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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