Expression of a Dominant Interfering Dynamin Mutant in 3T3L1 Adipocytes Inhibits GLUT4 Endocytosis without Affecting Insulin Signaling*

(Received for publication, April 27, 1998, and in revised form, July 6, 1998)

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To examine the role of clathrin-coated vesicle endocytosis in insulin receptor signaling and GLUT4 trafficking, we used recombinant adenovirus to express a dominant interfering mutant of dynamin (K44A/dynamin) in 3T3L1 adipocytes. Functional expression of K44A/dynamin, as measured by inhibition of transferrin receptor internalization, did not affect insulin-stimulated insulin receptor autophosphorylation, Shc tyrosine phosphorylation, or mitogen-activated protein kinase activation. Although the tyrosine phosphorylation of insulin receptor substrate-1 was slightly reduced, correlating with a 25% decrease in insulin receptor substrate-1-associated phosphatidylinositol 3-kinase activity, insulin-stimulated Akt kinase activation was unaffected. In contrast, expression of K44A/dynamin resulted in the cell-surface accumulation of GLUT4 under basal conditions and an inhibition of GLUT4 endocytosis without affecting insulin-stimulated GLUT4 exocytosis. These data demonstrate that disruption of clathrin-mediated endocytosis does not significantly perturb insulin receptor signal transduction pathways. Furthermore, K44A/dynamin expression causes an accumulation of GLUT4 at the cell surface, suggesting that GLUT4 vesicles exist in at least two distinct intracellular compartments, one that undergoes continuous recycling and a second that is responsive to insulin.

The insulin-responsive glucose transporter, GLUT4, is expressed at high levels in adipose tissue, skeletal muscle, and cardiac myocytes (1–4). Unlike other glucose transporter isoforms such as GLUT1, GLUT4 is primarily distributed to poorly defined intracellular compartments under basal conditions (5–7). GLUT4 slowly recycles between these intracellular compartments and the plasma membrane, with the majority of steady state GLUT4 residing in a vesicle population distinct from the endosomal compartments (8–11). In response to insulin, plasma membrane GLUT4 is enhanced 10–20-fold, an amount sufficient to account for the majority of insulin-stimulated glucose uptake, with a concomitant decrease in the low density microsome-associated GLUT4 protein (5, 7, 12, 13).

Concurrent with the insulin-dependent increase in GLUT4 vesicle exocytosis, insulin also reduces the rate of GLUT4 endocytosis up to 3-fold (8–10). Although the exact mechanism of this regulation is unknown, several lines of evidence suggest that GLUT4 internalization occurs via clathrin-coated vesicles. For example, disassembly of clathrin lattices by potassium depletion results in the accumulation of GLUT4 at the cell surface (14). Immunofluorescence and electron microscopy studies have localized GLUT4 to coated pits both in the basal and insulin-stimulated states (6, 13, 15). Furthermore, internalization of a chimeric transferrin receptor/GLUT4 protein was inhibited by acidification of the cytosol, a technique for blocking clathrin-coated vesicle endocytosis (16).

One protein important in the formation of clathrin-coated vesicles is dynamin, a 100-kDa GTPase implicated in the membrane scission step of vesicle formation (17, 18). Three different dynamin genes have been identified with each having multiple splice variants (19). These isoforms share a high degree of homology in the amino-terminal GTPase domain but have distinct proline-rich carboxyl-terminal tails that have been shown in vitro to bind various SH3-containing proteins (20–25). Expression of a dominant interfering form of dynamin I with a mutation in the conserved GTP-containing motif has been shown to prevent the constriction and budding of clathrin-coated vesicles (26, 27). Under these conditions, the inhibition of clathrin-coated vesicle formation interferes with the recycling of cell-surface receptors such as the transferrin receptor, the β-adrenergic receptor, and the epidermal growth factor receptor (26–29).

In this study, we examined the requirement of dynamin-mediated endocytosis for insulin-dependent signal transduction events in 3T3L1 adipocytes using adenoviral expression of a dominant interfering K44A/dynamin mutant. Our data demonstrate that inhibition of clathrin-mediated endocytosis does not impair either insulin receptor signaling or insulin-stimulated GLUT4 translocation. However, it does inhibit the clearance of GLUT4 from the cell surface, both in the basal state and following insulin stimulation, suggesting that dynamin may function in the regulation of GLUT4 endocytosis.

EXPERIMENTAL PROCEDURES

Materials—Chemical reagents were purchased from Sigma except protase inhibitors aprotinin, pepstatin, leupeptin, and Pefabloc purchased from Boehringer Mannheim, and as indicated. Antibodies were purchased from Transduction Laboratories (Lexington, KY) except polyclonal Akt1 (C20) antibody from Santa Cruz Biotechnology (Santa Cruz, CA), Akt2 antibody generously provided by Dr. Morris Birnbaum (Philadelphia, PA), phospho-Akt and phospho-p44/42 MAP kinase antibodies from New England Biolabs (Beverly, MA), and polyclonal antibody against rat GLUT4 (IA02) which was obtained by immunizing a rabbit with GLUT4 carboxyl-terminal peptide, SATFRRTPSLLEQ-
Dynamin-dependent GLUT4 Endocytosis

Expression of K44A/Dynamin Inhibits Transferrin Receptor Endocytosis—Previous studies have demonstrated that expression of a GTPase-deficient dynamin such as K44A/dynamin I in mammalian cells inhibits clathrin-mediated endocytosis by precipitation with polyclonal anti-dynamin antibodies. The dynamin I or dynamin II proteins, whereas wild-type dynamin has no effect (26, 27, 38). Due to the difficulty of quantitatively transfecting 3T3L1 adipocytes by standard methods, we generated a recombinant adenovirus encoding K44A/dynamin I (K44A) and infected cells for 48 h. Dynamin expression, as assessed by a dynamin I/II antibody, was markedly increased.

RESULTS

Dynamin Expression Is Increased during 3T3L1 Adipocyte Differentiation—Differentiated 3T3L1 adipocytes represent an excellent model system to study GLUT4 trafficking since they share many of the characteristics of primary adipocytes, including movement of GLUT4 from intracellular stores to the plasma membrane in response to insulin (36, 37). To examine the potential role of dynamin in insulin signaling and GLUT4 translocation, we initially examined the expression of dynamin during 3T3L1 adipocyte differentiation. By using a dynamin II-specific antibody, we observed a 6–9-fold increase in dynamin expression during differentiation of 3T3L1 fibroblasts into adipocytes (Fig. 1A, lanes 1–4). The expression of clathrin heavy chain was not significantly affected by adipocyte differentiation (Fig. 1B, lanes 1–4). In contrast, GLUT4 expression was highly induced following 3T3L1 adipocyte differentiation (Fig. 1C, lanes 1–4).

Expression of K44A/Dynamin Inhibits Transferrin Receptor Endocytosis—Previous studies have demonstrated that expression of a GTPase-deficient dynamin such as K44A/dynamin I in mammalian cells inhibits clathrin-mediated endocytosis by precipitation with polyclonal anti-dynamin antibodies. The dynamin I or dynamin II proteins, whereas wild-type dynamin has no effect (26, 27, 38). Due to the difficulty of quantitatively transfecting 3T3L1 adipocytes by standard methods, we generated a recombinant adenovirus encoding K44A/dynamin I (K44A) and infected cells for 48 h. Dynamin expression, as assessed by a dynamin I/II antibody, was markedly increased.
in K44A/dynamin adenovirus-infected 3T3L1 adipocytes compared with uninfected cells or cells infected with a LacZ-encoding adenovirus (LacZ) (Fig. 2A, top panel, lanes 1–3). Immunoblotting of these extracts with a dynamin II-specific antibody demonstrated no significant change in endogenous dynamin expression (Fig. 2A, middle panel, lanes 1–3). Since dynamin II expression was unchanged, the increase in dynamin I/II protein levels was due to the expression of K44A/dynamin I. Insulin receptor β (IRβ) expression was also unchanged in K44A/dynamin expressing cells compared with uninfected or LacZ (Fig. 2A, bottom panel, lanes 1–3). Under these conditions, greater than 95% of the cell population was infected with adenovirus with no observable changes in cell morphology by light microscopy (data not shown).

Having demonstrated increased expression of K44A/dynamin, we next determined its effect on clathrin-mediated endocytosis. This was assessed by examining the internalization of the transferrin receptor in uninfected, LacZ-, and K44A/dynamin-infected 3T3L1 adipocytes (Fig. 2B). In control adipocytes approximately 70% of the cell-surface transferrin receptor was internalized over a 60-min period (squares). Adenovirus infection slightly reduced the extent of transferrin receptor endocytosis, with approximately 60% internalized over the same time course (circles). In contrast, expression of K44A/dynamin resulted in only 30% of the cell-surface transferrin receptors being internalized with a significant decrease in initial rate of internalization compared with uninfected or LacZ-infected cells (triangles). Thus, increased expression of K44A/dynamin results in an inhibition of transferrin receptor internalization and therefore functions in a dominant interfering manner to reduce coated vesicle endocytosis in 3T3L1 adipocytes.

**Relationship between K44A/Dynamin Expression and Insulin Receptor Downstream Signaling**—Several studies have suggested that endosome localized insulin receptor provides the major pathway leading to insulin signal transduction and biological responsiveness (39–42). Since expression of K44A/dynamin in other cell lines has been shown to interfere with signaling events downstream of the epidermal growth factor and β-adrenergic receptors (28, 29), we investigated the insulin receptor signaling characteristics in 3T3L1 adipocytes expressing K44A/dynamin. The dose response for insulin-stimulated insulin receptor β subunit tyrosine autophosphorylation was similar in uninfected, LacZ-, and K44A/dynamin-expressing cells (Fig. 3A). In each case, autophosphorylation of the insulin receptor was not observed in the absence of or following stimulation with 0.1 nM insulin (Fig. 3A, lanes 1, 2, 6, 7, 11 and 12). Insulin receptor autophosphorylation in response to 1 nM insulin remained low (Fig. 3A, lanes 3, 8 and 13) but became more readily observable at 10 and 100 nM insulin (Fig. 3A, lanes 4, 5, 9, 10, 14 and 15). In contrast, tyrosine phosphorylation of IRS1 was significantly more sensitive to insulin, reflecting downstream amplification of insulin receptor kinase activity (Fig. 3B). Tyrosine phosphorylation of IRS1 was clearly discernible at 0.1 nM insulin (Fig. 3B, lanes 1, 2, 6, 7, 11 and 12) and increased in a dose-dependent manner up to 100 nM insulin (Fig. 3B, lanes 3–5, 8–10 and 13–15). Relative to uninfected and LacZ-expressing cells, K44A/dynamin overexpression had only a minor effect on IRS1 tyrosine phosphorylation in response to varying doses of insulin (Fig. 3B, compare lanes 1–5 with lanes 6–10 and lanes 11–15). Tyrosine phosphorylation of the 52-kDa Shc adapter protein in response to insulin was also unaffected by K44A/dynamin expression (data not shown).

To examine downstream insulin receptor signaling pathways, we next determined the effect of K44A/dynamin expression on the ERK family of mitogen-activated protein (MAP)
kinases (Fig. 4). Uninfected, LacZ-, and K44A/dynamin-expressing cells exhibited essentially identical insulin dose-response patterns when immunoblotted with a phospho-specific ERK1/ERK2 antibody (Fig. 4A, lanes 1–15). Similarly, the time dependence of insulin-stimulated ERK activation was unaffected by adenovirus-mediated expression of K44A/dynamin (Fig. 4B, lanes 1–18). These data are consistent with Shc tyrosine phosphorylation providing the predominant pathway for the insulin activation of ERK1 and ERK2.

In contrast to Shc, the IRS family of docking proteins is thought to provide the major pathways leading to the metabolic actions of insulin (43). As observed in Fig. 3, insulin stimulation of IRS1 tyrosine phosphorylation was slightly attenuated following expression of K44A/dynamin compared with uninfected and LacZ-expressing cells. To examine further the potential effect of decreased IRS1 tyrosine phosphorylation, we performed PI 3-kinase assays in IRS1 immunoprecipitates from LacZ- and K44A/dynamin-expressing cells. Following insulin stimulation, IRS1-associated PI-3 kinase was decreased by 25% in K44A/dynamin-expressing cells relative to the LacZ-expressing cells (Fig. 5, A and B). Since maximal activation of the PI-3 kinase may not be required for full biological responsiveness to insulin, we further examined the ability of insulin to stimulate Akt kinase activity. Akt is a serine-threonine kinase involved in the metabolic actions of insulin whose activity appears to be dependent upon PI-3 kinase (44–46). Akt activation is associated with phosphorylation on serine and threonine residues resulting in a characteristic decrease in SDS-polyacrylamide gel electrophoretic mobility. Relative to unstimulated cells, insulin caused an Akt gel shift in the uninfected and both LacZ- and K44A/dynamin adenovirus-infected cells (Fig. 6A). When insulin-stimulated Akt protein kinase activity was directly measured, no significant difference was found between the LacZ- and K44A/dynamin-expressing adipocytes (Fig. 6B). To confirm these findings, we also examined the insulin dose and time dependence of Akt activation by using a phosphoserine-specific Akt antibody and by Akt gel shift (Fig. 7). Under these conditions, uninfected, LacZ-, and K44A/dynamin-expressing cells exhibited similar patterns of Akt activation in response to increasing doses or times of insulin stimulation. Thus, expression of K44A/dynamin does not have a significant effect on the major proximal insulin receptor signaling pathways examined.

K44A/Dynamin Expression Results in the Accumulation of GLUT4 at the Plasma Membrane—Morphological studies have demonstrated that in the basal state, the small amount of GLUT4 present at the cell surface is localized to coated pits (5, 6). We therefore examined the effect of K44A/dynamin on the plasma membrane localization of GLUT4 by immunofluorescence microscopy of isolated plasma membrane sheets (Fig. 8). In the absence of insulin, membrane sheets exhibited a low level of background GLUT4 immunofluorescence which markedly increased following insulin stimulation (Fig. 8, panels 1–3). Similarly, following 48 h of infection with the LacZ-encoding adenovirus, there was also a low level of plasma membrane-associated GLUT4 immunofluorescence in the basal
state that dramatically increased in response to insulin (Fig. 8, panels 4–6). Surprisingly, expression of K44A/dynamin resulted in a significant increase in basal plasma membrane-associated GLUT4 immunofluorescence, although the cells remained responsive to insulin stimulation (Fig. 8, panels 7–9).

To confirm these findings, 3T3L1 adipocytes were allowed to express K44A/dynamin for various times, and the relative amount of plasma membrane-associated GLUT4 was determined (Fig. 9). K44A/dynamin adenovirus infection resulted in a time-dependent accumulation of GLUT4 at the plasma membrane (Fig. 9, panels 1–3). After 24 and 48 h of K44A/dynamin expression, insulin was still capable of inducing GLUT4 translocation to the plasma membrane (Fig. 9, panels 4 and 5). Following 72 h of K44A/dynamin expression, there was an additional elevation of basal plasma membrane-associated GLUT4. Although at 72 h insulin stimulation was able to increase further cell-surface GLUT4, the fold increase was lower due to the elevated basal plasma membrane GLUT4 (Fig. 9, panels 3 and 6).

Expression of K44A/Dynamin Inhibits GLUT4 Endocytosis—Since expression of K44A/dynamin did not appear to alter insulin-stimulated GLUT4 translocation (Figs. 8 and 9), the increased basal amount of GLUT4 at the plasma membrane was most likely due to an inhibition of GLUT4 endocytosis. To address this issue, we examined the rate of GLUT4 internalization in uninfected, LacZ, and K44A/dynamin-infected 3T3L1 adipocytes (Fig. 10). Insulin stimulation resulted in a marked translocation of GLUT4 to the plasma membrane in uninfected cells (Fig. 10, panels 1 and 2). Following removal of insulin, there was a time-dependent decrease in plasma membrane-associated GLUT4 indicative of endocytosis (Fig. 10, panels 3 and 4). Similarly, insulin stimulation of LacZ-expressing adipocytes caused an increase in cell-surface GLUT4 which decreased subsequent to removal of insulin (Fig. 10, panels 5–8). As previously observed, expression of K44A/dynamin resulted in increased levels of plasma membrane-associated GLUT4 which were further enhanced following insulin stimulation (Fig. 10, panels 9 and 10). However, there was no apparent endocytosis of GLUT4 following insulin removal (Fig. 10, panels 11 and 12). These data demonstrate that the expression of

![Fig. 6. Akt kinase activity in cells overexpressing K44A/dynamin. A, 3T3L1 adipocytes were either uninfected (Uninf) (lanes 1–2) or infected with adenovirus encoding LacZ (lanes 3 and 4) or K44A/dynamin (lanes 5 and 6). Cells were then serum-starved and incubated in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 100 nM insulin for 5 min at 37 °C. Whole cell lysates were collected, subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane, and immunoblotted (IB) with an Akt1-specific antibody. B, LacZ- or K44A/dynamin-infected 3T3L1 adipocytes were serum-starved and stimulated with or without 100 nM insulin for 5 min, followed by generation of whole cell lysates and Akt immunoprecipitation. Akt immunoprecipitates were incubated with [γ-32P]ATP and 2 mg/ml histone 2B as kinase substrate. The amount of 32P incorporation was determined and normalized for the amount of Akt immunoprecipitated as determined by densitometric scanning of Akt immunoblots. C, control; I, insulin.

![Fig. 7. Insulin dose and time dependence of Akt activation in 3T3L1 cells overexpressing K44A/dynamin. A, 3T3L1 adipocytes were left uninfected (Uninf) (lanes 1–6), infected with adenovirus encoding LacZ (lanes 6–10), or K44A/dynamin (lane 11–15). The cells were then serum-starved and incubated in the absence (lanes 1, 6, and 11) or presence of 0.1 (lanes 2, 7, and 12), 1 (lanes 3, 8, and 13), 10 (lanes 4, 9, and 14), and 100 nM (lanes 5, 10, and 15) insulin for 5 min at 37 °C. B, 3T3L1 adipocytes were left uninfected (lanes 1–6), infected with adenovirus encoding LacZ (lanes 7–12), or K44A/dynamin (lane 13–18). The cells were then serum-starved and incubated in the absence (lanes 1, 7, and 13) or presence of 100 nM insulin for 1 (lanes 2, 8, and 14), 5 (lanes 3, 9, and 15), 15 (lanes 4, 10, and 16), 30 (lanes 5, 11, and 17), and 60 (lanes 6, 12, and 18) min at 37 °C. Whole cell lysates were generated, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-phospho-Akt- specific antibody. B, 3T3L1 adipocytes were left uninfected (lanes 1–6), infected with adenovirus encoding LacZ (lanes 7–12), or K44A/dynamin (lane 13–18). The cells were then serum-starved and incubated in the absence (lanes 1, 7, and 13) or presence of 100 nM insulin for 1 (lanes 2, 8, and 14), 5 (lanes 3, 9, and 15), 15 (lanes 4, 10, and 16), 30 (lanes 5, 11, and 17), and 60 (lanes 6, 12, and 18) min at 37 °C. Whole cell lysates were generated, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an Akt2-specific antibody.

![Fig. 8. Overexpression of K44A/dynamin does not affect insulin-stimulated GLUT4 exocytosis. Uninfected (Uninf) cells (panels 1–3) or cells infected for 48 h with adenovirus encoding LacZ (panels 4–6) or K44A/dynamin (panels 7–9) were serum-starved and stimulated for 5 or 30 min with 100 nM insulin at 37 °C as indicated. Plasma membrane sheets were prepared and processed for GLUT4 immunofluorescence. Bar, 50 μm.

![Fig. 9. Overexpression of K44A/dynamin results in increased plasma membrane-associated GLUT4. A, 3T3L1 adipocytes were left uninfected (Uninf) (lanes 1–3), infected with adenovirus encoding LacZ (lanes 4–6), or K44A/dynamin (lanes 7–9). Plasma membrane sheets were prepared and processed for GLUT4 immunofluorescence. Bar, 50 μm.

![Fig. 10. Overexpression of K44A/dynamin results in increased plasma membrane-associated GLUT4. A, 3T3L1 adipocytes were left uninfected (Uninf) (lanes 1–3), infected with adenovirus encoding LacZ (lanes 4–6), or K44A/dynamin (lanes 7–9). Plasma membrane sheets were prepared and processed for GLUT4 immunofluorescence. Bar, 50 μm.
K44A/dynamin inhibits GLUT4 endocytosis and are consistent with the continuous recycling of GLUT4 to and from the plasma membrane even in the absence of insulin.

To assess further the relative levels of cell-surface GLUT4, isolated plasma membrane sheets were subjected to GLUT4 immunoblotting (Fig. 11). In uninfected, control 3T3L1 adipocytes, GLUT4-immunoreactive protein was detected in isolated plasma membrane sheets (Fig. 11, lane 1). Thirty min of insulin stimulation resulted in a dramatic increase in the amount of plasma membrane-associated GLUT4 which recovered to nearly basal levels 2 h after insulin removal (Fig. 11, lanes 2 and 3). Similarly, plasma membrane sheets from cells infected with the LacZ-encoding virus contained low levels of basal GLUT4 that increased following insulin stimulation (Fig. 11, lanes 4 and 5). The amount of plasma membrane GLUT4 also significantly decreased 2 h after insulin removal, although the effect was not as dramatic as in the uninfected cells (Fig. 11, lane 6). Even though expression of K44A/dynamin did not affect the insulin-stimulated translocation of GLUT4 to the cell surface, we observed an inhibition of GLUT4 endocytosis from the plasma membrane subsequent to insulin removal (Fig. 11, lanes 7–9).

K44A/Dynamin Does Not Affect GLUT4 Expression or Prevent Insulin Receptor Inactivation—In addition to the specific inhibition of dynamin-dependent endocytosis, it remained possible that expression of K44A/dynamin resulted in increased expression of GLUT4 protein and/or persistent insulin signaling following insulin removal. To examine the effect of K44A/dynamin expression on GLUT4 protein levels, whole cell extracts were immunoblotted for GLUT4 (Fig. 12A). Expression of K44A/dynamin had no significant effect on the cellular content of GLUT4 protein compared with uninfected and LacZ-expressing cells (Fig. 12A, lanes 1–3). To assess the possibility of persistent insulin signaling, whole cell lysates from basal, insulin-stimulated, and insulin-stimulated cells in which the insulin was removed were subjected to phosphotyrosine immunoblotting (Fig. 12B). In both the LacZ and K44A/dynamin cells, insulin stimulation resulted in equal amounts of insulin receptor (IRβ) and IRS1 tyrosine phosphorylation (Fig. 12B, compare lanes 1 and 2 with lanes 4 and 5). Following insulin removal, the LacZ- and K44A/dynamin-expressing adipocytes demonstrated a parallel loss of insulin receptor and IRS1 tyrosine phosphorylation (Fig. 12B, lanes 3 and 6). Thus, the retention of GLUT4 at the plasma membrane of K44A/dynamin-expressing cells was not due either to increased expression of the GLUT4 protein or prolonged insulin receptor activation.

DISCUSSION

Since its identification as the insulin-responsive glucose transporter, the mechanism and regulation of GLUT4 trafficking have been areas of intense investigation. GLUT4 expression is predominantly limited to striated muscle and adipose tissue (1–4, 47). In addition, GLUT4 vesicles undergo regulated exocytosis in response to a number of stimuli including insulin, exercise, and osmotic shock (7, 48, 49). Although the precise biochemical pathway by which GLUT4 is recruited from intracellular vesicles has not been fully delineated, this vesicle pool must be replenished following each round of insulin stimulation and withdrawal. In order to replenish intracellular GLUT4 stores after post-prandially elevated circulating insulin has declined, GLUT4 must be removed from the plasma membrane and sorted back into the insulin-responsive vesicle pool.

Specific sequence motifs in the amino- and carboxyl-terminal domains of GLUT4 have been shown to be responsible for directing appropriate trafficking of the GLUT4 protein. The carboxyl-terminal domain appears to function as a sequestration signal directing and/or inducing retention of GLUT4 to the insulin-responsive vesicle pool (50–53). In contrast, the amino-terminal FQQI motif appears to be necessary for the internalization of the plasma membrane-localized GLUT4 although the carboxyl-terminal dileucine motif may also contribute to GLUT4 endocytosis (53–56). Although the major effect of insulin is to increase the rate of GLUT4 exocytosis, insulin also reduces the rate of GLUT4 endocytosis suggesting that these two pathways are coordinately regulated (8–10).

One molecule implicated in endocytic coated-vesicle formation is the GTPase dynamin (for reviews, see Refs. 57 and 58). In addition to its highly conserved amino-terminal GTPase domain, dynamin contains a central pleckstrin homology domain and a carboxyl-terminal proline-rich region which negatively and positively regulate the dynamin self-assembly and GTPase activities, respectively (59). These domains can functionally interact with several signaling intermediates, including Grb2 (21–23, 60), Shc (61), inositol phospholipids (24), and amphipathins (25). In this regard, insulin has been observed to induce the association of a dynamin-Grb2 complex with tyrosine-phosphorylated IRS1, the binding of which can modulate the rate of dynamin GTPase activity in vitro (21). Together, these data suggest insulin may regulate the endocytic rate of constitutively recycling cell-surface proteins such as GLUT4 through a dynamin-dependent mechanism.

Previous studies have demonstrated that expression of the GTPase-defective K44A/dynamin I mutant in non-neuronal cells blocks coated vesicle formation and budding by function in a dominant, interfering manner over endogenous dynamin II (27, 38). These same studies have shown that expression of wild-type dynamin I has no effect on steady state rates of endocytosis. Since dynamin assembles into tetrameric complexes (59), overexpressed K44A/dynamin I likely interferes with endogenous dynamin II function by formation of heterotetrameric complexes. Consistent with previous studies, we have observed in 3T3L1 adipocytes that overexpressing K44A/dynamin I interferes with endogenous dynamin II functions by assessing transferin receptor endocytosis.

Based upon the kinetics and degree of insulin receptor kinase activation and subcellular localization, others (40–42, 62) have suggested that endosome-localized insulin receptor is the major site of insulin receptor signal transduction and biological responsiveness. Likewise, inhibition of epidermal growth factor receptor endocytosis by expression of K44A/dynamin I has been reported to interfere with several specific signaling pathways,
whereas wild-type dynamin I had no effect (29). In contrast, insulin receptor autophosphorylation and IRS1 tyrosine phosphorylation still occur when insulin receptor endocytosis was inhibited by incubation of cells at 4 °C, suggesting that plasma membrane-localized insulin receptor was sufficient for these proximal signaling events (62, 63).

We therefore directly assessed the ability of insulin to activate several signaling pathways in K44A/dynamin-expressing 3T3L1 adipocytes. Our data demonstrate that with the exception of a small but probably physiologically irrelevant decrease in IRS1-associated PI 3-kinase activity, inhibition of dynamin-dependent endocytosis does not affect insulin-stimulated signal transduction pathways leading to either Akt or MAP kinase activation. In addition, we have demonstrated that expression of a dominant interfering dynamin mutant does not affect the dephosphorylation and inactivation of the insulin receptor kinase or IRS1 following insulin removal. These data are consistent with a recent study demonstrating that insulin receptor signaling in the rat hepatoma H4IIE cell line is largely independent of insulin receptor internalization (64). However, these findings are in disagreement with epidemial growth factor receptor signal transduction events where expression of K44A/dynamin was observed to enhance DNA synthesis and tyrosine phosphorylation of Shc and phospholipase Cγ but to inhibit ERK1 and phosphatidylinositol 3-kinase activation (29). We are currently unable to explain this discrepancy, but it may reflect a fundamental difference in the specificity of insulin receptor versus epidermal growth factor receptor signaling mechanisms.

Nevertheless, consistent with the presence of functional signal transduction pathways, inhibition of clathrin-mediated endocytosis did not affect the ability of insulin to induce GLUT4 translocation to the plasma membrane. Expression of K44A/dynamin did, however, result in the accumulation of GLUT4 at the plasma membrane in the basal state. This gradual accumulation of GLUT4 at the cell surface suggests that the population of continuously recycling GLUT4 was unable to internalize from the plasma membrane. While this study was in progress, it has also been reported that expression of a mutant dynamin in Chinese hamster ovary cells co-expressing both the insulin receptor and GLUT4 results in a redistribution of GLUT4 from an intracellular location to the cell surface (65). Similarly, microinjection of a dynamin inhibitory peptide also
resulted in appearance of GLUT4 at the cell surface concomitant with a reduction in GLUT4 internalization (66). Together these data are consistent with the presence of at least two intracellular GLUT4 vesicle populations, one which is undergoing continuous recycling to and from the plasma membrane and a separate pool that is responsive to insulin stimulation.

In summary, our data demonstrate that insulin receptor signal transduction events in 3T3L1 adipocytes, particularly the activation of GLUT4 translocation, can occur in the absence of clathrin-mediated endocytosis. We conclude that GLUT4 endocytosis is dependent upon dynamin, and when its function is inhibited, GLUT4 accumulates at the plasma membrane. The ability of insulin to fully induce the translocation of the remaining intracellular GLUT4 vesicles strongly suggests the presence of two independent GLUT4 vesicle populations, one which undergoes constitutive recycling and a second which is uniquely responsive to insulin action. Although these two populations probably equilibrate with each other at a slow rate, the physical nature of these vesicles remains to be determined. These findings underscore the importance of dynamin function in GLUT4 endocytosis and suggest a potential mechanism by which insulin is able to regulate the rate of GLUT4 endocytosis.

Acknowledgments—We thank Diana Boeglin and Robert Brown for excellent technical assistance. Additionally, we thank Daniel Short for sharing expertise in making adenoviral constructs and Tom Monninger at the University of Iowa Central Microscopy Facility for invaluable aid in collecting confocal images.

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