Perturbation of Dynamin II with an Amphiphysin SH3 Domain Increases GLUT4 Glucose Transporters at the Plasma Membrane in 3T3-L1 Adipocytes

DYNAMIN II PARTICIPATES IN GLUT4 ENDOCYTOSIS*

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The GLUT4 glucose transporter continuously recycles between the cell surface and an endosomal compartment in adipocytes. Insulin decreases the rate of GLUT4 endocytosis in addition to increasing its exocytosis. Endocytosis of the transporter is thought to occur at least in part via the clathrin-mediated endocytic system. The protein dynamin is involved in the final stages of clathrin-coated vesicle formation. Here we show that the dynamin II isoform is enriched in 3T3-L1 adipocytes and is present in isolated plasma membrane and low density microsomal fractions. Insulin reduced the levels of dynamin II associated with the plasma membrane by about half, raising the possibility that the hormone may reduce GLUT4 endocytosis by removing dynamin from the cell surface. A fusion protein containing the amphiphysin SH3 domain selectively bound dynamin II from 3T3-L1 adipocyte cell lysates. Microinjection of the fusion protein into these cells inhibited transferrin endocytosis and increased the levels of GLUT4 at the cell surface. Glutathione S-transferase alone, the SH3 domains of spectrin and Crk, and a mutated amphiphysin SH3 domain unable to bind dynamin II did not affect GLUT4 distribution. However, a peptide containing the dynamin II sequence that binds amphiphysin increased the surface presence of GLUT4. Moreover, in cells first treated with insulin to externalize GLUT4, the dynamin peptide, but not an unrelated control peptide, inhibited GLUT4 internalization upon insulin removal. These results suggest that interactions of dynamin II with amphiphysin may play an important role in GLUT4 endocytosis. We hypothesize that insulin may reduce GLUT4 endocytosis by regulating the function of dynamin II at the cell surface, as part of the mechanism to increase glucose uptake.

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The GLUT4 glucose transporter is the major insulin-responsive transporter of muscle and fat tissues. In the basal state, it is largely sequestered in an intracellular compartment(s); however, several studies have shown that the transporter constitutively recycles between the cell surface and the intracellular loci (1–3).

There is ample evidence suggesting that the GLUT4 glucose transporter is internalized via clathrin-coated pits. GLUT4 has been localized to clathrin-coated regions by immunocytochemistry of plasma membrane lawns (4) and by immunoblotting of isolated clathrin-coated vesicles (5). Furthermore, lowering the intracellular K+ concentration, which disassembles clathrin lattices and prevents their reassembly, causes an accumulation of GLUT4 at the cell surface, presumably as a result of inhibiting the endocytic arm of its continuous recycling (6). Within its N-terminal domain, GLUT4 contains a putative internalization motif (FQIQ) that is similar to the internalization consensus sequence XXXΦ (where Φ represents any amino acid and Φ represents bulky hydrophobic residues) present in numerous recycling proteins (7). GLUT4 also contains a dileucine motif near its C terminus. Both motifs have been shown to contribute to the internalization of GLUT4 (8–13).

Upon insulin stimulation, GLUT4-containing vesicles are mobilized to the cell surface (14–16). In addition to increasing the rate of exocytosis of transporters, insulin also increases GLUT4 surface levels by reducing their endocytosis. Using an impermeant photoactivable glucose analog, it was observed that insulin lowered the rate of internalization of GLUT4 in rat adipocytes by 2.8-fold (1). A similar conclusion was reached when the amount of GLUT4 at the cell surface was measured by taking advantage of its exofacial trypsin-sensitive site in 3T3-L1 adipocytes (17). Moreover, insulin reduced the amount of GLUT4 associated with plasma membrane-derived clathrin-coated vesicles in the same cells (4, 5).

The final event in the formation of the clathrin vesicles at the plasma membrane is the periplasmic fusion at the neck of the newly formed pit. This is thought to involve the 100-kDa GTPase dynamin since transfection of a dominant-negative mutant dynamin I, unable to bind and hydrolyze GTP, results in inhibition of clathrin-mediated endocytosis in mammalian cells (18–21). Furthermore, GTPγS1 was found to arrest synaptic vesicle endocytosis at the stage of invaginated clathrin-coated pits and to induce an accumulation of dynamin at the

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1 The abbreviations used are: GTPγS, guanosine 5′-O-(3-thiotriphosphate); PM, plasma membrane(s); LDM, low density microsome(s); HDM, high density microsome(s); GST, glutathione S-transferase; PBS, phosphate-buffered saline solution.
neck of these structures (22). In addition to a GTP-binding domain, dynamin I contains a pleckstrin homology domain and a proline-rich region at its C terminus that binds a specific subset of SH3 domain-containing proteins (23–29). One of the proteins that binds with great specificity to the proline-rich region of dynamin is amphiphysin (27). This interaction with amphiphysin is thought to play a role in the recruitment of dynamin to sites of endocytosis (27, 28, 30). In this study, we have investigated the effect of disrupting SH3-mediated interactions of dynamin on the steady-state distribution of GLUT4 in 3T3-L1 adipocytes by microinjecting the SH3 domain of amphiphysin or a peptide of dynamin containing the amphiphysin-binding region.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—Mouse monoclonal anti-dynamin II-specific antibody was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-GLUT4 antibody was from Charles River Laboratories (Southbridge, MA). Monoclonal anti-clathrin heavy chain antibody was from Boehringer Mannheim (Laval, Quebec, Canada). Monoclonal anti-α-,α-Na/K-ATPase antibody 6H was a gift from Dr. M. Caplan (Yale University, New Haven, CT). Rhodamine-dextran (M, 10,000) was from Molecular Probes, Inc. (Eugene, OR).

**Tissue Culture and Subcellular Fractionation**—3T3-L1 fibroblasts were grown and induced to differentiate as described previously (31). Four to six days after differentiation, cultures were deprived of serum for 2 h and stimulated with 100 nm insulin for 20 min at 37 °C, as indicated in the figure legends. Total membranes and subcellular fractions (PM, LDM, HDM, and cytosol) from control and insulin-stimulated cells were prepared as described previously (31), except that cell breakage was performed with 10 cycles through a ball-bearing cell cracker (32) with a clearance of 0.0016 inches using 5 ml of homogenization buffer/10-cm dish. 3T3-L1 cell fractions and rat brain microsomes (33) were resolved by SDS-polyacrylamide gel electrophoresis and immobiloblated essentially as described earlier (34) using dynamin II, GLUT4, and clathrin heavy chain antibodies at 1:250, 1:1000, and 1:500 dilutions, respectively.

**Generation of Fusion Proteins and Peptides**—A fusion protein comprising the SH3 domain of amphiphysin 1 linked to GST (construct V (residues 545–695), here called GST-AmphiSH3) was generated as described previously (27, 35). A fusion protein expressing two mutations (G684R and P867L) in the amphiphysin 1 SH3 domain (GST-AmphiSH3M) was generated as described above for GST-AmphiSH3. Proteins encoding the SH3 domains of spectrin and Crk were generous gifts from Dr. A. Pawson (Mount Sinai Hospital, Toronto) (23). A 15-oligomer peptide (PPPQVPSRPNRAPG) representing amino acids 828–842 of dynamin II was a gift from Dr. A. Pawson (Mount Sinai Hospital, Toronto) (23). A 15-oligomer peptide (CVRRASEPGNRKGRLGNEK) (generous gift synthesized (30). This sequence is highly conserved in dynamin II, and is thought to be an amphiphysin-binding region.

**Dynamin Participates in GLUT4 Endocytosis**

Three mammalian dynamin genes have been cloned, each with several alternatively spliced forms (37, 38). Dynamin I is expressed exclusively in brain (39); dynamin II is present in brain, testis, and lung (40, 41); and dynamin II is found to varying degrees in all tissues tested (42, 43). Dynamin I could not be detected in 3T3-L1 adipocytes using an isoform-specific antibody (data not shown). Using an antibody specific to dynamin II, we examined whether this protein is present in these cells. In Fig. 1A, the content of dynamin II is compared in total membranes from

Dynamin was microinjected into 3T3-L1 adipocytes on glass coverslips placed in 6-well dishes. Coverslips were placed in coverslip chambers (Medical Systems Corp., Greenvale, NY) containing 1 ml of RPMI 1640 medium (R 4130 Sigma) supplemented with 20 mM HEPES and 4% fetal bovine serum. A region of ~1 mm² on the bottom of the coverslip was marked for subsequent microinjection on the stage of a Nikon fluorescence microscope resting on a Newport BenchTop vibration isolation system fitted with an Eppendorf microinjection unit (Micromanipulator 5171 and Transjector 5246). Bicarbonate microinjection pipettes (World Precision Instruments, Inc.) were pulled using a Sutter Instrument Flaming/Brown micropipette puller (Model P-97). The majority (~90%) of the cells in the marked area (typically 100–150 cells) were microinjected with a solution containing 2.0 mg/ml GST, GST-AmphiSH3, GST-AmphiSH3m, GST-spectrinSH3, or GST-CrkSH3, plus rhodamine-dextran (1.1 mg/ml) in microinjection buffer (110 mM potassium acetate, pH 7.2, 10 mM HEPES, 1 mM EDTA). The molar concentrations of these proteins in the microinjection solution were 75, 42, 41, 61, and 61 μg/ml, respectively. When the dynamin peptide or the unrelated peptide was microinjected, the concentration of the peptide in this buffer was 17 mg/ml. The medium was then changed to Dulbecco’s minimal essential medium containing 10% fetal bovine serum, and cells were incubated at 37 °C for 30 min, followed by a 2-h incubation in Dulbecco’s minimal essential medium alone. Cells in the injected and non-injected areas of the same coverslip were photographed 1.5 h after microinjection with phase-contrast and fluorescence optics under a 40× objective.

**Plasma Membrane Lawns and Confocal Microscopy**—Plasma membrane lawns (sheaths) were prepared by a modification of the procedure of Robinson et al. (44). Following the various treatments, cells were placed on ice and washed twice in ice-cold PBS. Hypotonic swelling buffer (23 mM KCl, 10 mM HEPES, 2 mM MgCl₂, 1 mM EDTA, pH 7.5) was added in three quick rinses. Five milliliters of breaking buffer (70 mM KCl, 30 mM HEPES, 5 mM MgCl₂, 3 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonfluoride, 1 μM pepstatin A, pH 7.5) was added, and the solution was aspirated up and down using a 1.0-ml pipetter to promote cell breakage. The adhered plasma membrane lawns were washed three times in breaking buffer and incubated with cold 3% paraformaldehyde in breaking buffer for 10 min on ice. The coverslips were washed three times in PBS, and excess fixative was quenched with 50 ml NH₄CUBPS for 5 min, followed by three washes with PBS at room temperature. The lawns were subsequently blocked by a 1-h incubation in 5% goat serum in PBS at room temperature. Labeling with rabbit anti-GLUT4 antisera (1:150) for 30 min at room temperature ensued, followed by three washes with PBS and labeling with fluorescein isothiocyanate-conjugated goat anti-rabbit (1:250 dilutions) and anti-sense (1:50) for 30 min. Immunolabeled lawns were rinsed four times with PBS and mounted with ProLong Antifade mounting solution (Molecular Probes, Inc.). Confocal images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 63× objective. All images were collected under identical gain settings established in pre-experiments. Confocal images were processed with Adobe Photoshop and analyzed with Metamorph software (Universal Imaging) with NIH Image software (45). For quantification, the fluorescence/unit area of each lawn was measured in two to four fields of microinjected and non-injected cells (each field contained 8–15 lawns). The results were collected in arbitrary units, and the S.E. for each experimental condition was calculated. Because each experimental condition had its own control (basal non-injected cells), once the S.E. values were calculated for the fluorescence intensity/unit area, the data were normalized to that control. Statistical analysis was by analysis of variance.

**Transferrin Endocytosis**—Monolayers of 3T3-L1 fibroblasts or adipocytes on glass coverslips were microinjected with GST-AmphiSH3 or GST-AmphiSH3m as described above, except that detection of injection was achieved by co-injection of lucifer yellow (0.5 mg/ml). Following microinjection, cells were serum-deprived for 1 h and then exposed to 0.25 μg/ml rhodamine-labeled transferrin for 1 h at 37 °C (18, 36). Control experiments were performed at 4 °C. Coverslips were washed three times in PBS, fixed in 3% paraformaldehyde/PBS for 1 h, and mounted as described above. Confocal images were obtained as above using a 63× objective.

**RESULTS**

Three mammalian dynamin genes have been cloned, each with several alternatively spliced forms (37, 38). Dynamin I is expressed exclusively in brain (39); dynamin II is present predominantly in testis, with some microinjection present in lung and brain (40, 41); and dynamin II is found to varying degrees in all tissues tested (42, 43). Dynamin I could not be detected in 3T3-L1 adipocytes using an isoform-specific antibody (data not shown). Using an antibody specific to dynamin II, we examined whether this protein is present in these cells. In Fig. 1A, the content of dynamin II is compared in total membranes from...
Dynamin Participates in GLUT4 Endocytosis

Fig. 1. Subcellular distribution of dynamin II in 3T3-L1 cells. A, total membranes (TM) from undifferentiated 3T3-L1 fibroblasts (F) and differentiated 3T3-L1 adipocytes (A) were prepared, resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the anti-dynamin II-specific antibody. B, 3T3-L1 adipocytes, either unstimulated (−) or stimulated with insulin for 20 min (+), were subfractionated as described under “Experimental Procedures.” Equal protein concentrations (20 μg) of LDM, PM, HDM, and cytosolic proteins (cyto) were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted to detect the proteins indicated on the right. Rat brain microsomes (Br) were also probed as indicated. The numbers on the left represent molecular masses (in kilodaltons). The figure illustrates one of three to four independent fractionation experiments. HC, heavy chain.

undifferentiated 3T3-L1 fibroblasts and differentiated 3T3-L1 adipocytes. The protein was readily detected at both stages of differentiation to comparable extents. By comparison, relatively little dynamin II was detected in total brain microsomes.

Dynamin II localization in 3T3-L1 adipocytes was further analyzed by subcellular fractionation. Fig. 1B shows the presence of different proteins in subcellular fractions isolated from unstimulated (basal) and insulin-stimulated 3T3-L1 adipocytes. Clathrin was found largely in the cytosol and to varying degrees in the membrane fractions. Its distribution was not affected by insulin stimulation. As expected, the cell-surface marker α1-Na/K-ATPase was found almost exclusively in the PM, and its distribution was also not affected by insulin stimulation. Dynamin II was detected in all membrane fractions, but was scarce in the cytosol. The concentration of dynamin II/unit protein was similar in the PM, LDM, and HDM fractions from unstimulated cells. Interestingly, insulin stimulation resulted in a decrease in dynamin II content in the PM of 44 ± 10% (n = 4), with small gains in both the LDM and HDM. In contrast, and confirming previous reports, the GLUT4 glucose transporter was most abundant in the LDM fraction in unstimulated cells, and its concentration dropped in this fraction and increased in the PM as a result of insulin stimulation.

To assess whether dynamin plays a role in GLUT4 traffic, we attempted to interfere with its action by using the fusion protein GST-AmphiSH3, a construct that was recently shown to block synaptic vesicle endocytosis in lamprey axons (30). To this effect, it was important to establish that this fusion protein, which encompasses the SH3 domain of the human amphiphysin 1 cDNA, can indeed bind the dynamin isoform present in 3T3-L1 adipocytes. Therefore, Triton X-100 lysates of 3T3-L1 adipocytes were incubated with glutathione-containing agarose beads bound to either GST or GST-AmphiSH3. The 45- and 30-kDa bands in the GST-AmphiSH3 lane reacted with an antibody to GST and are therefore likely to be degradation products of the fusion protein. The 100-kDa band did not react with this antibody. B, immunoblot of 3T3-L1 adipocyte proteins bound to GST and GST-AmphiSH3 using anti-dynamin II-specific antisera. C, immunoblot of 3T3-L1 adipocyte proteins bound to GST-AmphiSH3 and GST-AmphiSH3m using anti-dynamin II-specific monoclonal antibody. The mutant AmphiSH3 domain is unable to interact with dynamin II in vivo.

with the anti-GST antibody and are therefore likely degradation products of the fusion protein. The experiment was repeated using GST-AmphiSH3m (containing the point mutations G684R and P687L). No dynamin was sedimented by this construct (Fig. 2C), demonstrating the specificity of GST-AmphiSH3 to bind this protein.

To verify that GST-AmphiSH3 interferes with endocytosis of proteins that internalize via clathrin-coated pits, we incubated 3T3-L1 adipocytes with fluorescently labeled transferrin. The presence of intracellular transferrin was used as a measure of its endocytosis. Under the conditions of this assay, very little transferrin is left at the cell surface upon washing prior to fixation (18, 36). The internalized fluorescence was examined in cells that were microinjected with GST-AmphiSH3 as well as in the non-injected cells on the same coverslips (Fig. 3). On coverslips incubated at 37 °C, the distribution of transferrin in
the non-injected cells was punctate within the cytoplasm and concentrated in the perinuclear region. On the same coverslips, cells microinjected with GST-AmphiSH3 showed a marked reduction in intracellular transferrin; the perinuclear staining was no longer observed; and the cytoplasmic staining was equal to that observed in non-injected cells incubated at 4 °C. Control experiments with 3T3-L1 fibroblasts confirmed that GST-AmphiSH3 prevented transferrin endocytosis and that this effect was not reproduced by GST-AmphiSH3m (data not shown).

GST-AmphiSH3 was then microinjected into 3T3-L1 adipocytes to assess its effects on cell-surface GLUT4 levels. The microinjected 3T3-L1 adipocytes were allowed to recover for 30 min and deprived of serum for 2 h prior to generation of plasma membrane lawns. Fig. 4A illustrates one experiment representative of three. As anticipated, in a non-injected region of the coverslip (first column), the level of GLUT4 immunofluorescence on plasma membrane lawns was low (bottom panel), indicating that only a small amount of GLUT4 is present at the surface of unstimulated cells. Approximately 80% of the microinjected cells in the 1-mm² marked region of the coverslip clearly retained the microinjected rhodamine-dextran marker and were thus considered to be intact and retaining microinjected material (second column, middle panel). In the plasma membrane lawns of unstimulated cells that were microinjected with GST-AmphiSH3, the GLUT4 immunofluorescence was higher in many of the lawns compared with those of non-injected cells (second column, bottom panel). This suggests that the fusion protein elevated the surface content of GLUT4. Microinjection of GST did not affect the GLUT4 immunofluorescence compared with non-injected cells (third and fourth columns, bottom panels). Fig. 4B shows one experiment representative of three in which GLUT4 immunofluorescence was detected in the lawn of cells microinjected with GST-AmphiSH3m. As with GST alone, the signal was not different from that in control cells. By comparison, insulin stimulation of cells resulted in a marked gain in surface GLUT4. The quantitated results of all experiments are shown in Fig. 4C. Microinjection of GST-AmphiSH3 significantly doubled the amount of GLUT4 at the plasma membrane (p < 0.005). In contrast, neither GST nor GST-AmphiSH3m significantly affected the amount of surface GLUT4. Insulin stimulation of intact cells increased the amount of GLUT4 at the cell surface by ~3.5-fold relative to unstimulated, non-injected cells. This value is in good agreement with the 4-fold gain recently reported by Tellam et al. (44) using a similar approach.

Additional controls were designed to test the specificity of GST-AmphiSH3 to increase the presence of GLUT4 at the cell surface. 3T3-L1 adipocytes were microinjected with fusion proteins containing the SH3 domains of spectrin and Crk (Fig. 5A), which do not bind dynamin (23). Fig. 5B shows the expected increase in GLUT4 on lawns from 3T3-L1 adipocytes microinjected with GST-AmphiSH3, but not on lawns from 3T3-L1 adipocytes microinjected with GST-spectrinSH3 or GST-CrkSH3. In the two latter cases, the amount of GLUT4 on lawns from microinjected cells was similar to that on lawns from non-injected cells on the same coverslip.

We further examined whether the interaction of dynamin with amphiphysin is responsible for the retention of GLUT4 at the cell surface. Fig. 6A shows that a peptide containing the sequence of dynamin that binds amphiphysin interferes with the ability of GST-AmphiSH3 to pull the endogenous dynamin II out of 3T3-L1 adipocyte lysates. In contrast, a control peptide did not compete with the amph GST-SH3 dynamin II interaction. Microinjection of the dynamin peptide into 3T3-L1 adipocytes caused a marked increase in the amount of GLUT4 present at the cell surface, as revealed by immunodetection on plasma membrane lawns (Fig. 6B, panels a and b). This effect was similar to that produced by GST-AmphiSH3 seen in Fig. 4A. In contrast, microinjection of the control peptide had no effect on GLUT4 levels (Fig. 6B, panels c and d).

To directly address whether the amphiphysin-dynamin interaction is required for GLUT4 endocytosis, cells were first treated with insulin (100 nM for 20 min) and then injected with the dynamin peptide, followed by insulin removal for 30 min. Following insulin removal, the amount of GLUT4 in non-injected cells returned to near basal levels (Fig. 6B, panel f), as expected, since GLUT4 is internalized (12). However, in cells microinjected with the dynamin peptide, the amount of surface GLUT4 failed to return to basal levels (Fig. 6B, panel g) and resembled the amount of surface GLUT4 in cells continuously exposed to insulin (panel e). In contrast, microinjection of the control peptide did not prevent GLUT4 endocytosis after insulin removal (Fig. 6B, panels i and h). Similar to the effect of the dynamin peptide, microinjection of GST-AmphiSH3 also prevented GLUT4 endocytosis upon insulin removal (data not shown).

DISCUSSION

Numerous studies have examined the composition of the intracellular compartments containing GLUT4 glucose transporters (45–47) and the insulin signals that may regulate their exocytosis (48, 49). In contrast, little is known about the mechanism of GLUT4 endocytosis and of its potential regulation, despite the well documented, continuous recycling of this protein from the cell surface to the intracellular compartment and of the demonstration that insulin reduces GLUT4 internalization (1–3, 17). There is ample evidence supporting the notion that GLUT4 is internalized by clathrin-coated pits (4, 5). Recent evidence has shown that the internalization sequences on membrane proteins bind to the mu2-subunit of the AP-2 adapter complex of the clathrin coats (50–52). Although it is unknown whether GLUT4 can interact with AP-2, mutation of its endocytosis sequence (FQQI) causes accumulation of the transporter at the cell surface in 3T3-L1 adipocytes (11) and reduces the association of GLUT4 with plasma membrane clathrin lattices (53). Furthermore, a chimeric protein comprising the amino terminus of GLUT4 mutated in the internalization sequence attached to the transferrin receptor exhibited reduced endocytosis compared with the wild-type GLUT4 amino terminus in Chinese hamster ovary cells (8). These results support the notion that GLUT4 may interact with components of the clathrin-coated pit.
In the nervous system, fission of clathrin-coated pits from the plasma membrane to generate endocytic vesicles is thought to involve the GTPase dynamin (54). In the present study, we used a dynamin II-specific antibody to show that this protein is expressed in 3T3-L1 adipocytes and is found associated with membranes (Fig. 1). Insulin stimulation diminished the amount of dynamin II associated with the plasma membranes. The mechanism by which insulin reduces dynamin association

![Fig. 4. Microinjection of GST-AmphiSH3 into 3T3-L1 adipocytes elevates plasma membrane GLUT4 levels. Cells in an outlined region of the 3T3-L1 adipocyte monolayer were microinjected with GST-AmphiSH3 (GST-ASH3) or GST in microinjection buffer containing rhodamine-labeled dextran. Images were taken from these regions or from adjacent regions within the same coverslips containing non-injected cells (ni) as indicated. A: top panels, phase-contrast image of the cells; middle panels, fluorescence of rhodamine-dextran (rhod dex), confirming that the majority of cells within the microinjected region remain fluorescent after 1.5 h; bottom panels, confocal images of indirect GLUT4 immunofluorescence on plasma membrane lawns from these regions. B, GLUT4 immunofluorescence of membrane lawns derived from 3T3-L1 adipocytes that were non-injected (first panel), injected with the double point mutant GST-AmphiSH3m on the same coverslip (second panel), non-injected (third panel), and non-injected but stimulated with 100 nM insulin for 20 min (fourth panel). C, fluorescence intensity/unit area of the plasma membrane lawns of three independent experiments similar to those illustrated in A and B quantitated using NIH Imaging software as described under "Experimental Procedures." On average, >100 cells were microinjected per coverslip of each experimental replicate. Fields within and outside of the microinjected area were imaged for quantitation of each coverslip. The combined number of lawns (cells) scored from each experimental condition is indicated (# of lawns). The results were collected in arbitrary units, and the S.E. for each experimental condition was calculated. Because each experimental condition had its own control (basal non-injected cells), once the S.E. values were calculated for the fluorescence intensity/unit area, the results were normalized to that control. The results were statistically significant (analysis of variance test) as follows: non-injected versus GST-AmphiSH3-injected, p < 0.005; and insulin (Ins)-stimulated versus basal non-injected cells, p < 0.001.

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with the cell surface is unknown, but we note that this decrease is not paralleled by a decrease in clathrin associated with the plasmalemma. It is conceivable that loss of dynamin from the surface may occur upon internalization of occupied insulin receptors since receptor internalization is increased upon insulin binding (55). We hypothesize that insulin may remove dynamin as one of the steps to limit internalization of proteins such as GLUT4. This could potentially lead to the accumulation of GLUT4 proteins with components of the coated pits, as suggested by electron microscopy and biochemical observations (4, 5).

To address the potential role of dynamin in GLUT4 traffic, we adopted an approach that was recently used to inhibit synaptic vesicle endocytosis (30). By microinjecting GST-AmphiSH3 into axons, interaction of the endogenous dynamin with amphiphysin and possibly other SH3 domain-containing partners was prevented, and the recruitment of dynamin to coated pits was diminished (30). In the present study, microinjection of GST-AmphiSH3 interfered with clathrin-dependent endocytosis as demonstrated by the fact that transferrin internalization was largely inhibited in both 3T3-L1 fibroblasts and adipocytes. These results are in agreement with a recent report showing that transfection of a similar construct into COS-7 cells prevents transferrin endocytosis (36). As a control, transfection of other SH3 domains such as those of GRB2, phospholipase Cγ, and spectrin were without effect on endocytosis in that study. Our results further demonstrate that a double mutation in GST-AmphiSH3m that results in loss of ability to bind dynamin II from cell lysates also strips the construct of its ability to interfere with endocytosis.

Microinjection of GST-AmphiSH3 into 3T3-L1 adipocytes resulted in higher basal GLUT4 levels on plasma membranes compared with the levels in vicinal non-injected cells. GLUT4 levels were measured 2.5 h after microinjection to allow the continuous recycling of GLUT4 to reach its new steady state. The results are consistent with the interpretation that the fusion protein reduced GLUT4 endocytosis to reach a new steady state characterized by higher surface levels of GLUT4 protein. It is less likely that GST-AmphiSH3 caused increased exocytosis of GLUT4 from the intracellular compartment since there was little change in surface GLUT4 levels within the first 30 min following microinjection (data not shown). This suggests that within this short time, the amount of GLUT4 that is delivered to the surface from the intracellular compartment is not sufficient to produce a significant accumulation when endocytosis is prevented by the fusion protein. Microinjection of GST alone failed to alter the surface GLUT4 levels, confirming that the increase in surface GLUT4 is due to the amphiphysin SH3 portion of the fusion protein. Moreover, microinjection of the mutant GST-AmphiSH3m, shown here to be unable to interact with the endogenous dynamin II, was also unable to increase surface GLUT4 levels. Furthermore, GST-AmphiSH3 was unique among other SH3 domains tested (GST-spectrinSH3 and GST-CrkSH3) in that the latter two did not increase GLUT4 presence at the cell surface. These results suggest that amphiphysin-dynamin interactions are required for GLUT4 endocytosis. It is likely that microinjected GST-AmphiSH3 interferes with the binding of dynamin II to endogenous amphiphysin(s), as a second isoform of amphiphysin with various splice versions has recently been described in non-neuronal tissues (60–66). Confirming this possibility, microinjection of a peptide containing the dynamin region that binds amphiphysin also increased the permanence of GLUT4 at the cell surface.

Although the simplest interpretation of the effects of GST-AmphiSH3 and the dynamin peptide on GLUT4 surface levels is inhibition of GLUT4 endocytosis, a more direct proof of GLUT4 endocytosis was desirable. To this end, cells were first treated with insulin to externalize the glucose transporters, and then GLUT4 levels were monitored upon insulin removal. This experimental paradigm has been shown to lead to rapid GLUT4 endocytosis from the cell surface (12). Microinjection of the dynamin peptide, but not of an unrelated peptide, prior to insulin removal resulted in marked retention of GLUT4 at the cell surface compared with non-injected cells.

In addition to endocytosis, dynamin II may have other functions in 3T3-L1 adipocytes. Indeed, its presence in intracellular membranes (LDM and HDM) suggests that it may participate in budding events in intracellular compartments. This is con-
consistent with recent biochemical and electron microscopic data showing dynamin II in the Golgi complex area of HepG2 cells (67) and a dynamin-like protein associated with the Golgi complex area in cultured fibroblasts and melanocytes (68). In addition, dynamin-studded budding membranes have been observed in internal vacuoles of lysed nerve terminals treated with GTP\_S (69). It is conceivable that dynamin-like proteins could participate in insulin-induced exocytosis. However, it is not likely that GST-AmphiSH3 promoted GLUT4 exocytosis since microinjection of this construct did not potentiate the acute effect of insulin to increase GLUT4 at the cell surface (data not shown).

In summary, by preventing the interaction of the proline-rich domain of dynamin with the SH3 domain-containing protein amphiphysin, the endocytosis of the GLUT4 glucose transporter was reduced. Therefore, GLUT4 endocytosis is mediated by a mechanism that appears to involve dynamin. Since 3T3-L1 adipocytes lack the neuronal dynamin I, these results suggest participation of dynamin II in endocytosis. Since, in addition to transporter exocytosis, insulin also reduces GLUT4 internal-
ization, we propose that this effect may be achieved, at least in part, by decreasing the plasmalemma-bound pool of dynamin II, thereby contributing to raising the levels of the transporter at the cell surface and consequently elevating glucose uptake.

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