MOLECULAR AND CELLULAR BIOLOGY, Dec. 1996, p. 6879–6886 Vol. 16, No. 12
0270-7306/96/$04.00+0

Potential Role of Rab4 in the Regulation of Subcellular Localization of Glut4 in Adipocytes

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A role for Rab4 in the translocation of the glucose transporter Glut4 induced by insulin has been recently proposed. To study more directly the role of this small GTPase, freshly isolated adipocytes were transiently transfected with the cDNAs of both an epitope-tagged Glut4-myc and Rab4, a system which allows direct measurement of the concentration of Glut4 molecules at the cell surface. When cells were cotransfected with Glut4-myc and Rab4, the concentration of Glut4-myc at the cell surface decreased in parallel with the increased expression of Rab4, suggesting that Rab4 participates in the intracellular retention of Glut4. In parallel, the amount of Rab4 associated with the Glut4-containing vesicles increased. When Rab4 was moderately overexpressed, the number of Glut4-myc molecules recruited to the cell surface in response to insulin was similar to that observed in mock-transfected cells, and thus the insulin efficiency was increased. When Rab4 was expressed at a higher level, the amount of Glut4-myc present at the cell surface in response to insulin decreased. Since the overexpressed protein was predominantly cytosolic, this suggests that the cytosolic Rab4 might complex some factor(s) necessary for insulin action. This hypothesis was strengthened by the fact that Rab4 ΔCT, a Rab4 mutant lacking the geranylgeranylation sites, inhibited insulin-induced recruitement of Glut4-myc to the cell surface, even when moderately overexpressed. Rab3D was without effect on Glut4-myc subcellular distribution in basal or insulin-stimulated conditions. While two mutated proteins unable to bind GTP did not decrease the number of Glut4-myc molecules in basal or insulin-stimulated conditions at the plasma membrane, the behavior of a mutated Rab4 protein without GTPase activity was similar to that of the wild-type Rab4 protein, indicating that GTP binding but not its hydrolysis was required for the observed effects. Altogether, our results suggest that Rab4, but not Rab3D, participates in the molecular mechanism involved in the subcellular distribution of the Glut4 molecules both in basal and in insulin-stimulated conditions in adipocytes.

Insulin stimulates glucose uptake in muscle and adipose tissues by promoting the translocation of intracellular vesicles containing the glucose transporter Glut4 to the plasma membrane (37, 41). In the absence of insulin, Glut4 is largely excluded from the cell surface and is located in tubulovesicular elements clustered either in the *trans*-Golgi reticulum or in the cytoplasm close to the plasma membrane (38, 39). Insulin leads to the redistribution of approximately 50% of this internal pool of Glut4 and therefore induces an important increase in Glut4 at the cell surface. Although the exact mechanism of this insulin-induced Glut4 translocation is still unclear, several lines of evidence indicate that one or more GTP-binding proteins are involved in the insulin stimulation of glucose transport. Indeed, nonhydrolyzable GTP analogs (such as GTPγS) induce Glut4 translocation to the plasma membranes and stimulate glucose transport in adipocytes (3, 31, 32). More recently, GTPγS was shown to stimulate exocytosis and to inhibit reinternalization of Glut4 (35). The exact nature of the GTP-binding proteins involved in the translocation process is not known, but Rab proteins have been recently proposed for such a role. The Rab proteins are a family of low-molecular-mass GTP-binding proteins (20 to 30 kDa) located along the exocytic and endocytic organelles, and they are implicated at different levels of intracellular vesicular traffic (24, 51). One of these proteins, Rab3D, which is induced during adipogenic differentiation (2), could have a specific role in some adipocyte function, although there is no evidence for its involvement in Glut4 translocation (4). A series of observations suggest that Rab4, previously found at the cytosolic surface of endosomes in CHO cells (48), could be important in Glut4 translocation. First, Rab4 is present in internal microsomes, partly associated with the vesicles containing Glut4 in adipocytes and skeletal muscle (1, 12, 33). Second, insulin modifies its subcellular redistribution (1, 12, 33, 46), an effect which is suppressed by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (19). Third, there is a tight correlation between the insulin-induced movements of Glut4 and Rab4 (30). These observations led to the suggestion that Rab4 could play a role in insulin-induced Glut4 translocation.

The present study was undertaken to directly address the potential roles of Rab4 and Rab3D in the Glut4 translocation. To this aim, adipocytes were transiently cotransfected with Rab4 and with a Glut4 transporter tagged with a myc epitope (Glut4-myc) added in the first extracellular loop of the protein. This construct allows for the direct measurement of Glut4 at the cell surface by the binding of an antibody to myc (26–28). Thus, Glut4-myc translocation can be studied in the small proportion of cells which have been transfected. Using this system, we looked at the effect of the overexpression of the wild-type Rab4 protein (Rab4 WT) and of various forms of Rab4 in which its ability to bind and hydrolyze GTP has been modified.

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MATERIALS AND METHODS

Materials. Bovine serum albumin was from Intergen (Purchase, N.Y.). Collagenase was from Boehringer (Mannheim, Germany). ^{125}I-immunoglobulins against mouse antibodies were from Amersham (Little Chalfont, Buckingham-

shire, United Kingdom). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein assay were from Bio-Rad (Richmond, Calif.) and Pierce (Rockford, Ill.). DNA preparation kits and molecular biology reagents were from Biolabs (Richmond, Calif.), Clontech (Palo Alto, Calif.), and Bio 101 (La Jolla, Calif.). Oligonucleotides were from Eurogentech (Seraing, Belgium). All other chemical and biochemical products were from Sigma (St. Louis, Mo.) and Merck (Darmstadt, Germany).

Antibodies. Antibodies to myc used for binding experiments were monoclonal antibodies (9E10). Antibodies to Glut4 were obtained by immunizing a rabbit with a peptide corresponding to the last 12 amino acids of the Glut4 C terminus (20). Antibodies against human Rab4a were produced against the protein purified from *Escherichia coli* as described previously (11). Antibodies against Rab3D were obtained after immunization of a rabbit with a peptide corresponding to the sequence amino acids 192 to 205 of the protein (2).

DNA vector constructions. (i) pCIS2 and pCIS2 Glut 4-myc. pCIS2 is an expression vector containing a cytomegalovirus promoter and enhancer with a generic intron located upstream from the multiple cloning site. This vector gives a high level of protein expression in transiently transfected adipocytes (29). A unique *Stu*I site was introduced into the nucleotide sequence of the rat Glut4 cDNA by changing GGTCCT to AGGCCT (coding for Glut4 Gly-65–Pro-66) by site-directed mutagenesis (Transformer kit; Clontech). The cDNA was cut with *Stu*I to permit the insertion of the oligonucleotides coding for the myc epitope. The sense oligonucleotide was 5′-GCA-GAG-GAG-CAA-AAG-CTT-ATT-TC T-GAA-GAG-GAC-TTG-CTT-AAG-3′ (the antisense was 5′-CTT-AAG-CAA -GTC-CTC-TTC-AGA-AAT-AAG-CTT-TTG-CTC-CTC-TGC-3′). This resulted in a fusion gene encoding the peptide sequence AEEQKLISEEDLLK inserted into the first exofacial loop of Glut4 between amino acids 65 and 66. The construction was verified by sequencing the regions surrounding and including the oligonucleotide insert (42). Glut4-myc was subcloned into the pCIS2 vector.

(ii) pCIS2 Rab4 WT and pCIS2 mutated Rab4. The cDNA coding for human Rab4a (wild type) (50) was purified from pGEX-2T Rab4 (6) and subcloned into pCIS2. Mutations of Rab4a into pGEX-2T Rab4 were performed by site-directed mutagenesis. The residues S-22, Q-67, and N-121 were changed into N, L, and I with the following oligonucleotides: GGA-ACT-GGC-AAA-AAT-TGC-TTA-CTT-CAT-CAG, G-GAT-ACA-GCA-GGA-CTA-GAA-CGA-TTC-AG, and C-CTT-TGT-GGA-ATC-AAG-AAG-GAC-CTG-G, respectively. Rab4 ΔCT, in which the C-terminal Cys-Gly-Cys sequence was deleted, was obtained by introducing a stop codon in position 211 with the following oligonucleotide: C-GCT-CAG-GAG-TGA-GGT-TGT-TAG-G. The underlined nucleotides are the changed nucleotides which allow one to obtain the desired mutations. The mutations were verified by sequencing the regions of interest, and the cDNA coding for the mutated Rab4 was subcloned into pCIS2.

The plasmid DNAs were purified with a maxi kit (Qiagen), and their concentrations were determined by measuring the A_{260}.

Preparation of isolated rat adipocytes and electroporation. Adipocytes were isolated from epididymal fat pads of male Wistar rats (170 to 200 g) by collagenase digestion (10). Isolated adipocytes were transiently transfected by electroporation as described previously (26, 28, 29) with some modifications (42). Isolated adipocytes were resuspended as a 50% (vol/vol) cell suspension in Dulbecco modified Eagle medium. Cell suspensions (400 μl) were placed in a 0.4-cm-gap cuvette along with the plasmid DNAs (2 μg of pCIS2 Glut4-myc and 9 μg of pCIS2, pCIS2 Rab4 WT, or pCIS2 mutated Rab4). The total amount of DNA was kept constant (11 μg) under all conditions by the addition of pCIS2. Electroporation was performed with double electric shock (800 V, 25 μF; 200 V, 1,050 μF) by using an Easyject electroporator system (Eurogentec). Cells were diluted in 1.5 ml of Dulbecco modified Eagle medium containing 5% (wt/vol) bovine serum albumin (BSA), 25 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 200 nM (*R*)-*N*6-(1-methyl-2 phenylethyl)adenosine, and 100 μg of gentamicin per ml. The cells were incubated for 16 to 24 h at 37°C in 5% CO_2–95% air prior to further studies.

Assay for cell surface epitope-tagged Glut4 measurement. Electroporated adipocytes were washed twice with Krebs-Ringer bicarbonate buffer containing 30 mM HEPES (pH 7.4) (KRBH) and resuspended in KRBH–1% BSA at a suspension of 10% (vol/vol). Cells were then incubated for 30 min at 37°C in the absence or presence of insulin (100 nM). Then, KCN (2 mM final concentration) was added for 5 min to prevent Glut4 redistribution and adipocytes were incubated for 1 h at 25°C with 0.5 μg of monoclonal antibodies to myc (9E10) per ml. Cells were washed three times with KRBH–1% BSA and incubated in triplicate for 1 h at 25°C with ^{125}I-labeled sheep anti-mouse immunoglobulin (10 μCi/μg; final dilution, 1/200). Then samples (300 μl) were placed on 100-μl dinonylphthalate and centrifuged to separate cells from the medium. The fat cell cake was boiled in Laemmli buffer (3% SDS, 70 mM Tris [pH 7.4], 10% glycerol), and radioactivity associated with the cells was counted in a gamma counter. Radioactivity was normalized by measuring the protein concentration in each sample by a bicinchoninic acid assay (Pierce). Nonspecific binding of the antibodies, which represented 20% of the total binding observed in cells transfected with pCIS2 Glut4-myc in the absence of insulin stimulation, was obtained with cells transfected with pCIS2 alone and subtracted from all the values.

Preparation of total crude membranes and cytosol. Adipocytes were transfected as described in the legends to the figures. Twenty-four hours later, they were washed 3 times with KRBH buffer and homogenized in 2.5 volumes of 50 mM Tris (pH 7.4)–1 mM EDTA–250 mM sucrose in the presence of protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin) by using a Thomas Potter homogenizer (pestle C). Total crude membranes were obtained by centrifuging the homogenates for 20 min at 300,000 × *g* (TL100; Beckman). Protein amounts in each fraction were determined as described previously. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF), and immunoblotting of Glut4, Rab4, and Rab3D was performed with the appropriate antibodies (12).

Immunoprecipitation and immunoblotting of Glut4-myc. Adipose cells were cotransfected with pCIS2 Glut4 and the cDNAs indicated in the legends to the figures, incubated for 24 h, washed, and homogenized. Total membranes were prepared and solubilized in HEPES (30 mM, pH 7.4), NaCl (30 mM), 1% Triton X-100, and protease inhibitors. Glut4-myc was immunoadsorbed by using antibodies to myc (9E10; 2 μg) coupled to protein G-Sepharose beads. After being washed, the pellets were boiled in Laemmli buffer, and proteins were separated by SDS-PAGE and transferred onto PVDF sheets. Glut4-myc was then immunodetected with a rabbit antipeptide antibody directed against the C-terminal sequence of Glut4.

Immunopurification of Glut4-containing vesicles. The immunopurification of Glut4-containing vesicles was performed by using antibodies to Glut4 as previously described with some modifications (12, 49). Adipocytes were prepared, transfected (for each immunoprecipitation condition, 2 ml of adipocytes pooled from 10 electroporation cuvettes was used), and homogenized as described above. The homogenates were centrifuged at 40,000 × *g*, and the supernatant (containing nonsedimented low-density microsomes) was immediately used for immunopurification. The fraction, supplemented with protease inhibitors, was incubated with affinity-purified immunoglobulins against Glut4 (160 μg) preadsorbed on protein A-Sepharose (80 μl) or with the same amount of nonimmune immunoglobulins. Following immunoadsorption (4 to 5 h at 4°C), pellets were washed twice with phosphate-buffered saline and three times with a HEPES buffer (50 mM, pH 7.4) containing 50 mM NaCl. Pellets were treated with 3% (wt/vol) SDS–70 mM Tris (pH 7.4)–11% glycerol–0.7 M 2-mercaptoethanol–0.05% (vol/vol) bromophenol blue (18). Samples were resolved by SDS-PAGE using a 10% polyacrylamide resolving gel containing 8 M urea. Transfer to PVDF sheets and immunodetection of Glut4 and Rab4 were performed as described above.

Purification and characterization of WT and mutated GST-Rab4. Bacteria transformed with the pGEX-2T Rab4 constructs of interest were cultured until the A_{600} reached 0.6. The production of the glutathione *S*-transferase (GST)–Rab4 was then induced by the addition of 50 μM isopropyl-β-D-thiogalactoside. Proteins were purified on glutathione-Sepharose columns, and the GTPase activity of the proteins was determined by measuring [α-^{32}P]GTP hydrolysis into [α-^{32}P]GDP (6). Results of GTPase activity were expressed as a percentage of GTP hydrolyzed into GDP.

Statistics. Experiments were repeated 3 to 6 times with different adipocyte preparations. Statistical significance was assessed by the Student *t* test for paired data.

RESULTS

Effect of Rab4 overexpression on Glut4-myc subcellular localization. To investigate whether Rab4 WT plays a role in the subcellular localization of Glut4, rat adipocytes were cotransfected with pCIS2 Glut4-myc without (mock conditions) or with pCIS2 Rab4 WT (Fig. 1). Under all conditions, the total DNA amount used for the transfection was adjusted to a similar level by the addition of pCIS2 (the empty expression vector). In mock-transfected cells, insulin increased by 2- to 2.5-fold the concentration of Glut4-myc at the cell surface, indicating that insulin was able to promote Glut4-myc translocation, an effect similar to that previously described (26–28). The transfection of 1 μg of pCIS2 Rab4 WT led to three- to fourfold overexpression of Rab4 in total homogenates (Fig. 1B). Since about 10% of the cells were transfected under the electroporation conditions used (42), Rab4 overexpression can be estimated to be 20- to 30-fold in the transfected adipocytes. This overexpression was associated with a 50% decrease in the concentration of Glut4-myc present at the cell surface of the unstimulated adipocytes without any change in the amount of Glut4-myc at the cell surface following a treatment with 100 nM insulin (Fig. 1A). When 9 μg of plasmid Rab4 WT was used, a condition which led to 20- to 30-fold overexpression of the protein in total homogenates and thus to 200- to 300-fold overexpression in transfected adipocytes, an effect was observed under both basal and insulin-stimulated conditions. Indeed, the concentration of Glut4-myc at the cell surface under

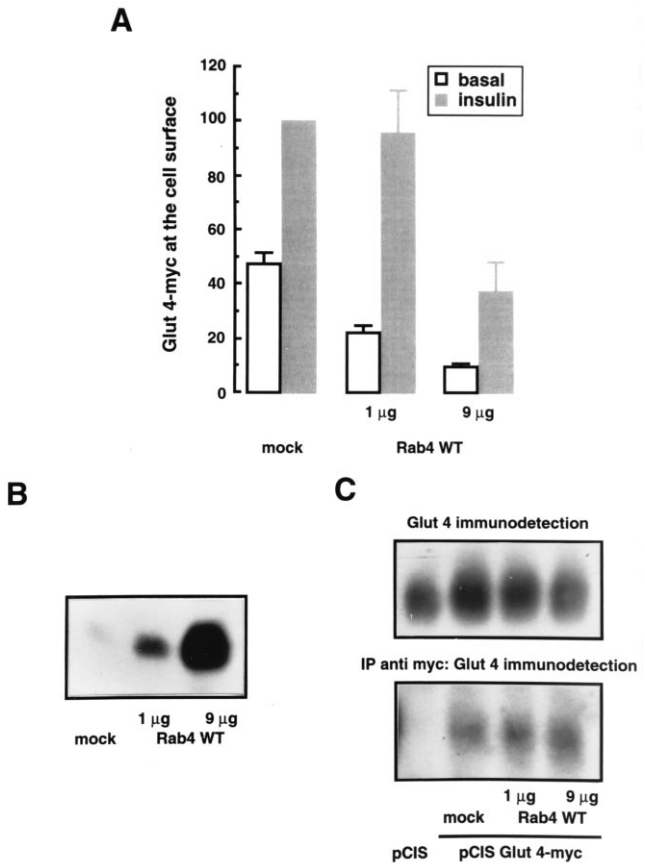

FIG. 1. Effect of the overexpression of Rab4 WT on Glut4-myc subcellular distribution. Rat adipocytes (400-μl suspension) were transiently transfected with pCIS2 Glut4-myc (2 μg) together with pCIS2 (mock) or with pCIS2 Rab4 WT (1 or 9 μg as indicated). (A) Twenty-four hours later, cells were incubated for 30 min in the absence (open bars) or in the presence (shaded bars) of 100 nM insulin before binding of antibodies to myc at the cell surface was measured as described in Materials and Methods. Results are expressed as a percentage of the cell surface binding measured under insulin-stimulated conditions for the mock-transfected cells and are presented as the means ± standard errors of the mean (SEM) for four experiments performed with different cell populations. (B) Under each experimental transfection condition, 30 μg of adipocyte homogenates was analyzed by SDS-PAGE, transferred to PVDF sheets, and probed with antibodies to Rab4. (C) Membranes, prepared from transfected adipocytes, were directly analyzed (25 μg of proteins) for Glut4 content (top) and immunoprecipitated with antibodies to myc (bottom). The immune pellets were analyzed by SDS-PAGE and immunoblotted with an antibody to Glut4 as described in Materials and Methods. The autoradiograms are representative of at least 3 experiments.

basal conditions decreased further to reach 20% of the level observed under basal mock conditions. Furthermore, insulin recruited only 40% the amount of Glut4-myc compared with mock-transfected cells (Fig. 1A). The amount of Glut4-myc present at the cell surface under insulin-stimulated conditions was decreased by more than 80% when we transfected 18 μg of pCIS2 Rab4 WT (data not shown). Since both basal and stimulated values were modified, the factor of insulin stimulation was calculated as an index of the ability of insulin to promote Glut4-myc translocation (Table 1). Under both conditions of transfection (1 or 9 μg), we observed an increased efficiency of insulin to recruit Glut4-myc to the cell surface.

To verify that the overexpression of Rab4 did not affect the expression of the Glut4-myc, immunoprecipitation from the adipocytes transfected in the various conditions was performed with an antibody to myc before immunoblotting with an anti-

TABLE 1. Efficiency of insulin to promote Glut4-myc translocation in Rab4 WT and Rab4 ΔCT transfected adipocytes[a]

Transfection conditions	Insulin effect on Glut4-myc translocation (fold stimulation)	
	Rab4 WT	Rab4 ΔCT
Mock	2.1 ± 0.2	2.6 ± 0.3
1 μg	4.2 ± 0.5*[b]	1.8 ± 0.1*
9 μg	5.6 ± 1.2*	2.4 ± 0.3

[a] Adipocytes were transfected with pCIS2 (mock), pCIS2 Rab4 WT, or pCIS2 Rab4 ΔCT at 1 or 9 μg in combination with pCIS2 Glut4-myc as described in the legends to Fig. 1 and 4. Cells were or were not treated with 100 nM insulin, and Glut4-myc translocation was measured by binding of anti-myc antibodies as described in Materials and Methods. Results are expressed as the fold stimulation induced by insulin compared to basal conditions and represent the means ± SEM for four experiments.

[b] *, results are significantly different, with a P of <0.025, from those under respective mock conditions.

body to Glut4 (Fig. 1C). As expected, the amount of Glut4-myc was not significantly different for cells transfected with a pCIS2 vector alone (mock) or with pCIS2 Rab4 WT. In parallel, immunodetection on total membrane fraction with antibodies to Glut4 was performed (Fig. 1C). The overexpression of Glut4 was 1.5- to 2.0-fold in total membranes and thus 5- to 10-fold in the transfected adipocytes. This series of experiments allowed us to conclude that the overexpression of Rab4 leads to a change in the subcellular distribution of Glut4-myc in basal conditions. Further, the ability of insulin to recruit the same number of Glut4-myc molecules was decreased when the larger amount of Rab4 was overexpressed, but under both overexpression conditions, the efficiency of insulin to recruit the epitope-tagged transporters to the cell surface was increased.

Subcellular distribution of the overexpressed Rab4 WT protein. To understand the differences between the effects obtained at different levels of Rab4 overexpression, we decided to look at the subcellular distribution of the overexpressed Rab4 protein and compare it with that of the endogenous protein. Total membranes and cytosol were prepared from adipocytes transfected with the mock vector or various concentrations of pCIS2 Rab4 WT as described in Materials and Methods. Pro-

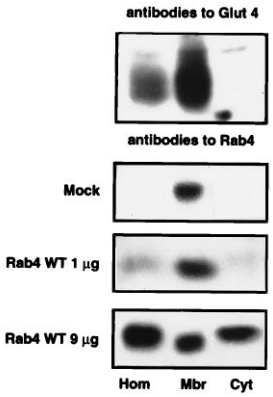

FIG. 2. Subcellular distribution of the overexpressed Rab4 WT protein. Rat adipocytes were transfected with pCIS2 (Mock) or pCIS2 Rab4 WT (1 or 9 μg). Twenty-four hours later, cells were homogenized and the homogenates (Hom) were fractionated into total membranes (Mbr) and cytosol (Cyt) as described in Materials and Methods. Proteins from each fraction (80 or 30 μg in mock or Rab4 WT, respectively) were analyzed by SDS-PAGE, transferred to PVDF sheets, and probed with antibodies to Glut4 (top panel) or Rab4 as described in the legend to Fig. 1. The autoradiograms were exposed for 24 h (mock) or 5 h (1- and 9-μg conditions) for Rab4 blotting.

TABLE 2. Relative subcellular distribution of transfected Rab4 WT and Rab4 ΔCT[a]

Transfection conditions (μg)	Fraction	Rab4 concn[b] (%)	Total protein content (relative amt [%])
Mock	Membranes	Present	25 ± 6
	Cytosol	NQ	75 ± 6
Rab4 WT (1)	Membranes	100	27 ± 3
	Cytosol	20 ± 8	73 ± 2
Rab4 WT (9)	Membranes	100	26 ± 3
	Cytosol	132 ± 16	74 ± 3
Rab4 ΔCT (1)	Membranes	100	24 ± 1
	Cytosol	237 ± 48	75 ± 1
Rab4 ΔCT (9)	Membranes	100	27 ± 2
	Cytosol	693 ± 108	73 ± 2

[a] Adipocytes were transfected with pCIS2 Rab4 WT or pCIS2 Rab4 ΔCT at 1 or 9 μg as described in Materials and Methods and were fractionated 24 h later into total crude membranes and cytosol. Protein contents were determined and expressed as the percentage of the sum of membrane and cytosol contents. Rab4 WT and Rab4 ΔCT concentrations were determined after immunodetection with antibodies to Rab4, and quantification was performed with a molecular imager (Bio-Rad). Values are the means ± SEM of 3 to 5 experiments.

[b] Cytosolic concentrations of Rab4 were expressed as the percentage of the membrane Rab4 concentration. NQ, under the limit of quantification.

teins from each fraction (homogenates, membranes, and cytosol) were separated on SDS-PAGE and immunoblotted with antibodies to Glut4 and Rab4. A characteristic autoradiogram is shown in Fig. 2, and the quantification of three to four experiments is presented in Table 2. Glut4 was found only in membranes and was absent from the cytosol, indicating that the fractionation procedure was correct. In mock-transfected cells, Rab4 was largely enriched in membranes, an expected result, since the Rab proteins are anchored to the membranes through their geranylgeranyl moiety (51). Its detection was too faint in the cytosol to allow for a precise quantification. When pCIS2 Rab4 WT was transfected at the lowest concentration (1 μg), Rab4 was also enriched in the membranes, since its concentration was three- to fivefold larger in the membranes than in the cytosol. By contrast, when adipocytes were transfected with 9 μg of pCIS2 Rab4 WT, the concentration of Rab4 WT was similar in membrane and cytosol. Further, the apparent mobility of the protein was lower in the cytosol than in the membranes, a likely index of a defective isoprenylation of Rab4. When the total membrane and cytosol protein amounts were taken into account, we observed that at the lowest concentration, Rab4 WT was equally distributed between membranes and cytosol. By contrast, when highly overexpressed, Rab4 WT accumulated into the cytosol.

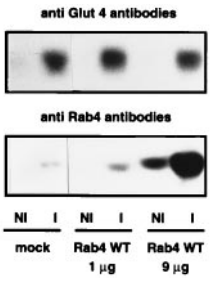

FIG. 3. Localization of the overexpressed Rab4 WT protein in the Glut4-containing vesicles. Rat adipocytes were transfected with pCIS2 (mock) or pCIS2 Rab4 WT (1 of 9 μg). Twenty-four hours later, microsomes were prepared and Glut4-containing vesicles were immunopurified with nonimmune immunoglobulins (NI) or immunoglobulins to Glut4 (I) as described in Materials and Methods. The immune pellets were analyzed by SDS-PAGE and immunoblotted with antibodies to Glut4 and Rab4. The autoradiograms were exposed 10 times longer in mock transfection than in Rab4 overexpressing adipocytes for Rab4 blotting.

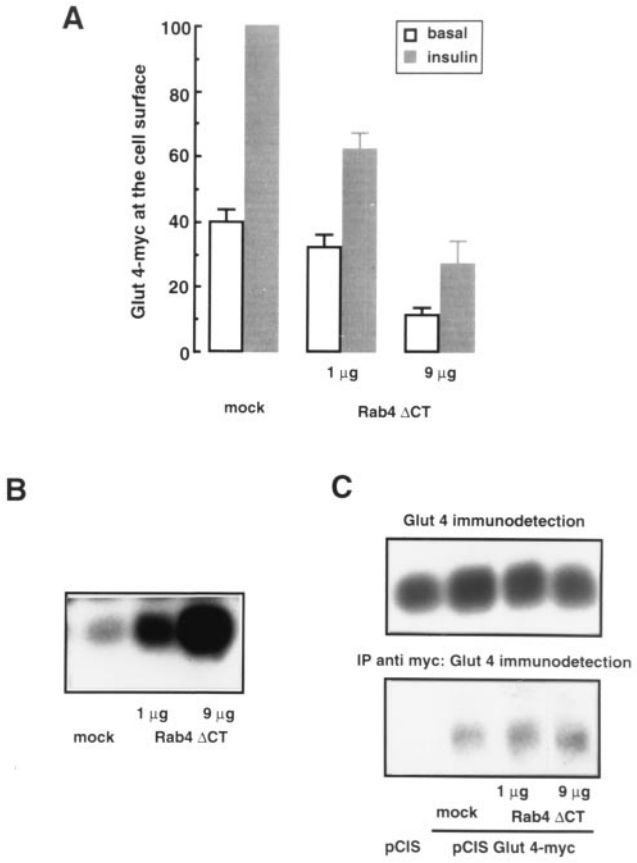

FIG. 4. Effect of the overexpression of Rab4 ΔCT on Glut4-myc subcellular distribution. Rat adipocytes (400-μl suspension) were transiently transfected with pCIS2 Glut4-myc (2 μg) together with pCIS2 (mock) or with pCIS2 Rab4 ΔCT (1 or 9 μg as indicated). (A) Glut4-myc present at the cell surface was quantified as described in the legend to Fig. 1, and results are expressed as a percentage of the cell surface binding measured under insulin-stimulated conditions for the mock-transfected cells and are presented as the means ± SEM of 4 experiments performed with different cell populations. (B and C) The expression of Rab4 ΔCT (B) and Glut4 and Glut4-myc (C) was determined as described in the legend to Fig. 1. The autoradiograms are representative of at least 4 experiments.

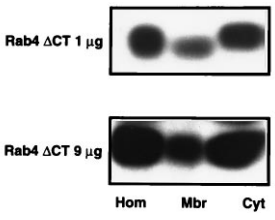

FIG. 5. Subcellular distribution of the overexpressed Rab4 ΔCT protein. Rat adipocytes were transfected with pCIS2 (mock) or pCIS2 Rab4 ΔCT (1 or 9 μg). Twenty-four hours later, cells were homogenized and the homogenates (Hom) were fractionated into total membranes (Mbr) and cytosol (Cyt) as described in the legend to Fig. 2. Proteins from each fraction (100 μg) were analyzed by SDS-PAGE, transferred to PVDF sheets, and probed with antibodies to Rab4 as described in the legend to Fig. 2. The autoradiograms were exposed for 24 h.

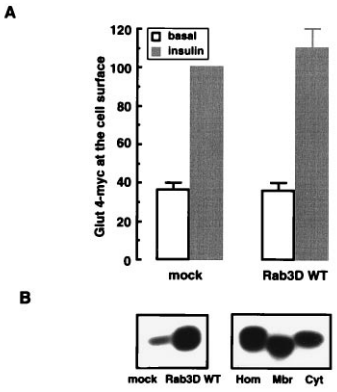

FIG. 6. Effect of the overexpression of Rab3D on Glut4-myc subcellular distribution. Rat adipocytes (400-μl suspension) were transfected with pCIS2 Glut4-myc (2 μg) together with 9 μg of pCIS2 (mock) or 9 μg of pCIS2 Rab3D WT. (A) Twenty-four hours later, cells were treated for 30 min in the absence (basal) or presence of 100 nM insulin before binding of antibodies to myc at the cell surface was measured as described in the legend to Fig. 1. Results are presented as a percentage of the cell surface binding measured under insulin-stimulated conditions for the mock-transfected cells and are presented as the means ± SEM of 3 experiments performed with different adipocyte preparations. (B) (Left) Adipocyte homogenates (100 μg) were analyzed by SDS-PAGE, transferred to PVDF sheets, and probed with antibodies to Rab3D. (Right) Fractionation of adipocytes transfected with Rab3D was performed as described in the legend to Fig. 2. Proteins of homogenate (Hom; 50 μg), membranes (Mbr; 100 μg), and cytosol (Cyt; 20 μg) were analyzed by SDS-PAGE and immunoblotted with antibodies to Rab3D.

Overexpressed Rab4 WT was found in Glut4-containing vesicles. In adipocytes, Rab4 is found associated in part with the Glut4-containing vesicles (12, 13). We verified that overexpressed Rab4 WT was also present in those vesicles. Glut4-containing vesicles were immunopurified from adipocytes transfected with pCIS2 alone (mock vector) or pCIS2 Rab4 WT (1- and 9-μg conditions) as follows. A 40,000 × *g* supernatant of adipocyte homogenates was incubated with nonimmune (NI) or anti-Glut4 (I) immunoglobulin. As shown in Fig. 3, Rab4 was present in Glut4-containing vesicles under all conditions. However, the concentration of Rab4 in the vesicles markedly increased, in parallel with the amount of Rab4 associated with the low-density microsomes (data not shown).

Effect of Rab4 ΔCT overexpression on Glut4-myc translocation. The inhibition of Glut4-myc translocation induced by insulin at a high level of Rab4 WT overexpression might be due to the accumulation of the small GTPase into the cytosol. To investigate this hypothesis, we overexpressed a Rab4 protein (Rab4 ΔCT) in which the geranylgeranylation site, the main site of membrane anchorage, was deleted. Rat adipocytes were cotransfected with pCIS2 Glut4-myc without (mock conditions) or with pCIS2 Rab4 ΔCT (Fig. 4). Similar to the results observed with the WT protein, the transfection of 1 μg of pCIS2 Rab4 ΔCT led to three- to fourfold overexpression of Rab4 in total homogenates (Fig. 4B), but as expected, the overexpressed Rab4 ΔCT was present only in the cytosol (Fig. 5 and Table 2), since the membrane-associated Rab4 corresponded exclusively to the endogenous protein (data not shown). The Rab4 ΔCT overexpression very slightly affected the amount of Glut4-myc present at the cell surface under basal conditions (Fig. 4A). By contrast, a 40% decrease in the amount of Glut4-myc transporters present at the cell surface was observed under insulin-stimulated conditions. Thus the efficiency of insulin to stimulate Glut4-myc translocation was only 1.8-fold compared to 2.6-fold in mock-transfected cells (Table 1). When highly overexpressed, Rab4 ΔCT further accumulated into the cytosol (Fig. 5 and Table 2), but a small proportion was also found to be associated with the membranes, where the Rab4 concentration was increased two- to threefold (Fig. 5). This was accompanied with a 50% decrease in the amount of Glut4-myc at the cell surface under basal conditions and a further decrease under insulin-stimulated conditions (Fig. 4A). By contrast with the results observed with Rab4 WT, the efficiency of insulin to stimulate Glut4-myc translocation was not increased when Rab4 ΔCT was overexpressed (Table 1).

Effect of Rab3D WT overexpression on Glut4-myc subcellular localization. To determine whether the effects of Rab4 on the subcellular distribution of Glut4-myc were specifically observed with this small GTPase, we studied the effects of Rab3D transfection on Glut4-myc translocation. This Rab protein was chosen since it has been proposed to play a crucial role in adipocytes because of its markedly increased level of expression after differentiation (2). When cells were cotransfected with pCIS2 Rab3D WT and pCIS2 Glut4-myc, no differences in the concentration of Glut4-myc were observed between these and control cells transfected with pCIS2 Glut4-myc alone, either under basal or under insulin-stimulated conditions (Fig. 6). This lack of effect was not due to an absence of Rab3D expression, since it was increased more than 20-fold compared to the endogenous protein in total homogenates. The Rab3D was abundant in the cytosol, and, similar to Rab4 WT, the cytosolic Rab3D migrated with a lower mobility into the gel, indicating that it might be incompletely processed.

Effects of overexpression of various mutated Rab4 proteins on Glut4-myc subcellular localization. The next series of experiments was undertaken to determine which function of the small GTPase Rab4 was important for the observed effects on Glut4 subcellular localization. Three mutations were chosen; these were supposed to affect the ability of the proteins to bind or to hydrolyze GTP. The residues Q-67, S-22, and N-121 were changed by site-directed mutagenesis to L, N, and I, respectively. We first characterized the mutated proteins to test whether the mutations did indeed affect Rab4 functions. The mutations were created in the prokaryotic expression vector pGEX-2T Rab4 WT, which allowed for the synthesis of the

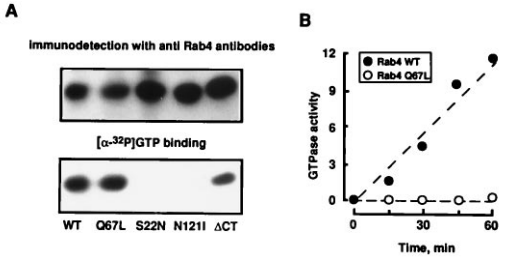

FIG. 7. Characterization of the Rab4 mutated proteins. (A) GTP binding properties of the mutated proteins. *E. coli* transformed with pGEX-2T Rab4 WT, pGEX-2T Rab4 Q67L, pGEX-2T Rab4 S22N, pGEX-2T Rab4 N121I, or pGEX-2T Rab4 ΔCT was grown, centrifuged, and treated with Laemmli buffer. An aliquot was analyzed by SDS-PAGE, transferred to PVDF sheets, and probed successively with [α-^{32}P]GTP for binding determination (lower panel) and then with antibodies to Rab4 for protein immunodetection (upper panel). (B) GTPase activity of the Rab4 WT and Rab4 Q67L mutant. GST fusion proteins were purified from *E. coli* by affinity chromatography on glutathione-Sepharose as described in Materials and Methods. Fusion proteins (2 μg) were loaded with [α-^{32}P]GTP for 30 min at 30°C; GTP hydrolysis was then induced by the addition of $MgCl_2$ (10 mM). Aliquots of the mixture were removed after various incubation durations and adsorbed on glutathione-Sepharose beads, and the nucleotides (GTP and GDP) bound to GST-Rab4 were separated by thin-layer chromatography. Results of GTPase activity are expressed as a percentage of GTP hydrolysis as described in Materials and Methods.

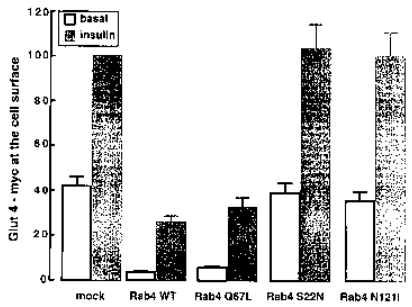

FIG. 8. Effect of the overexpression of the various mutated Rab4 proteins on Glut4-myc subcellular distribution. Rat adipocytes (400-μl suspension) were transiently transfected with pCIS2 Glut4-myc (2 μg) together with 9 μg of pCIS2 (mock), pCIS2 Rab4 WT, or the various mutated pCIS2 Rab4 proteins as indicated. Twenty-four hours later, cells were incubated for 30 min in the absence (open bars) or in the presence (shaded bars) of 100 nM insulin before binding of antibodies to myc at the cell surface was measured as described in the legend to Fig. 1. Results are expressed as a percentage of the cell surface binding measured under insulin-stimulated conditions for the mock-transfected cells and are presented as the means ± SEM for 3 experiments performed with different adipocyte preparations. The effect of Rab4 WT and Rab4 Q67L was significant compared to that under the mock conditions both under basal and insulin-stimulated conditions, with a P of <0.01.

fusion proteins GST-Rab4 WT and GST-Rab4 Q67L, S22L, N121I, and ΔCT. Lysates were obtained from bacteria transformed with the various vectors following induction with 50 μM isopropyl-β-D-thiogalactopyranoside, and proteins were analyzed by SDS-PAGE. Following transfer to PVDF, the proteins were probed with [α-^{32}P]GTP for their ability to bind the nucleotides and were immunoblotted with antibodies to Rab4. As shown in Fig. 7A, although the wild-type and mutated proteins were similarly expressed, only GST-Rab4 WT, GST-Rab4 Q67L, and GST-Rab4 ΔCT bound GTP. There was no [α-^{32}P]GTP binding to the S22N and N121I mutated proteins, indicating that these two mutations induced a defect in the ability of the proteins to bind the nucleotides. We then tested GST-Rab4 WT and GST-Rab4 Q67L for their abilities to hydrolyze GTP (Fig. 7B). Both purified proteins were loaded with [α-^{32}P]GTP and then incubated in the presence of $MgCl_2$ for increasing periods before adsorption onto glutathione-Sepharose beads. The nucleotides bound to the GST fusion proteins were analyzed by thin-layer chromatography. The spots corresponding to GTP and GDP were counted, and the GTPase activity of the proteins was expressed as a percentage of GTP hydrolyzed to GDP as previously described (6). We observed that GST-Rab4 WT hydrolyzed GTP, thus behaving as a GTPase (6). By contrast, GST-Rab4 Q67L did not possess any GTPase activity.

We then tested those mutated Rab4 proteins lacking either GTP binding ability (Rab4 S22N and N121I) or GTPase activity (Rab4 Q67L) for their effect on Glut4 localization in adipocytes (9 μg of plasmid DNA was used under all conditions). The overexpression of the two mutated proteins unable to bind GTP, Rab4 S22N and Rab4 N121I, did not modify the amount of Glut4-myc detected at the cell surface under either basal or insulin-stimulated conditions compared with that under the mock conditions (Fig. 8). In contrast, the overexpression of Rab4 Q67L led to effects similar to those of the Rab4 WT, i.e., the level of Glut4-myc at the cell surface under basal conditions was markedly inhibited compared with that under mock conditions, and insulin recruited only 30 to 40% of the Glut4-myc molecules compared with mock-treated cells. All mutated proteins were overexpressed at a similar level, and none of them affected the expression of Glut4-myc (data not shown).

DISCUSSION

The present work was designed to study whether the small GTPase Rab4 plays a role in the subcellular distribution of the Glut4 glucose transporter in adipocytes, which are highly responsive to insulin for glucose transport. Toward this aim, we used the system recently established by Quon et al. (26–28) consisting of adipocytes transiently transfected with the cDNA of Glut4 in which a myc epitope has been introduced in its first ectodomain. This generates a direct, sensitive method to detect Glut4 at the cell surface without any subcellular fractionation. Further, a cDNA of interest can be cotransfected with Glut4-myc to study how it affects the behavior of the Glut4-myc in the small percentage of transfected cells (10%) (42) independently of the endogenous Glut4 (28, 29). By cotransfecting Glut4-myc with Rab4, we observed that the overexpression of Rab4 affects the subcellular distribution of the transporter, while the overexpression of Rab3D had no effect. However, the effects of Rab4 depended on the degree of the protein overexpression, pointing to two different roles of Rab4, which are substantiated by the results obtained with the overexpression of the Rab4 ΔCT. The Rab4 protein associated with membrane structures would participate in the intracellular retention of the Glut4-containing vesicles, while an excess of cytosolic Rab4 protein would inhibit insulin action on Glut4 translocation.

In adipocytes, Glut4 is sequestered intracellularly and is nearly absent from the plasma membrane (14, 41). It has been proposed that part of the intracellular Glut4 is present in a specialized intracellular compartment, not yet characterized but distinct from the endosomal system and unique to cells in which insulin acutely regulates Glut4 translocation (16, 23). Most of those Glut4-containing vesicles do not contain Glut1, IGF II receptors, and transferrin receptors (10, 23, 41, 52). In transfected adipocytes, a substantial amount of Glut4-myc is present at the plasma membrane under basal conditions, i.e., in the absence of insulin (26, 27). This is the likely consequence of the 5- to 10-fold overexpression of Glut4-myc, since an identical phenomenon was observed with transgenic mice overexpressing Glut4 in adipose tissue (44) or muscle (7, 15, 45), in which basal glucose transport is markedly increased in the corresponding tissues. This suggests that the mechanism responsible for the intracellular sequestration of Glut4 is saturated when Glut4 is overexpressed. However, even under those conditions, Glut4 appears to be targeted to the same unique type of structurally defined vesicles as in native cells (43). When Rab4 was overexpressed together with Glut4-myc, the number of the Glut4-myc transporters present at the cell surface in the absence of insulin decreased without any change in their total number. This decrease was associated with the higher concentration of Rab4 associated with the Glut4-containing vesicles. This result is in marked contrast with the observation that overexpression of Rab4 in CHO cells led to an increase in the number of transferrin receptors at the cell surface (47). Our result could indicate that Rab4 participates in the sequestration or the targeting of the Glut4 molecules to a specialized compartment. The Glut4-myc molecules are probably targeted to the correct compartment when Rab4 is moderately overexpressed, since the epitope-tagged transporters were recruited to the cell surface in response to insulin. The level of Glut4-myc molecules present at the cell surface following insulin stimulation was similar to the level reached in mock-transfected cells, and thus the efficiency of insulin to promote Glut4-myc translocation, as judged by the fold stimulation induced by the hormone, was increased in cells overexpressing Rab4. Although the mechanism by which Rab4 acts in the maintenance of this specific pool remains to be deter-

mined, the membrane localization of the protein seems to be important, considering the results obtained with the Rab4 ΔCT. At the lowest expression level, Rab4 ΔCT, which was only cytosolic, did not increase the intracellular retention of the Glut4-myc. When Rab4 ΔCT was overexpressed at a higher level, it diminished the basal membrane localization of Glut4-myc, but it was also partially associated with membranes. Such a presence of Rab4 ΔCT in membrane fractions when 200- to 300-fold overexpression was induced could be explained by the observation that some domains other than the C-X-C terminus participate in the targeting of the Rab proteins to correct membrane compartments (5). It should also be noted that the overexpression of two mutated proteins in which the ability to bind GTP was altered did not affect the subcellular localization of Glut4-myc. This indicates that the GTP-binding function of Rab4 is crucial for its function, as observed for all GTP-binding proteins. In contrast, the GTPase activity of the protein was not necessary, as proven by the effect of a GTPase-deficient Rab4 on Glut4-myc subcellular localization. In a similar fashion, the corresponding mutation (Q79L) on Rab5 did not affect Rab5 function (22).

Our observations that insulin was no longer able to normally recruit Glut4-myc to the cell surface when Rab4 ΔCT was moderately overexpressed, or when the overexpression of Rab4 WT was increased, suggest that Rab4 is also playing a role in insulin-induced glucose transport. This is in accordance with the following two observations: (i) insulin induces concomitantly the departure of Glut4 to the plasma membranes and that of Rab4 to the cytosol (12), and (ii) a synthetic peptide corresponding to the highly variable region of the C terminus (positions 191 to 210) of Rab4, but not that of Rab3D, inhibits insulin-induced glucose transport and Glut4 translocation into freshly isolated adipocytes (34). These effects of Rab4 WT overexpression could be linked to its cytosolic accumulation. This soluble Rab4 protein has a lower electrophoretic mobility in SDS-PAGE, a likely consequence of defective geranylgeranylation, a process required for correct protein attachment to the membranes (17, 21). It is, however, unlikely that the saturation of the geranylgeranylation machinery could be responsible for this observation, for example, in decreasing the possibility of posttranslational modifications of other Rab proteins. Indeed, the overexpression of Rab3D which possesses the same C-X-C motif did not induce any inhibitory effect on insulin-induced Glut4-myc translocation. In a similar fashion, a depletion of the intracellular GTP pool can probably be excluded, since the overexpression of Rab3D did not affect the distribution of Glut4-myc. The presence of an excess of cytosolic Rab4 might complex some regulatory factor(s) needed for the insulin-induced Glut4 recruitment to the cell surface. It is unlikely that the observed blockade of insulin action results from a complexation of guanosine dissociation inhibitors (GDIs) with the cytosolic Rab4. Indeed, the interaction with GDIs requires the Rab proteins to be geranylgeranylated (51). Further, GDIs can interact with any Rab protein, and overexpression of Rab3D, which also accumulates in the cytosol, does not induce such an inhibitory effect. The factors interacting with Rab4 are still unknown. However, this putative factor(s) would interact with Rab4-GTP, since the overexpression of the GTP-binding-deficient Rab4 proteins had no effect. These factors could be analogous to rabphilin 3a, a Rab3a-binding protein identified as a possible effector protein for Rab3a (9, 36). Rabphilin 3a is recruited to synaptic vesicles by Rab3a-GTP, a required event for the stimulated exocytosis of synaptic vesicles (40). In a similar fashion, Ras-GTP recruits Raf to the plasma membrane preceding the subsequent activation of the effector cascade (8). The overexpression of a cytosolic Ras inhibits the recruitment of Raf to the plasma membrane and thus inhibits its activation (25). If such a protein interacting with Rab4 exists, it is conceivable that the overexpression of Rab4 which remains cytosolic would exert a dominant negative effect.

In summary, the present study suggests that Rab4 is involved in the regulation of Glut4 subcellular distribution. First, Rab4 would be involved in the intracellular sequestration of the Glut4-containing vesicles. Second, insulin could interplay with Rab4 to allow the recruitment of Glut4, although the exact molecular events remain to be determined.

ACKNOWLEDGMENTS

This work was supported by grant 194149 from the Juvenile Diabetes Foundation, International, and grants from the Institut National de la Santé et de la Recherche Médicale and the Université de Nice. M.N.B. received support from the Fondation de la Recherche Médicale (Paris, France). M.C. received support from the Institut Danone.

We thank J. F. Tanti for critical reading of the manuscript and scientific discussion during the course of this study, T. Grémeaux for the Glut4-myc cDNA construct, and G. Visciano for illustration work. We are indebted to A. Zahraoui for the Rab4 cDNA and to J. Baldini and H. Lodish for the Rab3D cDNA. We thank Genentech for the gift of the pCIS2 vector.

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