0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 11
Printed in U.S.A.

The Small Guanosine Triphosphate-Binding Protein Rab4 Is Involved in Insulin-Induced GLUT4 Translocation and Actin Filament Rearrangement in 3T3-L1 Cells*

PETER VOLLENWEIDER, STUART S. MARTIN, TETSURO HARUTA, AARON J. MORRIS, JAMES G. NELSON, MIREILLE CORMONT, YANNICK LE MARCHAND-BRUSTEL, DAVID W. ROSE, AND JERROLD M. OLEFSKY

Department of Medicine, University of California, San Diego, La Jolla, California 92093; Veterans Administration Research Service (J.M.O.), San Diego, California 92161; and INSERM U-145, Faculté de Médecine (M.C., Y.L.M.-B.), 06107 Nice, France

ABSTRACT

Insulin's stimulation of glucose transport involves the translocation of vesicles containing the glucose transporter GLUT4 to the plasma membrane. Small GTP-binding proteins have been implicated in the regulation of vesicular traffic. We studied the effects of microinjection of wild-type Rab4 glutathione *S*-transferase fusion protein (WT Rab4), a GTP-binding defective mutant (Rab4 N121I), a guanosine triphosphatase-defective mutant (Rab4 Q67L), and a Rab4 antibody on insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. Microinjection of Rab4 N121I and Rab4 antibodies had no effect on basal GLUT4 staining, but inhibited insulin-induced GLUT4 translocation by 50% compared with that in control IgG-injected cells. WT Rab4 and Rab4 Q67L microinjection had no effect on either basal or insulin-induced GLUT4 translocation. Premixing and coinjection of the Rab4 antibody with WT Rab4 almost completely abolished its inhibitory effect on insulin-induced GLUT4 translocation.

In contrast, microinjection of an antibody directed against the highly conserved region of Rab3 proteins had no effect on insulin-induced GLUT4. These results point to a direct role of Rab4 in insulin-induced GLUT4 translocation, and that this effect is dependent on nucleotide binding to the protein. We also studied the effect of microinjection of the same proteins on insulin-induced actin filament rearrangement (membrane ruffling) in the same cell line. Microinjection of Rab4 N121I and Rab4 antibodies inhibited insulin-induced membrane ruffling by 40%, whereas WT Rab4 or a Rab3 antibody injection had no effect on cytoskeletal rearrangement. In summary, 1) Rab4 is a necessary component of the insulin/GLUT4 translocation signaling pathway; 2) the function of Rab4 in this pathway requires GTP binding; 3) Rab4 also participates in the process of insulin-induced membrane ruffling; and 4) Rab3 proteins do not seem to be involved in these processes. (*Endocrinology* **138:** 4941–4949, 1997)

ONE OF THE major effects of insulin is to promote glucose uptake by muscle cells and adipocytes. These cells express the glucose transporter GLUT4, insulin-sensitive glucose transporter isoform, and upon insulin stimulation, GLUT4 containing vesicles are translocated from an intracellular compartment to the cell surface (1, 2). The increased number of glucose transporters at the plasma membrane level triggers glucose uptake. The exact molecular mechanisms by which insulin induces GLUT4 translocation are still mainly unknown. This process appears to be closely related to regulated exocytosis, in which small GTP-binding proteins have been implicated (3, 4). In support of this, guanosine 5′-*O*-(3-thiotriphosphate), a nonhydrolyzable GTP analog, induces GLUT4 translocation or/and increased glucose uptake in rat adipocytes (5), 3T3-L1 adipocytes (6), and cardiac myocytes (7).

The Rab protein family of Ras-related small GTP-binding proteins has been implicated in the regulation of intracellular vesicular traffic (3, 4, 8, 9). These proteins, with a molecular mass between 20–30 kDa, are highly homologous to the yeast YPT1 and SEC4 proteins, which play a crucial role in endocytosis and exocytosis. More than 30 members have been identified in mammalian cells, and individual Rab proteins are localized to distinct compartments of both the endocytotic and exocytotic pathways. All members contain highly conserved domains required for guanine nucleotide binding, GTP/GDP exchange, and GTP hydrolysis (10). Nucleotide binding and subsequent hydrolysis are essential for proper targeting and function of the Rab molecules (11). For example, when Rab5 mutants that are unable to bind GTP are overexpressed, they act as dominant negative mutants for endocytosis *in vitro* and *in vivo* (12, 13).

The small GTP-binding protein Rab4 is expressed in rat adipocytes (14) and 3T3-L1 adipocytes (15) and is closely associated with GLUT4-containing vesicles (14, 16, 17). Insulin stimulation induces a redistribution of Rab4 from the GLUT4-containing vesicles to the cytoplasm (14). This effect is reversible after insulin withdrawal. It is, therefore, possible, that Rab4 is involved in insulin-induced GLUT4 translocation. In this regard, it has been recently shown that elec-

Received March 25, 1997.
Address all correspondence and requests for reprints to: Jerrold M. Olefsky, M.D., Department of Medicine (0673), University of California, 9500 Gilman Drive, La Jolla, California 92093-0673.
* This work was supported in part by NIDDK, NIH Grant DK-33651, the V.A. Medical Research Service, a Swiss National Foundation fellowship (to P.V.), NIH Research Fellowship DK-09415 (to A.J.M.), a Mentor-Based fellowship grant from the American Diabetes Association (to T.H.), and the Claude Bernard Foundation (to M.C.).

troporation of a peptide corresponding to the hypervariable carboxy-terminal domain of Rab4 inhibits insulin-stimulated glucose uptake in rat adipocytes (18), but the effector molecules of such peptides are unknown.

Other members of the Rab protein family are highly expressed in insulin-sensitive tissues. In particular two isoforms of the Rab3 proteins, Rab3A and Rab3D, have been shown to be expressed in rat adipocytes and 3T3-L1 adipocytes (19, 20). Interestingly, messenger RNA levels and expression of Rab3A and Rab3D increase significantly during differentiation of 3T3-L1 fibroblasts into mature adipocytes (19, 20). It has therefore been proposed that these proteins could be involved in exocytotic or endocytotic processes induced by insulin. In contrast to Rab4, Rab3D does not associate with GLUT4-containing vesicles (21) and is not redistributed after insulin stimulation (21). By sucrose density gradient centrifugation in 3T3-L1 adipocytes, Rab3A localizes in a different fraction than GLUT4-containing vesicles (20), but potential functions of theses two proteins in insulin-induced vesicular trafficking remain to be determined.

Besides their role in endocytotic and exocytotic pathways, other potential functions seem to be played by the small GTP-binding proteins of the Rab family. For example, Rab8 has been implicated in actin filament rearrangement in fibroblasts (22). As insulin induces cytoskeletal rearrangement (membrane ruffling) in 3T3-L1 cells (23), we wondered whether Rab4 could also play a role in insulin-induced actin filament rearrangement.

Therefore, to further assess whether Rab4 functions in insulin-induced GLUT4 translocation and actin filament rearrangement, we used single cell microinjection of 3T3-L1 adipocytes coupled with immunofluorescence microscopic detection of GLUT4 or actin filament localization. Here we show that injection of a glutathione *S*-transferase (GST)-Rab4 protein with a point mutation in the GTP binding site (Rab4 N121I) as well as a Rab4 antibody directed against the C-terminal portion of the protein inhibit both insulin-induced GLUT4 translocation and actin filament rearrangement in 3T3-L1 adipocytes. Microinjection of an antibody directed against a highly conserved region of the Rab3 isoforms had no effect on insulin-induced GLUT4 translocation and actin filament rearrangement. This suggests a direct role for Rab4 in insulin-induced GLUT4 translocation and cytoskeletal rearrangement.

Materials and Methods

Materials

Porcine insulin was provided by Eli Lilly Co. (Indianapolis, IN). Polyclonal anti-GLUT4 antibody (F349) was described previously (24). Monoclonal anti-GLUT4 antibody (1F8) was obtained from East Acres Biologicals (Southbridge, MA) (25). Sheep IgG and fluorescein isothiocyanate- and 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated antirabbit, antimouse, and antisheep IgG antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). A rabbit polyclonal, affinity-purified, IgG antibody raised against a synthetic peptide corresponding to amino acids 191–210 in the C-terminus of Rab4, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal anti Rab3 antibody was purchased from Transduction Laboratories (Lexington, KY). A protein fragment corresponding to amino acids 60–220 of rat Rab3A was used as immunogen. Tetramethylrhodamine-conjugated phalloidin and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

DNA vector construction and GST fusion protein purification

The complementary DNA coding for wild-type (WT) Rab4 was subcloned in the expression vector pGEX-2T. Mutations of WT Rab4 into pGEX-2T were performed by site-directed mutagenesis. The residue N-121 was changed into I with the following nucleotide C-CTT-TGT-GGA-A*T*C-AAG-AAG-GAC-CTG-G as previously described (26). For the Q67L mutant, residue Q 67 was changed into L with the following nucleotide: G-GAT-ACA-GCA-GGA-C*T*A-GAA-CGA-TTC-AG (26).

Bacteria transformed with the pGEX-2T Rab4 constructs of interest were cultured until the A_{600} reached 0.6. Production of the GST-Rab4 was then induced by the addition of 50 μM isopropyl-β-D-thiogalactoside. Proteins were then purified as described previously (27).

Electrophoresis and immunoblotting

Differentiated 3T3-L1 adipocytes were lysed in a buffer containing 50 mM HEPES, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, 2 mM phenylmethylsulfonylfluoride, 10% glycerol, 4 mM Na_3VO_4, 400 mM sodium fluoride, and 20 mM sodium pyrophosphate, pH 7.4, at 4 C. Fifty micrograms of whole cell lysates were separated by SDS-PAGE (12% polyacrylamide), and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) in a Tris-glycine-methanol buffer. After transfer, the membranes were blocked with Tris-buffered saline-5% non fat milk (wt/vol) or PBS-5% BSA (wt/vol) overnight at 4 C, then incubated with anti-Rab4 (1:1000) and anti-Rab3 (1:2000) antibodies. Bound antibodies were visualized using an enhanced chemiluminescence detection kit (Pierce Chemical Co., Rockford, IL).

Cell culture and microinjection

Preparation of antibodies. For microinjection, antibodies were purchased without sodium azide or BSA and then concentrated by centrifugation through 30-kDa cut-off microconcentrators (Amicon Corp., Beverly, MA) with at least three washes with a buffer containing 5 mM sodium phosphate (pH 7.2) and 100 mM KCl (microinjection buffer).

GLUT4 translocation and membrane ruffling. 3T3-L1 cells were maintained and differentiated into adipocytes, then reseeded onto glass coverslips as previously described (28). Cells were serum starved 2 h before microinjection for GLUT4 detection and overnight for actin fiber staining. All reagents for microinjection were dissolved in microinjection buffer. All reagents, except Rab4 and Rab3 antibodies, were coinjected with preimmune sheep IgG (10 mg/ml) to allow the detection of injected cells. After a recovery period of 1 h, the cells were stimulated with 10 ng/ml insulin (1.7 nM) for 20 min unless otherwise specified. The cells were then fixed for staining.

Immunostaining and fluorescence microscopy

GLUT4 protein staining. Immunostaining of GLUT4 was performed essentially as previously described (28). The cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. After washing, the cells were permeabilized and blocked with 0.1% Triton X-100 and 2% FCS in PBS for 5–10 min. Cells were then incubated overnight at 4 C with F349 (1 μg/ml, final concentration) or 1F8 (4 μg/ml), which were diluted in PBS with 2% FCS. After washing with PBS for 10 min, cells were incubated with fluorescein-conjugated donkey antirabbit (1:100) or antimouse antibody (1:100) as appropriate, and with AMCA-conjugated antisheep, or antirabbit antibody (1:100) to detect injected cells.

Actin staining. Cells were washed and permeabilized as described above and then incubated in PBS with rhodamine-phalloidin (1 μg/ml) to visualize the location of polymerized actin (membrane ruffles) and AMCA-conjugated donkey antisheep or antirabbit antibody to detect injected cells.

Cell quantification. Slides were analyzed on a Zeiss Axiophot immunofluorescence microscope (Zeiss, New York, NY). The AMCA-positive

microinjected cells on each coverslip were evaluated for the presence of plasma membrane-associated GLUT4 staining or actin membrane ruffles. The observer was blinded to the experimental conditions. The percentage of injected cells displaying each phenotype is represented as GLUT4 translocation (percent positive cells) or the ruffling index, respectively.

Imaging. Images were captured using a CCD camera from Photometrics (Tucson, AZ) and were saved using Isee software from Inovision (Durham, NC).

Statistics

Statistical significance was assessed by Student's *t* test for paired data.

Results

The role of Rab4 in insulin-stimulated GLUT4 translocation was studied using single cell microinjection of 3T3-L1 adipocytes. After insulin stimulation, translocation of GLUT4 was visualized by immunofluorescence microscopy using anti-GLUT4 antibodies and FITC-labeled secondary antibodies (Fig. 1, A and B). Injected cells were identified by AMCA-labeled anti-IgG antibodies directed against IgG injected for this purpose (Fig. 1B). As previously shown, microinjection of IgG does not impair insulin's effects on 3T3-L1 adipocytes (28). Unstimulated cells display GLUT4 staining mostly localized around the nucleus, with some staining distributed around the cytoplasm (Fig. 1A, basal). After insulin stimulation, GLUT4 staining was seen at the plasma membrane level with a concomitant decrease in the cytoplasm (Fig. 1A, + insulin). The cells displaying staining predominantly at the plasma membrane were counted as positive, and typically, after insulin stimulation, about 50% of the cells were scored as positive.

Effect of microinjection of a GTP-binding defective Rab4 mutant (Rab4 N121I) on insulin-induced GLUT4 translocation

Rab4 N121I was injected at increasing concentrations of 2, 4.5, and 9 mg/ml into 3T3-L1 cells along with sheep IgG (10 mg/ml). There was no effect on GLUT4 distribution in unstimulated cells, and the number of positive cells for GLUT4 staining was comparable to that in control experiments. In contrast, Rab4 N121I microinjection at concentrations of 2, 4.5, and 9 mg/ml inhibited insulin-induced GLUT4 translocation by 34% ($P < 0.0005$ *vs.* control IgG), 51% ($P = 0.001$), and 50% ($P = 0.0003$), respectively, compared with control sheep IgG microinjection (Figs. 1B and 2).

Effect of microinjection of GST-WT Rab4 on insulin-induced GLUT4 translocation

Microinjection of WT Rab4 protein at concentrations of 7 and 14 mg/ml in 3T3-L1 adipocytes had no effect on the pattern of GLUT4 staining in basal or insulin-stimulated cells. After insulin stimulation, the percentage of positive cells after WT Rab4 injection was comparable to that of control injected cells (47% and 42% *vs.* 47%; $P = 0.64$ and $P = 0.78$, respectively; Fig. 2).

Effect of microinjection of a guanosine triphosphatase (GTPase)-defective mutant (Rab4 Q67L) on insulin-induced GLUT4 translocation

Microinjection of Rab4 Q67L protein at a concentration of 15 mg/ml in 3T3-L1 adipocytes had no effect on GLUT4 localization on either basal or insulin-stimulated cells. After insulin stimulation, the percentage of positive cells after Rab4 Q67L was comparable to that of control injected cells (50% *vs.* 52%; $P = 0.42$; Fig. 3).

Effect of microinjection of a Rab4 antibody or a Rab3 antibody on insulin-induced GLUT4 translocation

To further explore the role of Rab4 in GLUT4 translocation, we microinjected 3T3-L1 adipocytes with a rabbit affinity-purified IgG antibody raised against a synthetic peptide corresponding to amino acids 191–210 in the C-terminus of Rab4 at a concentration of 10 mg/ml. Injection of the Rab4 antibody had no effect on basal GLUT4 staining in 3T3-L1 adipocytes (Fig. 3). After insulin stimulation (10 ng/ml for 20 min), GLUT4 translocation was inhibited by 55% compared with that in control IgG-injected cells ($P = 0.0037$; Fig. 3). As Rab3A and Rab3D expression increased in 3T3-L1 fibroblasts during differentiation into adipocytes, we wondered whether these proteins could also be involved in insulin-induced GLUT4 translocation. To assess this question, we used a mouse monoclonal antibody raised against amino acids 60–220 of rat Rab3A in microinjection studies. This is a highly conserved region between the different Rab3 isotypes (>85% identity between Rab3A and Rab3D) (19). Microinjection of this antibody (6 mg/ml) had no effect on either basal or insulin-induced GLUT4 translocation in 3T3-L1 adipocytes (54% *vs.* 53% in control injected cells; $P = 0.57$; Fig. 4).

Effect of coinjection of Rab4 antibody and WT Rab4 on insulin-induced GLUT4 translocation

To address the specificity of the effect of the Rab4 antibody microinjection on GLUT4 translocation, we premixed the Rab4 antibody with WT Rab4. Rab4 antibody (10 mg/ml) was mixed 1:1 with WT Rab4 (7 mg/ml) and subsequently injected into 3T3-L1 adipocytes. Rab4 antibody microinjection alone at a concentration of 5 mg/ml inhibited GLUT4 translocation by 43% compared with that in control injected cells. When mixed and coinjected with WT Rab4, the inhibitory effect of the antibody was markedly reduced, indicating that exposure to the antigen (WT Rab4) quenched the ability of the Rab4 antibody to interfere with insulin-induced GLUT4 translocation (Fig. 4).

Western blotting of Rab4 and Rab3 proteins in 3T3-L1 adipocytes

After separation by SDS-PAGE and transfer to PVDF membranes, whole cell lysates were probed with Rab4 and Rab3 antibodies. Figure 5 shows that the Rab4 and Rab3 antibodies recognize a single band of the expected size in 3T3-L1 adipocyte whole cell lysates.

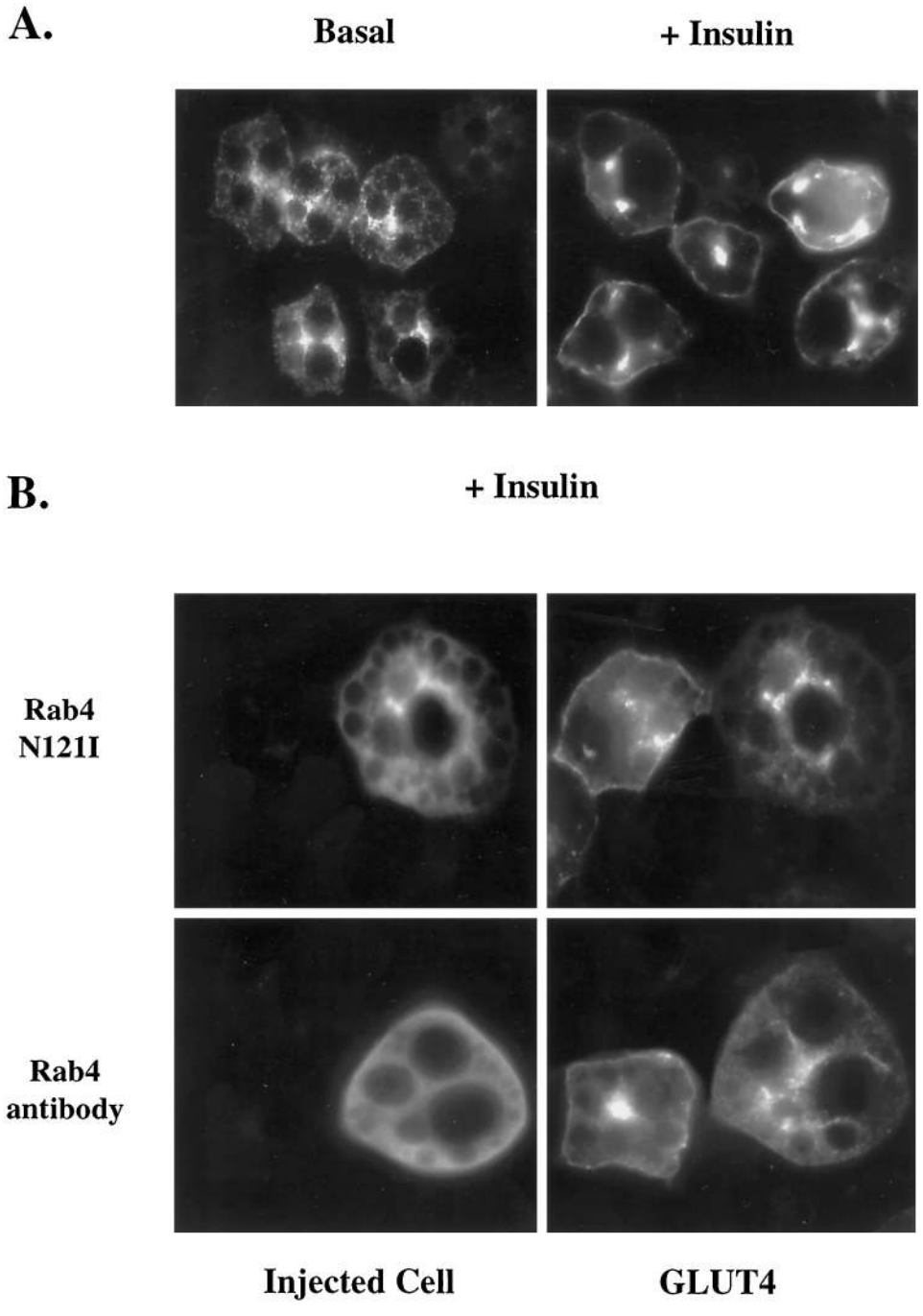

FIG. 1. Images of GLUT4 immunofluorescence staining in 3T3-L1 adipocytes. Serum-starved adipocytes on coverslips were microinjected, then treated in the absence or presence of insulin (10 ng/ml) for 20 min. After fixation, cells were stained with anti-GLUT4 antibody (F349 or 1F8), followed by incubation with FITC-conjugated second antibody. Injected cells were identified by staining with AMCA antisheep or anti-rabbit IgG (B, *left panels*) as appropriate. A, Typical GLUT4 staining in 3T3-L1 adipocytes in basal conditions and after insulin stimulation. B, Individual cells injected with either Rab4 N121I or Rab4 antibody. The *left panels* show injected cells, and the *right panels* demonstrate GLUT4 staining.

Effects of WT Rab4, Rab4 N121I, Rab4 antibody, and Rab3 antibody on membrane ruffling in 3T3-L1 adipocytes

WT Rab4, Rab4 N121I, Rab4, and Rab3 antibody were injected into quiescent 3T3-L1 adipocytes. One hour after microinjection, cells were either fixed or stimulated with insulin (100 ng/ml) for 20 min and analyzed for actin filament rearrangement (Fig. 6). Rab4 antibody and Rab4 N121I inhibited insulin-induced membrane ruffling in 3T3-L1 adipocytes by 42% and 39%, respectively, compared with that in control injected cells ($P < 0.0004$ and $P < 0.05$; Fig. 7). WT Rab4 or Rab3 antibody microinjection had no effect on insulin-induced membrane ruffling ($P = 0.41$ and $P = 0.8$, respectively).

Discussion

One of the major effects of insulin is to increase glucose uptake into target tissues such as skeletal muscle, adipose tissue, and cardiac muscle. This effect is largely mediated by insulin-induced recruitment of insulin-sensitive glucose transporters (GLUT4) to the cell surface. In the basal state, GLUT4-containing vesicles are located throughout the cytoplasm, predominantly in the perinuclear region. After insu-

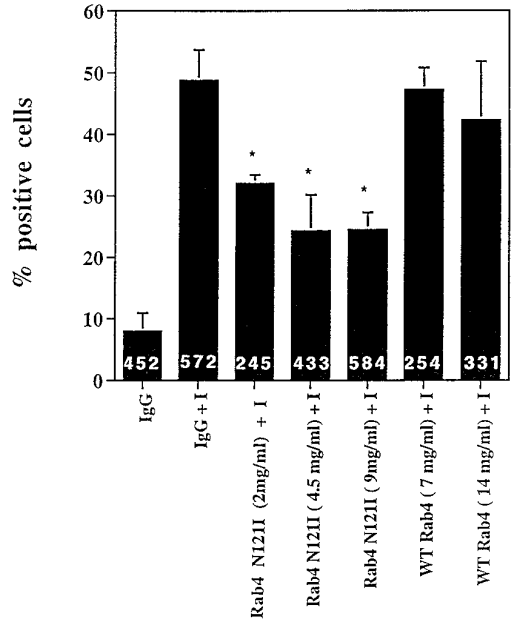

FIG. 2. Effect of microinjection of a GTP-binding defective Rab4 protein (Rab4 N121I) and WT Rab4 on insulin-induced GLUT4 translocation. Serum-starved 3T3-L1 adipocytes on coverslips were microinjected with GST-Rab4 N121I and GST-WT Rab4 at various concentrations (2, 4.5, and 9 mg/ml, and 7 and 14 mg/ml, respectively), then treated with insulin (10 ng/ml) for 20 min. Fixed cells were stained with anti-GLUT4 antibody (F349), followed by incubation with FITC-conjugated antirabbit IgG antibody and AMCA-conjugated antisheep IgG antibody. AMCA-positive injected cells were evaluated for GLUT4 translocation. Results are expressed as the percentage of positive cells for GLUT4 translocation. The total number of injected cells scored is indicated on each *bar*. *Error bars* represent the SE for four separate experiments. *, $P < 0.005$ *vs.* IgG control microinjected cells.

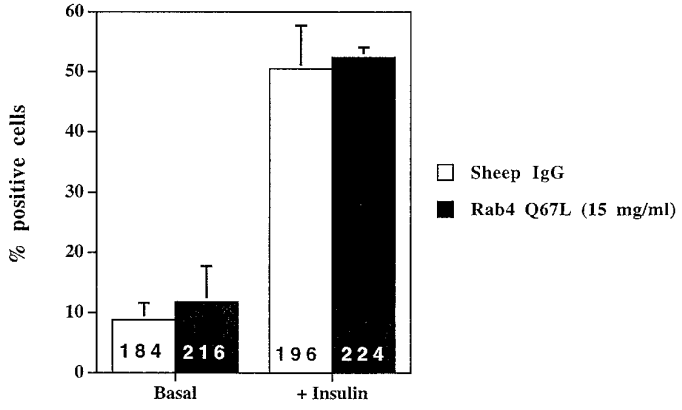

FIG. 3. Effect of microinjection of a GTPase defective mutant (Rab4 Q67L) on insulin-induced GLUT4 translocation. Serum-starved 3T3-L1 adipocytes were microinjected with either sheep IgG (10 mg/ml) as a control or Rab4 Q67L (15 mg/ml). Cells were treated without and with insulin (10 ng/ml) for 20 min and then fixed and stained for injected IgG. AMCA-positive injected cells were evaluated for GLUT4 translocation. Results are expressed as the percentage of positive cells. The total number of injected cells scored is indicated on each *bar*. *Error bars* represent the SE for three separate experiments.

lin stimulation, these vesicles are translocated to the cell surface by a mechanism thought to be very similar to regulated exocytosis. Despite the importance of the signaling pathway leading to this effect, the molecular mechanisms leading to GLUT4 translocation are incompletely understood. Here we show that microinjection of a GTP-binding defective mutant of Rab4 (Rab4 N121I) as well as antibodies directed against the carboxy-terminus of the Rab4 protein inhibit insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. In these same cells, we show for the first time an inhibitory effect of Rab4 N121I and Rab4 antibodies on insulin-induced actin filament rearrangement (membrane ruffling).

Inhibition of insulin-induced GLUT4 translocation

Rab4 is part of the Rab family of Ras-related small GTP-binding proteins that are essential components of exocytotic and endocytotic pathways (29). Originally, Rab4 had been shown to be associated with early endosomes in Chinese hamster ovary cells or HepG2 cells. In several cell lines, Rab4 controls recycling of transferrin receptors from early endosomes to the cell surface (30). More recently, Cormont *et al.* have shown that Rab4 is present on GLUT4-containing vesicles in rat adipocytes (14), and that insulin stimulation results in translocation of Rab4 from the vesicles to the cytosol. Importantly, similar results have been reported in rat skeletal muscle, another insulin-sensitive tissue (16, 17).

Rab proteins are small GTPases, and therefore, nucleotide binding as well as GTP/GDP cycling control shuttling and targeting of the protein as well as its function. Almost all Ras-related proteins have a highly conserved GTP-binding pocket (10), and mutations in this GTP/GDP binding motif can result in defective guanine nucleotide binding. We used a mutated protein with a substitution of N for I at position 121. Similar mutations of both YPT1 and SEC4 result in proteins that do not bind GTP and produce dominant lethal phenotypes and secretory defects when expressed in yeast (31, 32). Overexpression of a Rab5 N133I mutant, similar to Rab4 N121I, in BHK-21 cells leads to a complete inhibition of endocytosis and endosome fusion *in vitro* and *in vivo* (12). With this as a background, one could ask how could a GTP-binding defective Rab4 protein exerts an inhibitory effect on insulin-induced GLUT4 translocation? One possibility is that the microinjected Rab4 N121I sequesters certain factors required for endogenous Rab4 function. Rab proteins have to interact with a set of proteins, such as GTPase-activationg protein, guanine nucleotide dissociation stimulator proteins, and Rab effector proteins, to be activated and carry out their biological functions. Rab4 N121I could interact with such factors, fail to be activated by GTP binding, and prevent the interaction of endogenous Rab with those proteins.

Interestingly, it has recently been shown, that insulin stimulates GTP binding to Rab4 in rat adipocytes (33). This dose-dependent effect could be blocked by phosphatidylinositol 3-kinase inhibitors that also inhibit insulin-induced GLUT4 translocation (33). We show here that neither wild-type Rab4 nor Rab4 Q67L (a GTPase-defective mutant), which both bind GTP (26), had an effect on insulin-induced GLUT4 translocation. Taken together, our data indicate that GTP binding to Rab4 (rather than GTP hydrolysis) is required for its function in insulin signaling. We speculate that the various Rab4 proteins studied can engage with endogenous

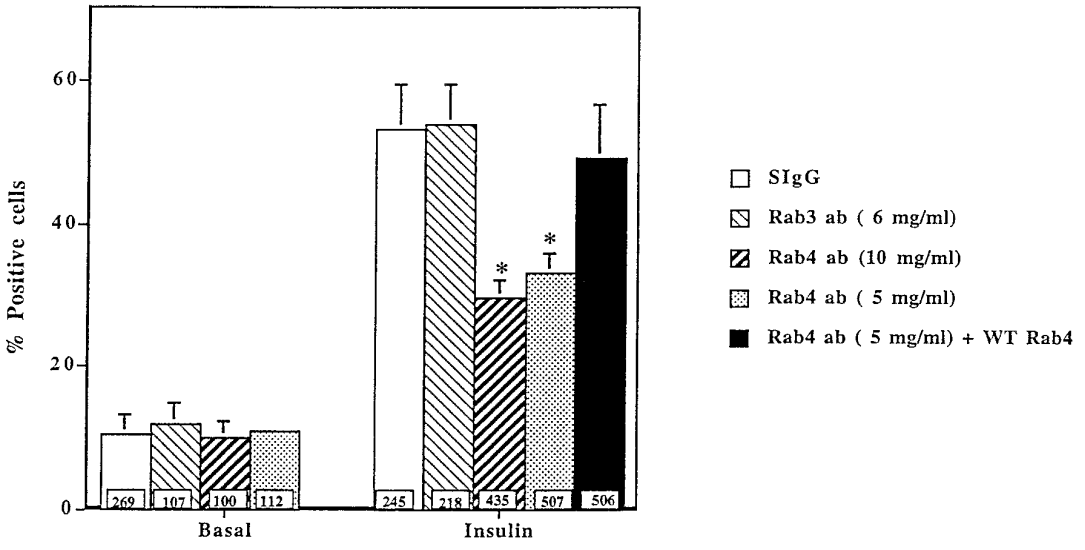

FIG. 4. Effects of microinjection of Rab4 antibody, premixed Rab4 antibody plus WT Rab4 or Rab3 antibody on insulin-induced GLUT4 translocation. Serum-starved 3T3-L1 adipocytes were microinjected with sheep IgG (10 mg/ml) as a control, Rab4 antibody at concentrations of 10 and 5 mg/ml, or with a 1:1 mix of Rab4 antibody (10 mg/ml) plus WT Rab4 (7 mg/ml) or Rab3 antibody (6 mg/ml). Cells were treated without and with insulin (10 ng/ml) for 20 min, then fixed and stained for GLUT4, and injected IgG. AMCA-positive injected cells were evaluated for GLUT4 translocation. Results are expressed as the percentage of positive cells. The total number of injected cells scored is indicated on each *bar*. *Error bars* represent the SE for four separate experiments with the antibody microinjection and three separate experiments for the mixing of Rab4 antibody and WT Rab4. *, $P < 0.005$ *vs.* IgG-injected cells.

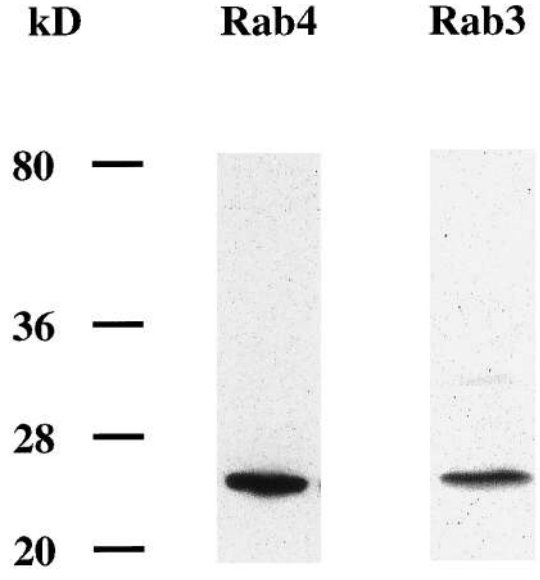

FIG. 5. Western blotting of Rab4 and Rab3 in whole cell lysates of 3T3-L1 adipocytes. Samples of 50 μg total cellular extract from 3T3-L1 adipocytes were applied to a 12% polyacrylamide gel. PVDF membranes were then probed with either Rab4 antibody or Rab3 antibody, and bound antibody was detected with enhanced chemiluminescence. The *arrows* indicate the position of the specific signal, and mol wt markers are indicated. Rab4 and Rab3 antibodies recognize a protein of the expected molecular mass in cell extracts of 3T3-L1 adipocytes.

effector molecules, but without GTP binding they are not functional; therefore, Rab4 N121I is inhibitory. Furthermore, the fact that WT Rab4 or Rab4 Q67L microinjection had no stimulatory effect on basal or insulin-induced GLUT4 distribution suggests that Rab4 proteins are required, but not sufficient, for GLUT4 translocation.

Vesicular trafficking in the cell requires a precise targeting and docking mechanism. It is now generally agreed that important partners in this process are multisubunit particles, known as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor complex (SNARE). The SNARE hypothesis proposes that this is accomplished by the partnering of specific markers, carried by transport vesicles, termed v-SNARES, with their cognate t-SNARES associated with the intended target membrane (34, 35). The SNARE complex assembly is essential for vesicle docking and fusion. Vesicle-associated membrane protein proteins (vesicle-associated membrane protein-2 and cellubrevin), which are part of the SNARE complex, have been localized to GLUT4 vesicles (36, 37), and it has been shown that Rab proteins are required for the assembly of SNARE complexes in the docking of transport vesicles (38, 39). Proteins of the SNARE complex could be effectors of Rab4. The potential role of Rab4 in insulin-induced GLUT4 translocation is further supported by the inhibitory effect observed after Rab4 antibody microinjection. This effect is specific, as premixing the antibody with WT Rab4 almost completely abolishes its inhibitory effect on insulin-induced GLUT4 translocation.

Baldini *et al.* have shown that two isoforms of the Rab3 proteins, Rab3A and Rab3D, are expressed in rat adipocytes as well as in 3T3-L1 adipocytes (19, 20). Furthermore, they showed that the expression levels of these isoforms of Rab3 increases severalfold during differentiation of 3T3-L1 fibroblasts into 3T3-L1 adipocytes (19, 20). It was speculated that Rab3D or Rab3A could be involved in insulin-regulated secretory pathways, but their function has not yet been characterized. Rab3D does not colocalize with GLUT4 vesicles and, unlike GLUT4, is not redistributed in response to insulin (21). In sucrose gradient fractionation experiments with 3T3-L1 adipocytes, Rab3A does not localize in the same pool

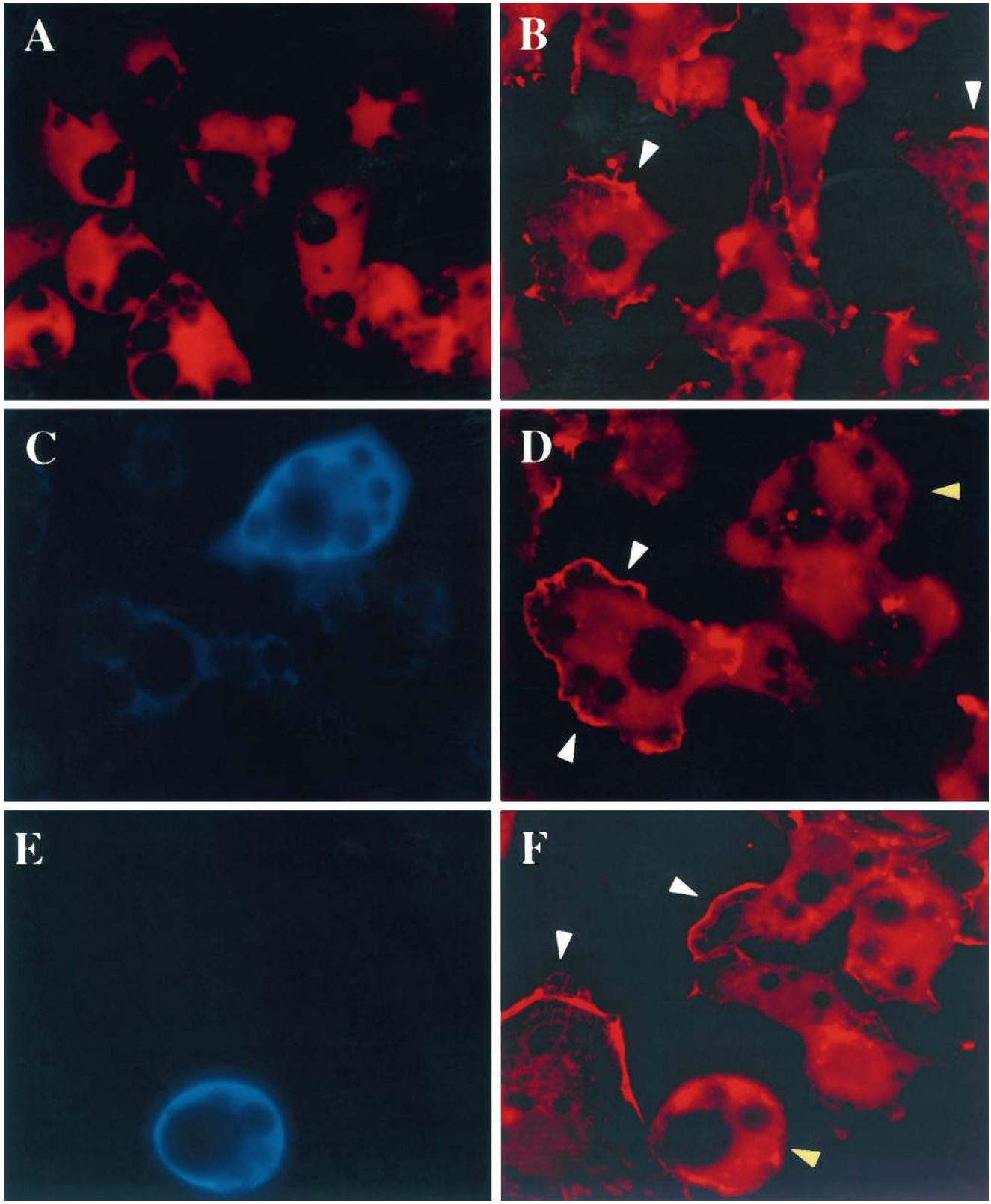

FIG. 6. Images of actin localization in 3T3-L1 adipocytes. Serum-starved adipocytes on coverslips were treated in the absence (A) or presence (B) of insulin (100 ng/ml) for 20 min, and actin localization was detected with tetramethylrhodamine-labeled phalloidin. Typical membrane ruffles are shown (*white arrows*). Individual adipocytes were microinjected with the appropriate reagents and 10 mg/ml of sheep IgG to allow detection of injected cells. After fixation, cells were stained for actin localization with tetramethylrhodamine-labeled phalloidin (*red*, D and F). Injected cells were identified by staining with AMCA antisheep IgG for coinjected sheep IgG or AMCA antirabbit IgG (*blue*, C and E). C and D, Individual 3T3-L1 cells microinjected with Rab4 N121I and treated with insulin. E and F, Individual 3T3-L1 cells microinjected with Rab4 antibody and treated with insulin. *White arrows* indicate membrane ruffles, and *yellow arrows* indicate injected cells.

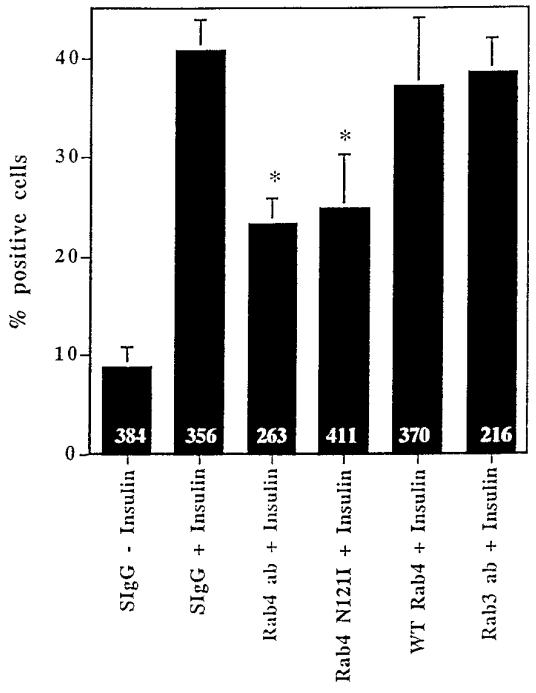

FIG. 7. Effects of microinjection of Rab4 antibody, Rab4 N121I, WT Rab4, and Rab3 antibody on insulin-induced actin filament rearrangement in 3T3-L1 adipocytes. Quiescent 3T3-L1 adipocytes were microinjected with preimmune IgG (10 mg/ml) as a control, Rab4 antibody (10 mg/ml), Rab4 N121I (4.5 mg/ml), WT Rab4 (7 mg/ml), or Rab3 antibody (6 mg/ml). Cells were then treated with and without 100 ng/ml insulin for 20 min, fixed, and stained for actin filament rearrangement (membrane ruffling) and injected IgG. AMCA-positive cells were evaluated for actin filament rearrangement. The total number of injected cells scored is indicated on each *bar*. Results are expressed as the percentage of positive cells. *Error bars* represent the SE for three separate experiments. *, $P < 0.05$.

as GLUT4-containing vesicles. Rab3A is expressed in neuronal and neuroendocrine cells, where it is associated with synaptic and synaptic-like vesicles. To investigate a potential role for Rab3 proteins in insulin-induced GLUT4 translocation and assess the specificity of the effect observed with the Rab4 antibody, we microinjected a Rab3 antibody, which had no effect on insulin-induced GLUT4 translocation. The Rab3 antibody is a monoclonal antibody against amino acids 60–220 of rat Rab3A, and this region is highly homologous between Rab3A and Rab3D (>85% amino acid identity) (19). This suggests that the antibody cross-reacts with the different isoforms of Rab3. Our results would indicate that Rab3 isoforms are probably not involved in insulin-induced GLUT4 translocation.

Using a different approach, Shibata *et al.* reported that electroporation into adipocytes of a 20-amino acid peptide corresponding to the hypervariable C-terminal region of the Rab4 protein can inhibit insulin-induced GLUT4 translocation and glucose transport by about 40–50% (18). As the carboxy-terminal domain of Rab proteins contains structural elements necessary for the association with specific target membranes, it was postulated that the peptide could compete with endogenous Rab at target sites. In contrast, electroporation of peptides corresponding to the Rab3C and Rab3D hypervariable region had no effect on insulin-induced glucose transport. This latter result supports our own data showing a lack of effect of Rab3 antibody on insulin-induced GLUT4 translocation. However, effector molecules of such peptides have yet to be characterized.

By cotransfecting freshly isolated rat adipocytes with Rab4 proteins and a GLUT4 transporter tagged with a *myc*-epitope (GLUT4-myc), Cormont *et al.* recently showed that Rab4 could play a role in GLUT4 subcellular localization (26). Overexpression of WT Rab4 resulted in a decrease in GLUT4-*myc* at the cell surface with normal or even increased responses to insulin-induced tagged transporter translocation. No effect on basal or insulin-induced membrane levels of GLUT4-*myc* was detected by cotransfecting adipocytes with Rab4 N121I, although they did find that expression of a C-terminal-deleted Rab4 inhibited insulin-induced GLUT4 translocation. It is possible that the observed differences in results from those of the current studies are related to the level of overexpression of the Rab4 proteins. Clearly, the responses vary depending on the amount of DNA transfected into the adipocytes. In our system, we measure localization of endogenous GLUT4 molecules present in 3T3-L1 adipocytes rather than transfected overexpressed GLUT4. This might explain why we did not observe an effect of WT Rab4 microinjection on basal GLUT4 localization. Another difference lies in the fact that we microinject GST fusion proteins, rather than expression vectors. Nevertheless, we believe that our results provide further evidence that Rab4 is involved in insulin-induced GLUT4 translocation in 3T3-L1 adipocytes and suggest the importance of nucleotide binding for the function of Rab4.

Actin filament rearrangement

We also studied the effect of the microinjected Rab4 proteins and Rab4 antibody on insulin-induced actin filament rearrangement (membrane ruffling) in the same cell line. Our results showed that the GTP-binding defective Rab4 mutant (Rab4 N121I) as well as the Rab4 antibody inhibit insulin-induced actin filament rearrangement by about 40% when injected into 3T3-L1 adipocytes. WT Rab4 as well as Rab3 antibody microinjection had no effect on membrane ruffling induced by insulin. To our knowledge, this is the first time that a potential role for Rab4 in insulin-induced actin filament rearrangement has been observed. Rac and Rho, which are also small GTP-binding proteins, are known to function in this pathway (40, 41). Interestingly, it has recently been shown that a member of the Rab protein family, Rab8, could be involved in cytoskeletal rearrangement. Transient as well as stable expression of WT Rab8 and an activated form of Rab8 (Rab8Q67L) in BHK cells induced reorganization of their actin filaments and formation of membrane ruffles (22). It was postulated that Rab8 could play a role in membrane delivery to membrane ruffles and possibly cross-talk with the small GTP-binding proteins Rho and Rac, leading to cytoskeletal rearrangement. Our results suggest that Rab4 is involved in insulin-induced actin filament rearrangement in 3T3-L1 cells and that GTP binding is important for that function. Rab4 protein could be a common signaling element in insulin-induced GLUT4 translocation and membrane ruffling in 3T3-L1 adipocytes.

In summary, our results demonstrate that microinjection of a GTP-binding defective Rab4 protein (Rab4 N121I) as well as Rab4 antibody can inhibit insulin-induced GLUT4 translocation, providing evidence that Rab4 is involved in this metabolic action. We further show that this effect is dependent on nucleotide binding, as WT Rab4 microinjection had no effect on insulin-induced GLUT4 translocation. In addition, we observed an inhibition of insulin-induced membrane ruffling after microinjection of Rab4 N121I and Rab4 antibody, suggesting, for the first time, that Rab4 could play a role in cytoskeletal rearrangement in 3T3-L1 cells.

Acknowledgments

We thank Michael Mueckler (Washington University School of Medicine) for providing F349 anti-GLUT4 antibody.

References

1. **Cushman SW, Wardzala LJ** 1980 Potential mechanims of insulin action on glucose transport in the isolated rat adipose cell. J Biol Chem 255:4758–4762
2. **Mueckler M** 1994 Facilitative glucose transporters. Eur J Biochem 219:713–725
3. **Ferro-Novick S, Novick P** 1993 The role of GTP-binding proteins in transport along the exocytic pathway. Annu Rev Cell Biol 9:575–599
4. **Novick P, Brennwald P** 1993 Friends and family: the role of the Rab GTPases in vesicular traffic. Cell 75:597–601
5. **Baldini G, Hohman R, Charron MJ, Lodish HF** 1991 Insulin and nonhydrolyzable GTP analogs induce translocation of GLUT4 to the plasma membrane in α-toxin-permeabilized rat adipose cells. J Biol Chem 266:4037–4040
6. **Robinson LJ, Pang S, Harris DS, Heuser J, James DE** 1992 Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3–L1 adipocytes: effects of ATP, insulin, and GTPGS and localization of GLUT4 to clathrin lattices. J Cell Biol 117:1181–1196
7. **Manchester J, Kong X, Lowry OH, Lawrence JC, Jr** 1994 Ras signaling in the activation of glucose transport by insulin. Proc Natl Acad Sci USA 91:4644–4648
8. **Nuoffer C, Balch WE** 1994 GTPases: multifunctional molecular switches regulating vesicular traffic. Annu Rev Biochem 63:949–990
9. **Balch WE** 1990 Small GTP-binding proteins in vesicular transport. Trends Biochem Sci 15:473–477
10. **Dever TE, Glynias MJ, Merrick WC** 1987 GTP-binding domain: three consensus sequence elements with distinct spacing. Proc Natl Acad Sci USA 84:1814–1818
11. **Ullrich O, Horiuchi H, Bucci C, Zerial M** 1994 Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature 368:157–160
12. **Li G, Stahl PD** 1993 Structure-function relationship of the small GTPase rab5. J Biol Chem 268:24475–24480
13. **Kurzchalia TV, Gorvel J-P, Dupree P, Parton R, Kellner R, Houthaeve T, Gruenberg J, Simon K** 1992 Interaction of Rab5 with cytosolic proteins. J Biol Chem 267:18419–18423
14. **Cormont M, Tanti J-F, Zahraoui A, Van Obberghen E, Tavitian A, Le Marchand-Brustel Y** 1993 Insulin and okadaic acid induce Rab4 redistribution in adipocytes. J Biol Chem 268:19491–19497
15. **Ricort J-M, Tanti J-F, Cormont M, Van Obberghen E, Le Marchand-Brustel Y** 1994 Parallel changes in GLUT4 and Rab4 movements in two insulin-resistant states. FEBS Lett 347:42–44
16. **Aledo JC, Darakhshan F, Hundal HS** 1995 Rab4, but not the transferrin receptor, is colocalized with GLUT4 in an insulin-sensitive intracellular compartment in rat skeletal muscle. Biochem Biophys Res Commun 215:321–328
17. **Sherman LA, Hirshman MF, Cormont M, Le Marchand-Brustel Y, Goodyear LJ** 1996 Differential effects of insulin and exercise on Rab4 distribution in rat skeletal muscle. Endocrinology 137:266–273
18. **Shibata H, Omata W, Suzuki Y, Tanaka S, Kojima I** 1996 A synthetic peptide corresponding to the Rab4 hypervariable carboxy-terminal domain inhibits insulin action on glucose transport in rat adipocytes. J Biol Chem 271:9704–9709
19. **Baldini G, Hohl T, Lin HY, Lodish HF** 1992 Cloning of a Rab3 isotype predominantly expressed in adipocytes. Proc Natl Acad Sci USA 89:5049–5052
20. **Baldini G, Scherrer PE, Lodish HF** 1995 Nonneuronal expression of Rab3A: induction during adipogenesis and association with different intracellular membranes than Rab3D. Proc Natl Acad Sci USA 92:4284–4288
21. **Guerre-Millo M, Baldini G, Lodish HF, Lavau Marcelle, Cushman SW** 1997 Rab 3D in rat adipose cells ant ist overexpression in genetic obesity (Zucker fatty rat). Biochem J 321:89–93
22. **Peranen J, Auvinen P, Virta H, Wepf R, Simons K** 1996 Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. J Cell Biol 135:153–167
23. **Morris AJ, Martin SS, Haruta T, Nelson JG, Vollenweider P, Gustafson TA, Mueckler M, Rose DW, Olefsky JM** 1996 Evidence for an insulin receptor substrate 1 independent insulin signaling pathway that mediates insulin-responsive glucose transporter (GLUT4) translocation. Proc Natl Acad Sci USA 93:8401–8406
24. **Haney PM, Slot JW, Piper RC, James DE, Mueckler M** 1991 Intracellular targetting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. J Cell Biol 114:689–699
25. **James DE, Brown R, Navarro J, Pilch PF** 1988 Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. Nature 333:183–185
26. **Cormont M, Bortoluzzi M-N, Gautier N, Mari M, Van Obberghen E, Le Marchand-Brustel Y** 1996 Potential role of Rab4 in the regulation of subcellular localization of Glut4 in adipocytes. Mol Cell Biol 16:6879–6886
27. **Bortoluzzi MN, Cormont M, Gautier N, Van Obberghen E, and Le Marchand-Brustel Y** 1996 GTPase activating protein activity for Rab4 is enriched in the plasma membrane of 3T3–L1 adipocytes. Possible involvement in the regulation of Rab4 subcellular localization. Diabetologia 39:899–906
28. **Haruta T, Morris AJ, Rose DW, Nelson JG, Mueckler M, Olefsky JM** 1995 Insulin stimulated GLUT4 translocation is mediated by a divergent intracellular signaling pathway. J Biol Chem 270:27991–27994
29. **Chavrier P, Parton RG, Hauri HP, Simons K, Zerial M** 1990 Localization of low molecular weight GTP binding proteins to exocytotic and endocytotic compartment. Cell 62:317–329
30. **van der Sluijs P, Hull M, Zahraoui A, Tavitian A, Goud B, Mellman I** 1991 The small GTP-binding protein Rab4 is associated with early endosomes. Proc Natl Acad Sci USA 88:6313–6317
31. **Schmidt HD, Puzicha M, Gallwitz D** 1988 Study of a temperature-sensitive mutant of the *ras* related YPT1 gene product in yeast suggests a role in the regulation of intracellular calcium. Cell 53:635–647
32. **Schmidt HD, Wagner P, Pfaff E, Gallwitz D** 1986 The *ras*-related YPT1 gene product in yeast: a GTP-binding protein that might be involved in microtubule organization. Cell 47:401–412
33. **Shibata H, Omata W, Kojima I** 1997 Insulin stimulates guanine nucleotide exchange on Rab4 via a wortmannin-sensitive signaling pathway in rat adipocytes. J Biol Chem 272:14542–14546
34. **Rothman JE, Orci L** 1992 Molecular dissection of the secretory pathway. Nature 355:409–415
35. **Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE** 1993 SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318–324
36. **Tamori Y, Hashiramoto M, Araki S, Kamata Y, Takahashi M, Kozaki S, Kasuga M** 1996 Cleavage of vesicle-associated membrane protein (VAMP)-2 and cellubrevin on GLUT4-containing vesicles inhibits the translocation of GLUT4 in 3T3–L1 adipocytes. Biochem Biophys Res Commun 220:740–745
37. **Cain CC, Trimble WS, Lienhard GE** 1992 Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat aipocytes. J Biol Chem 267:11681–11684
38. **Brennwald P, Kearns B, Champion K, Keranen S, Bankaitis V, Novick P** 1994 Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell 79:245–258
39. **Sogaard M, Tani K, Ye RR, Geromanos S, Tempst P, Kirchhausen T, Rothman JE, Sollner T** 1994 A Rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell 78:937–948
40. **Ridley AJ, Hall A** 1992 The small GP-binding protein rho regulates the assembly of focal adhesions and actin strees fiber in response to growth factors. Cell 70:389–399
41. **Ridley AJ, Paterson HF, Johnston CL, Dieckmann D, Hall A** 1992 The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. Cell 70:401–410