Functional comparison of the role of dynamin 2 splice variants on GLUT-4 endocytosis in 3T3L1 adipocytes

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Kao, Aimee W., Chunmei Yang, and Jeffrey E. Pessin. Functional comparison of the role of dynamin 2 splice variants on GLUT-4 endocytosis in 3T3L1 adipocytes. Am J Physiol Endocrinol Metab 278: E825–E831, 2000.—Previously, we reported that expression of a dominant-interfering neuronal-specific dynamin 1 (K44A/dynamin 1) inhibited the plasma membrane internalization of GLUT-4 in 3T3L1 adipocytes (15). To investigate the role of the ubiquitously expressed isoform of dynamin, dynamin 2, on adipocyte GLUT-4 internalization, and to determine whether dynamin splice variants have functional specificity, we expressed each of the four dynamin 2 isoforms (aa, ab, ba, and bb) as either wild-type proteins or GTPase-defective mutants. When expressed as enhanced green fluorescent protein (EGFP) fusions, these isoforms were found to have overlapping subcellular distributions being localized throughout the cell cytoplasm, on punctate vesicles and in a perinuclear compartment. This distribution was qualitatively similar to that of endogenous dynamin 2 and overlapped with GLUT-4 in the basal state. Expression of wild-type dynamin 2 isoforms had no effect on the basal or insulin-stimulated distribution of GLUT-4; however, expression of the dominant-interfering dynamin 2 mutants inhibited GLUT-4 endocytosis. These data demonstrate that dynamin 2 is required for GLUT-4 endocytosis in 3T3L1 adipocytes and suggest that, relative to GLUT-4 trafficking, the dynamin 2 splice variants have overlapping functions and are probably not responsible for mediating distinct GLUT-4 budding events.

These data support an important role for dynamin in cell surface receptor and GLUT-4 endocytosis. However, mammalian cells have three dynamin genes: dynamin 1 expression is restricted to neural tissue, dynamin 2 is ubiquitously expressed, and dynamin 3 is primarily found in testes, with lower levels in the lung and nervous tissue (8, 16, 22). Earlier studies of dynamin function utilized overexpression of dominant inhibitory dynamin 1 in nonneuronal cells. Thus the observed effects on plasma membrane budding events may not have accurately reflected the function of endogenous dynamin 2. Several reports have suggested a role for dynamin 2 in intracellular budding events. Immunolocalization of dynamin 2 by fluorescence microscopy and expression of dynamin 2 fused to the enhanced green fluorescent protein (EGFP) suggest that dynamin is localized in a trans-Golgi distribution (5, 12). Furthermore, in a cell-free assay of trans-Golgi network (TGN) vesicle formation, addition of dynamin-specific antibodies or immunodepletion of dynamin protein inhibited budding, whereas addition of a dynamin-enriched fraction restored budding (14). However, in marked contrast, it was also reported that expression of a dominant inhibitory dynamin 2 mutant in fibroblast and epithelial cell lines had no functional consequence on TGN vesicle formation, although it was a more potent inhibitor of receptor-mediated endocytosis at the basolateral surface of MDCK cells than dynamin 1 (2).

An additional layer of complexity to understanding dynamin function is conferred by the existence of multiple dynamin splice variants. Careful characterization of the expression and distribution of dynamin isoforms has revealed at least eight splice variants of dynamin 1, four of dynamin 2, and thirteen of dynamin 3, bringing the total number of potential dynamin isoforms to twenty-five (5). The dynamin 2 splice forms are designated aa, ab, ba, or bb. Because of the large number of potential splice variants and their differential tissue expression patterns, it is an attractive hypothesis to assign specific dynamin splice forms to different membrane budding events, analogous to Rab proteins (19). In particular, it has been suggested that dynamin 2aa and 2ab splice forms can exhibit dissimilar subcellular localizations (5).

Although adipocytes predominantly express the dynamin 2 isoform, there is currently no information with regard to the relative abundance and/or function of these different isoforms (6, 15, 27). Therefore, to evaluate the potential roles of dynamin 2 splice variants in the regulation of insulin-stimulated GLUT-4 traffick-
ing, we have examined the subcellular localization and functional consequences of wild-type and mutant dynamin 2aa, ab, ba, and bb in 3T3L1 adipocytes. Our data demonstrate that all four dynamin 2 splice variants display a similar pattern of intracellular localization in 3T3L1 adipocytes and have a role in GLUT-4 endocytosis but do not affect insulin-stimulated GLUT-4 exocytosis.

**EXPERIMENTAL PROCEDURES**

Materials. The IA02 rabbit polyclonal GLUT-4 antibody was prepared as previously described (15). The dynamin 2 rabbit polyclonal was purchased from Covance (formerly BABC0, Richmond, CA). The syntaxin 6 monoclonal antibody was purchased from Transduction Laboratories (West Grove, PA). 3T3L1 adipocytes were obtained from the American Type Culture Collection (Rockville, MD) and were grown and differentiated as previously described (18). Cells were maintained in DMEM with 10% fetal bovine serum for 1–2 days postdifferentiation.

cDNAs constructs. The wild-type rat dynamin 2aa, ab, ba, bb-EGFP and K44A/dynamin 2aa-EGFP fusion cDNAs were generously provided by Dr. Mark McNiven (Mayo Clinic and Foundation, Rochester, MN). K44A/dynamin 2ab, ba, and bb-EGFP fusion constructs were generated from their respective wild-type constructs by exchanging a Xho I fragment containing the nucleotide sequences encoding the wild-type lysine 44 with a homologous fragment encoding alanine 44 from the mutant dynamin 2aa-EGFP clone. GLUT4-EGFP was prepared as previously described (23). The plasmid vector pEGFP-C1 was purchased from Stratagene (La Jolla, CA). All the dynamin 2 and dynamin 2-EGFP fusion cDNAs were cloned into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA).

Electroporation. Fifteen-centimeter plates of 3T3L1 adipocytes days 9 or 10 postdifferentiation were washed with PBS, pH 7.4, and trypsinized. The cells were pelleted at 200 g in a Beckman tabletop centrifuge, washed 2 times with Dulbecco’s PBS, and resuspended in 0.5 ml/plate with Dulbecco’s PBS. Electroporation was performed on a Bio-Rad Gene Pulser II at 950 µF and 0.15 kV. Cells were allowed to rest for 10 min at room temperature before plating onto glass coverslips for whole cell fluorescence experiments. In parallel, cells used for endocytosis experiments were plated on collagen IV-coated tissue culture dishes to increase the adherence of the cells necessary for the washing conditions used to remove insulin. Fifty micrograms of dynamin 2-EGFP DNA were used for whole cell fluorescence experiments, and cells were examined 8 h posttransfection. Fifty micrograms of pEGFP and 150 µg of various wild-type or K44A/dynamin 2 DNA were utilized for endocytosis experiments.

GLUT-4 translocation/endocytosis and fluorescence microscopy. Ten hours postelectroporation, 3T3L1 cells were serum-starved for 2 h in DMEM containing 25 mM glucose. The cells were left untreated or stimulated with 100 nM insulin for 30 min. To remove the bound insulin from the insulin receptor, the cells were then washed in an acidic buffer (140 mM NaCl and 5 mM Na acetate, pH 5.0) followed by a PBS rinse and incubation at 37°C for an additional 2 h in fresh starvation media. The cells were then fixed and permeabilized in 2% paraformaldehyde-0.2% Triton X-100 in PBS, pH 7.45, for 10 min at room temperature. All subsequent steps in the whole cell immunofluorescence labeling were performed at room temperature. Fixed cells were rinsed with PBS three times and blocked with 1% BSA and 5% donkey serum in PBS for 1 h. Blocked cells were incubated with IAO2 anti-GLUT-4 antibody for 1 h diluted in blocking solution at 1:200. The cells were washed and incubated for 1 h with Texas Red-conjugated anti-rabbit antibody. The cells were washed again with PBS and placed in Vectashield mounting medium. Images were collected using a Bio-Rad laser confocal microscope, and the number of transfected cells positive for cell surface membrane fluorescence was determined by at least two individuals in a blinded manner. Cells scored positive for plasma membrane translocation were characterized by a complete or nearly complete (>90%) continuous plasma membrane fluorescence with an overall fluorescence intensity similar to that of cell interior.

**RESULTS**

Immunofluorescence localization of endogenous dynamin 2 in 3T3L1 adipocytes. In 3T3L1 adipocytes, we examined the distribution of these proteins by immunofluorescence microscopy (Fig. 1). Because only a rabbit dynamin 2 antibody was found to give a specific immunofluorescence signal, and the GLUT-4 antibody was also a rabbit antibody, we compared the localization of these proteins in separate cell populations. In the basal state, endogenous dynamin 2 was found localized throughout the cell and on punctate vesicles (Fig. 1a). In particular, a bright central area of immunofluorescence was observed near the nucleus. The pattern of staining was similar to endogenous GLUT-4, although the GLUT-4 perinuclear compartment tended to be somewhat more crescent-shaped, more typical of the TGN (Fig. 1, compare a and b). Insulin stimulation had little effect on the subcellular localization of dynamin 2 (Fig. 1, compare a and c).

![Fig. 1](image_url) Immunofluorescence localization of dynamin 2 and GLUT-4 in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were serum-starved for 2 h and incubated in the absence (a and b) or presence (c and d) of 50 nM insulin for 20 min at 37°C. Cells were then fixed and incubated with the dynamin 2 (a and c) or GLUT-4 (b and d) specific antibodies and subjected to confocal immunofluorescent microscopy.
In contrast, GLUT-4 traffics to the cell surface and exhibits a strong rim fluorescence after the addition of insulin (Fig. 1d).

To further evaluate the perinuclear distribution of dynamin 2, we next compared the distribution of dynamin 2 with that of an established resident TGN protein, syntaxin 6 (3, 4). As observed in Fig. 1, dynamin 2 was highly concentrated in regions juxtaposed to the nucleus (Fig. 2a). Similarly, syntaxin 6 was also predominantly distributed to the perinuclear region and displayed a near identical co-localization with that of dynamin 2 (Fig. 2, b and c). As expected, insulin had no effect on the distribution of either dynamin 2 or syntaxin 6, which maintained their co-localization (Fig. 2, d–f). The small amount of background fluorescence observed throughout the cytosol could represent a small amount of nonspecific immunoreactivity and/or labeling of the endoplasmic reticulum.

Subcellular localization of dynamin 2 splice variants. It was recently reported that dynamin 2 splice variants display distinct subcellular distributions when expressed in fibroblast cell lines as EGFP fusion proteins (5). To examine this phenomenon in 3T3L1 adipocytes, we analyzed the subcellular distribution of the dynamin 2 splice variants demonstrate qualitatively similar subcellular distributions (Fig. 3). Each of the four dynamin 2-EGFP fusion proteins was localized at the plasma membrane, in cytosolic or periplasma membrane vesicles, diffusely in the cell cytoplasm, and in a perinuclear compartment reminiscent of Golgi and TGN (Fig. 3, a–d). In several cells, the plasma membrane fluorescence was smooth, rather than punctate. Furthermore, the perinuclear and intracellular vesicle localization of the expressed dynamin 2-EGFP fusion proteins correlated with the distribution observed for the endogenous dynamin 2 protein (Figs. 1 and 2). Thus these data indicate that all four dynamin 2 splice variants display a similar but not identical overlapping subcellular distribution with that of endogenous dynamin 2 in 3T3L1 adipocytes.

Expression of K44A/dynamin 2aa induces the accumulation of GLUT-4 at the cell surface and inhibits GLUT-4 endocytosis. Despite the fact that they displayed a similar subcellular distribution, the dynamin 2 splice variants could still exhibit functional differences. Therefore, we determined the effect of each splice variant and its dominant-interfering mutant on insulin-stimulated GLUT-4 translocation and endocytosis. Because the addition of a large EGFP tag may interfere with dynamin 2 function, we transfected the dynamin 2 cDNAs into cells without an EGFP tag and co-transfected pEGFP to aid in identifying the cells that had taken up DNA. After electroporation of the 3T3L1 adipocytes, the cells expressing the dynamin 2 protein were visualized by confocal microscopy, and the subcellular localization of endogenous GLUT-4 was compared. Under basal conditions, expression of the wild-type dynamin 2aa splice variant had no effect on the distribution of GLUT-4 (Fig. 4A, a and b). Insulin stimulation resulted in the translocation of GLUT-4 to the plasma membrane, characterized by the appearance of a rim of GLUT-4 immunofluorescence (Fig. 4A, c and d). As expected, removal of insulin followed by a 2-h incubation resulted in the internalization of plasma membrane-localized GLUT-4 (Fig. 4A, e and f). In the cells expressing wild-type dynamin 2aa splice variant, the insulin-dependent GLUT-4 translocation to the plasma membrane and subsequent internalization after insulin washout are identical to those observed in the surrounding nontransfected cells (Fig. 4A, a–f) as well as in nonelectroporated cell populations (data not shown).

In comparison, expression of the dominant-interfering K44A/dynamin 2aa mutant resulted in a different

![Fig. 2. Immunofluorescence co-localization of dynamin 2 with syntaxin 6 in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were serum-starved for 2 h and incubated in the absence (a–c) or presence (d–f) of 50 nM insulin for 20 min at 37°C. Cells were then fixed and incubated with dynamin 2 (a and d) or syntaxin 6 (b and e) specific antibodies and subjected to confocal immunofluorescent microscopy. Merged images of a and b (basal) and d and e (insulin stimulated) are presented in c and f, respectively.](http://ajpendo.physiology.org/)

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pattern of GLUT-4 localization. In the basal state, K44A/dynamin 2aa caused an increase in the amount of GLUT-4 localized to the plasma membrane (Fig. 4B, a and b). This was in marked contrast to wild-type dynamin 2aa, which had no effect on the basal-state distribution of GLUT-4. In the cells expressing K44A/dynamin 2aa mutant, insulin stimulation caused a further increase in the number of cells displaying plasma membrane GLUT-4 (Fig. 4B, c and d). However, unlike wild-type dynamin, expression of K44A/dynamin 2aa prevented the internalization of plasma membrane GLUT-4 upon the removal of insulin (Fig. 4B, e and f). Thus the expression of this mutant dynamin 2 isoform increased the basal state accumulation of GLUT-4 at the cell surface and prevented GLUT-4 endocytosis in a manner similar to that previously observed for K44A/dynamin 1 (1, 15, 27).

Expression of K44A/dynamin 2bb, ab, and ba splice variants also induces the accumulation of GLUT-4 at the cell surface and inhibits GLUT-4 endocytosis. Similar to wild-type dynamin 2aa, expression of wild-type dynamin 2bb had no effect on the basal or insulin-stimulated translocation of GLUT-4 (Fig. 5A, a–d). In addition, 2 h after insulin removal, the GLUT-4 protein was nearly completely internalized away from the plasma membrane (Fig. 5A, e and f). Consistent with the K44A/dynamin 2aa results, expression of K44A/dynamin 2bb caused an increase in plasma membrane-associated GLUT-4 in the absence of insulin (Fig. 5B, a and b). Because the majority of K44A/dynamin 2bb-expressing cells already displayed cell surface GLUT-4, stimulation with insulin had little additional effect (Fig. 5B, c and d). More importantly, 2 h after insulin removal, GLUT-4 remained plasma membrane localized in all of the K44A/dynamin 2bb-expressing cells but not in the surrounding nonexpressing cells (Fig. 5B, e and f). Quantitation of the effect of dynamin 2aa and dynamin 2bb, as well as dynamin 2ab and dynamin 2ba, on GLUT-4 translocation is summarized in Fig. 6. These data show that the expression of any of the wild-type dynamin 2 splice variants had no significant effect on the distribution of GLUT-4 under basal, insulin-stimulated, or insulin washout states. In contrast, expression of all four dominant-interfering K44A/
Dynamin 2 splice variants increased the amount of cell surface-localized GLUT-4 in the absence of insulin and inhibited subsequent GLUT-4 endocytosis after insulin removal.

**DISCUSSION**

The protein dynamin was originally identified as the mammalian homologue of the Drosophila shibire gene product, which results in an animal with a temperature-sensitive paralytic phenotype due to an inability to recycle synaptic vesicles (7, 24). Subsequently, dynamin 1 has been extensively characterized and documented to play an essential role in the clathrin-mediated endocytosis of certain plasma membrane proteins (20). In particular, we and others have observed that expression of a dominant-interfering dynamin 1 mutant (K44A/dynamin 1) in adipocytes results in the accumulation of GLUT-4 at the cell surface because of a marked inhibition of GLUT-4 endocytosis (1, 15, 27). Adipocytes, however, do not express dynamin 1 but instead express the dynamin 2 isoform. Because dynamin 1 functions as a tetramer, the inhibition of GLUT-4 endocytosis by mutant dynamin 1 presumably results from the formation of inactive heterotetramers composed of both endogenous dynamin 2 and the expressed mutant K44A/dynamin 1.

The GTPase activities and regulatory properties of dynamin 1 and dynamin 2 are markedly different. Dynamin 2 has a higher intrinsic GTPase activity and propensity for self-assembly than dynamin 1 (29). Whereas dynamin 1 regulation is as yet poorly understood, dynamin 1 is known to be modulated through its carboxyl-terminal domain via interaction with a variety of adapter and effector proteins, including Grb2, amphiphysin, and phospholipase Cγ (10, 21, 28). In addition, when expressed in polarized MDCK cells, dominant-interfering dynamin 1 was a more potent inhibitor of endocytosis from the apical membrane, whereas mutant dynamin 2 was more effective at blocking endocytosis from the basolateral membrane (2). Although controversial, there have been published
reports supporting a role for dynamin 2 in budding from the TGN. For example, it was observed that a dynamin 2ab splice variant was localized exclusively at the plasma membrane, whereas a dynamin 2aa splice form was found at both the plasma membrane and the TGN (5). Consistent with dynamin 2 being present at the TGN, in vitro budding assays have demonstrated a requirement for dynamin function in the formation of constitutive and clathrin-coated vesicles from the TGN (14). However, it remains unclear which splice variants are expressed and/or their potential functions in adipocytes. To address these issues, we examined the functional role of dynamin 2 in the insulin regulation of GLUT-4 trafficking in 3T3L1 adipocytes.

Our data demonstrate that the endogenous dynamin 2 proteins are distributed throughout the cell cytoplasm, in variably sized vesicles and in a perinuclear compartment. This distribution partially overlaps but is distinct from the subcellular localization of GLUT-4. In addition, GLUT-4 undergoes a marked insulin-stimulated translocation to the plasma membrane, whereas the dynamin 2 distribution is unaffected. Because dynamin 2 isoform-specific antibodies do not exist, we further examined the subcellular localization of the four dynamin 2 splice variants by using EGFP fusion proteins as previously reported for Clone 9 fibroblasts (5). In contrast to the distinct localization of the different splice forms in Clone 9 cells, their distribution in all the 3T3L1 adipocytes surveyed was essentially indistinguishable.

Our major objective, however, was to determine the effect of dynamin 2 splice forms on GLUT-4 trafficking. Expression of wild-type dynamin 2 isoforms had no measurable effect on the insulin-stimulated exocytosis or subsequent plasma membrane endocytosis of GLUT-4. These data further document that the steady-state levels of dynamin 2 are not rate limiting for either the formation of transport vesicles or plasma membrane endocytosis. As expected, expression of the dominant-interfering dynamin 2 mutants enhanced the basal-state accumulation of GLUT-4 at the plasma membrane and completely prevented its subsequent endocytosis. The accumulation of GLUT-4 at the plasma membrane because of the inhibition of endocytosis is consistent with previous observations and reflects the continuous recycling of the GLUT-4 protein in the absence of insulin. The observation that all four of the dominant-interfering dynamin 2 splice variants inhibited GLUT-4 endocytosis also demonstrates that these proteins display overlapping functions, at least in regard to GLUT-4 trafficking in 3T3L1 adipocytes. Furthermore, the fact that none of the mutants appeared to prevent the insulin-stimulated plasma membrane accumulation of GLUT-4 suggests that dynamin 2 probably does not play a significant role in the process of GLUT-4 exocytosis. Thus, if insulin-stimulated GLUT-4 translocation requires an intracellular budding step, this event would be independent of dynamin 2 function. However, it also remains formally possible that this putative budding step is significantly less sensitive to the effects of the dominant-interfering dynamin 2 mutants than plasma membrane endocytosis. Future quantitative studies of GLUT-4 trafficking will be necessary to conclusively resolve this issue.

In any case, our data demonstrate that dynamin 2 plays an essential role in the regulation of GLUT-4 endocytosis in 3T3L1 adipocytes. Inhibition of clathrin-mediated plasma membrane internalization results in the accumulation of GLUT-4 at the cell surface consistent with the continuous recycling of GLUT-4 in the basal state. Furthermore, the four dynamin 2 splice variants have overlapping functions and do not appear to display distinct subcellular localization patterns or functional differences regarding the process of GLUT-4 trafficking.

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