to move GLUT4 at any one of the transit steps is not recycled because NSF is not active. This model could also explain the inhibitory effect of mutant NSF on basal cell-surface GLUT4 expression since GLUT4 has been proposed to constitutively cycle between endosomes and the GSC in the basal state in 3T3-L1 adipocytes [28].

It has been reported that insulin minimally affects the rate of TfR endocytic recycling [29], while insulin significantly inhibits the movement of GLUT4 through the endocytic recycling system [30,31]. To further confirm that NSF activity is important for the rapid and extensive endocytic recycling from ERC to GSC, insulin was used during 20 h of culture. Interestingly, the presence of insulin diminishes the appearance of GLUT4 in the perinuclear region in normal adipose cells (Fig. 3), indicating that recycling from endosome to GSC is inhibited. In this condition, however, the effect of NSF-D1EQ on cell-surface GLUT4 levels is not observed by morphological examination (Fig. 3) and an anti-HA antibody-binding assay (data not shown). This suggests that the NSF mutant loses its inhibitory effect on cell-surface GLUT4 expression when GLUT4 trafficking skips the rapid and extensive reactions which requires more NSF activity. These data further support that NSF ATPase activity is required for endocytic recycling of GLUT4 from ERC to GSC.

Taken together, the data presented here have led to a model for how NSF participates in the intracellular trafficking of GLUT4 vesicles in adipose cells. The distinct steps of GLUT4 vesicle trafficking display differential requirements for NSF activity possibly based on their different rates and the number of membrane fusion reactions. Trafficking of GLUT4 to the plasma membrane is less sensitive to loss of NSF activity, perhaps due to a slower rate and fewer number of membrane fusion reactions. Trafficking from endosomal system to GSC is more dependent on NSF activity, indicating that this movement is rapid and extensive, and requires more turnover of the SNARE proteins responsible for the membrane fusion steps. Despite the conceptual advance suggested by our data, it is clear that further studies are needed to directly assess the movement of GLUT4 vesicles between endosomes and GSC, and to determine whether the requirement for NSF depends upon the rate of vesicle movement or membrane fusion.

Acknowledgments

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