

## Expression of a prenylation-deficient Rab4 inhibits the GLUT4 translocation induced by active phosphatidylinositol 3-kinase and protein kinase B

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The small GTPase Rab4 has been shown to participate in the subcellular distribution of GLUT4 under both basal and insulin-stimulated conditions in adipocytes. In the present work, we have characterized the effect of Rab4  $\Delta$ CT, a prenylation-deficient and thus cytosolic form of Rab4, in this process. We show that the expression of Rab4  $\Delta$ CT in freshly isolated adipocytes inhibits insulin-induced GLUT4 translocation, but only when this protein is in its GTP-bound active form. Further, it not only blocks the effect of insulin, but also that of a hyperosmotic shock, but does not interfere with the effect of

zinc ions on GLUT4 translocation. Rab4  $\Delta$ CT was then shown to prevent GLUT4 translocation induced by the expression of an active form of phosphatidylinositol 3-kinase or of protein kinase B, without altering the activities of the enzymes. Our results are consistent with a role of Rab4  $\Delta$ CT acting as a dominant negative protein towards Rab4, possibly by binding to Rab4 effectors.

**Key words:** adipocyte, glucose transport, hyperosmolarity, insulin signalling, zinc.

### INTRODUCTION

Insulin stimulates glucose transport in muscle and adipose tissues by promoting the translocation of the GLUT4 glucose transporters from an intracellular compartment to the plasma membrane. Under basal conditions, GLUT4 is predominantly localized intracellularly [1]. Insulin leads to the redistribution of intracellular GLUT4 transporters to the plasma membrane. After insulin withdrawal, GLUT4 is re-internalized using the endosomal recycling pathway and sorted to a specialized storage compartment different from the constitutively recycling endosomal system [2–4]. Following insulin stimulation, insulin receptors are autophosphorylated and activated, allowing for phosphorylation of the proteins of the insulin receptor substrate (IRS) family. These proteins serve as docking proteins for adapters, including the p85 regulatory subunit of the heterodimeric (p85/p110) phosphatidylinositol 3-kinase (PI3-kinase) [5]. The activation of PI3-kinase is necessary for insulin-induced GLUT4 translocation [6–8]. Further, the overexpression of constitutively active PI3-kinase (p110\*) triggers the translocation of GLUT4 to the plasma membrane [9–12]. The activation of PI3-kinase leads to the formation of PtdIns(3,4,5) $P_3$ , which is a ligand for pleckstrin homology domains [5]. The protein kinase B (PKB) and/or atypical protein kinases C that possess pleckstrin homology domains then appear to be involved in insulin signalling to glucose transport, since the overexpression of their active forms triggers the translocation of GLUT4 [13–17].

Members of the Rab family of small GTPases play central roles in the regulation of vesicular traffic. Among the different Rab proteins expressed in adipocytes, Rab4 has been suggested to play a role in the regulation of GLUT4 traffic. Indeed, insulin modifies the subcellular distribution of Rab4 from its internal compartment to the cytosol [7,18–20] and stimulates the exchange of GDP to GTP, leading to the activation of Rab4 [20], two events that require the activation of PI3-kinase. The importance of Rab4 in GLUT4 subcellular movements has also emerged from two additional series of studies. First, Rab4 overexpression

increases the intracellular retention of GLUT4 in adipocytes and cardiomyocytes [21,22]. Second, insulin-induced GLUT4 translocation is altered following the electroporation of a peptide corresponding to the C-terminus of Rab4 [23], the microinjection of an antibody to Rab4 [23,24] or the expression of a prenylation-deficient, cytosolic form of Rab4 (Rab4  $\Delta$ CT) [21,25].

It was recently observed that, in 3T3-L1 adipocytes overexpressing Rab4  $\Delta$ CT, the tyrosine phosphorylation of IRS1 was reduced by 50% in response to insulin, causing a subsequent reduction in PI3-kinase and PKB activities [25]. The authors concluded that regulation of internal membrane trafficking plays a role in transduction of the insulin signal, in addition to any role Rab4 may play in the regulation of GLUT4 vesicle translocation. It is however possible that this attenuation in the insulin-signalling pathway induced by the expression of Rab4  $\Delta$ CT could not lead to the inhibition of insulin-induced GLUT4 translocation. Indeed the overexpression of protein-tyrosine phosphatase-1B (PTB) [26] or of the NPXY-binding domain of IRS1 [27] did not affect insulin-stimulated glucose uptake in 3T3-L1 adipocytes although the tyrosine phosphorylation of IRS1 and PI3-kinase activity were efficiently reduced. The aim of the present work was to look for a specific role of Rab4 in the final steps of GLUT4 translocation. In the present paper we show that expression of the cytosolic Rab4  $\Delta$ CT inhibits GLUT4 translocation induced by constitutively active forms of PI3-kinase and PKB. Further, we show that Rab4  $\Delta$ CT also inhibits GLUT4 translocation induced by an osmotic shock but does not affect zinc-induced GLUT4 translocation. The possibility that Rab4  $\Delta$ CT inhibits the sorting of GLUT4 from recycling endosomes to its sequestration compartment is discussed.

### EXPERIMENTAL

#### Materials

BSA was from Intergen (Purchase, NY, U.S.A.). Collagenase was purchased from Boehringer Mannheim (Mannheim, Germany). Reagents for SDS/PAGE and protein assays were from

Abbreviations used: Rab4  $\Delta$ CT, prenylation-deficient, cytosolic form of Rab4; PKB, protein kinase B; PI3-kinase, phosphatidylinositol 3-kinase; KRBH, Krebs–Ringer bicarbonate buffer containing 30 mM Hepes, pH 7.4; IRS, insulin receptor substrate.

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Bio-Rad (Richmond, CA, U.S.A.) and Pierce (Rockford, IL, U.S.A.). DNA-preparation kits and molecular-biology reagents were from New England Biolabs (Richmond, CA, U.S.A.), Clontech (Palo Alto, CA, U.S.A.) and Bio 101 (La Jolla, CA, U.S.A.). Oligonucleotides were from Eurogentec (Seraing, Belgium). All other chemical and biochemical products were from Sigma (St. Louis, MO, U.S.A.) and Merck (Darmstadt, Germany).

### Antibodies

Polyclonal antibodies to Myc used for binding experiments were obtained by immunizing a rabbit with the peptide sequence (AEEQKLISEEDLLK) corresponding to the Myc epitope. Monoclonal antibodies, 9E10, to Myc (Santa Cruz Biotechnology) were used for immunoprecipitation and immunodetection. Antibodies to GLUT4 were obtained against the C-terminus of GLUT4 [21]. Antibodies against human Rab4a were produced against the whole protein [28]. Antibodies to PKB are a gift of Dr P. J. Coffey (University Hospital, Utrecht, The Netherlands). Antibodies to phosphotyrosine, p85 and IRS1 used in this study were described in [29].

### DNA vector constructions

DNA vectors were obtained as described in [11,14,21] for pCIS, pCIS GLUT4-Myc, pCIS Rab4  $\Delta$ CT, pCIS Rab4 S22N, pCG-p110\* and pSG-gag-PKB. The DNA vector for pCIS Rab4 S22N  $\Delta$ CT was obtained by performing the  $\Delta$ CT and S22N mutations sequentially. A modified pcDNA3-Myc plasmid was obtained by inserting a Myc epitope into the *EcoRV* site of pcDNA3 (Invitrogen). The sense oligonucleotide was 5'-GCC-ACC-ATG-GCA-GAG-GAG-CAA-AAG-CTT-ATT-TCT-GAA-GAG-GAC-TTG-CTT-AAG-GAT-3'. The antisense oligonucleotide was 5'-ATC-CTT-AAG-CAA-GTC-CTC-TTC-AGA-AAT-AAG-CTT-TTG-CTC-CTC-TGC-CAT-GGT-GGC-3'. The cDNA coding for PKB was then subcloned into pcDNA3-Myc allowing for the expression of Myc-PKB. The mutations were verified by sequencing the regions of interest. The plasmid DNAs were purified using a Qiagen maxi kit (Qiagen) and their concentrations were determined by measuring the absorbance at 260 nm.

### Preparation of isolated rat adipocytes and electroporation

Adipocytes were isolated from epididymal fat pads of male Wistar rats (170–200 g) by collagenase digestion [18]. Isolated adipocytes were transiently transfected by electroporation as described in [11,21,30,31]. Briefly, isolated adipocytes were resuspended at a 50% (v/v) cell suspension in Dulbecco's modified Eagle's medium. Cell suspensions (400  $\mu$ l) were placed in a cuvette (0.4 cm diameter) along with the plasmid DNAs. The amount of transfected DNA was always kept constant by adding the corresponding empty plasmid to obtain 10  $\mu$ g of total DNA/400  $\mu$ l of adipocytes. Electroporation was performed with a double electric shock (800 V/25 mF, 200 V/1050 mF) using an Easyject electroporator system (Eurogentec). Cells were diluted in 1.5 ml of Dulbecco's modified Eagle's medium containing 5% BSA (w/vol), 25 mM Hepes, pH 7.4, and 100  $\mu$ g/ml gentamycin. The cells were incubated for 16–24 h at 37 °C in 5% CO<sub>2</sub>/95% air prior to further studies.

### Determination of cell-surface epitope-tagged GLUT4

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%

(v/v). Cells were then incubated for 30 min at 37 °C in the absence or presence of insulin (100 nM), as indicated in the Figure legends. After insulin treatment, KCN (2 mM final concentration) was added for 5 min before a 1 h incubation at 25 °C with rabbit antibodies to Myc (0.2  $\mu$ g/ml). Cells were washed three times with KRBH/1% BSA and incubated in triplicate for 1 h at 25 °C with <sup>125</sup>I-Protein A (500 000 c.p.m./ml). Samples (300  $\mu$ l) were placed in tubes containing 100  $\mu$ l of dinonyl phthalate and centrifuged to separate the cells from the medium. The fat cake was boiled in Laemmli buffer (3% SDS/70 mM Tris/HCl, pH 7.4/10% glycerol) and radioactivity associated with the cells was counted in a  $\gamma$ -counter. Radioactivity was normalized to the protein concentration measured in each sample using bicinchoninic acid assay (Pierce). Non-specific binding obtained with cells transfected with the appropriate empty vector was subtracted from all values.

### Immunoprecipitation and immunoblotting of GLUT4-Myc

Adipose cells, co-transfected with the various plasmids as detailed in the Figure legends, were incubated for 24 h, washed and then solubilized in 30 mM Hepes, pH 7.4, 30 mM NaCl, 1% Triton X-100 and protease inhibitors [11,21]. GLUT4-Myc was immunopurified using anti-Myc antibody 9E10 (2  $\mu$ g) adsorbed on Protein G–Sepharose beads. Pellets were washed and boiled in Laemmli buffer. Proteins were separated by SDS/PAGE and transferred on to PVDF sheets. GLUT4-Myc was then immunodetected with an anti-GLUT4 antibody.

### Determination of kinase activities

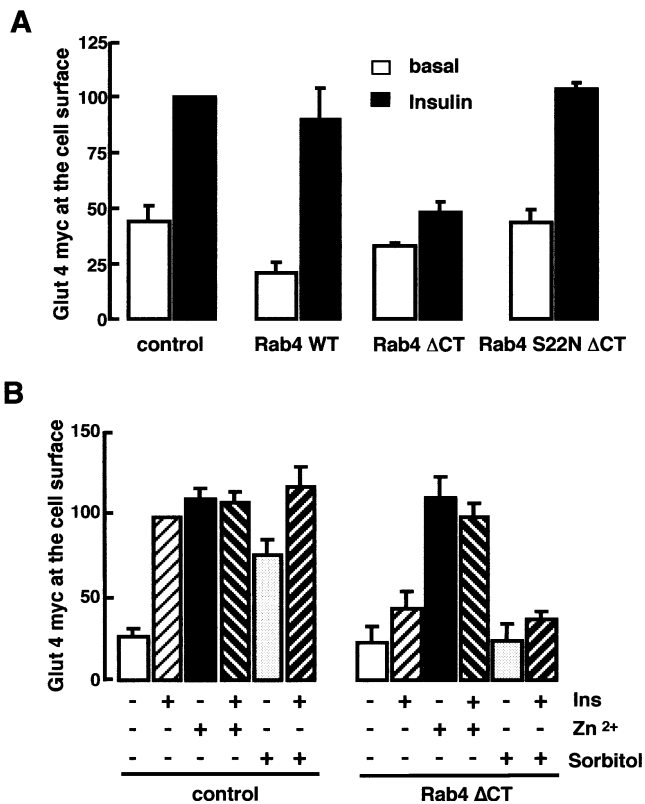
The PI3-kinase activity of the constitutively active p110\* was determined as follows. Adipocytes were transfected, as described above, with pCG-p110\*, incubated for 20–24 h and solubilized in 2 vol. of 20 mM Tris/HCl, pH 7.4/137 mM NaCl/100 mM NaF/10 mM EDTA/2 mM Na<sub>3</sub>VO<sub>4</sub>/10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>/1% Nonidet P40/1 mM PMSF/100 units/ml aprotinin for 40 min at 4 °C. Lysates were centrifuged for 10 min at 13 000 g. Supernatants were incubated for 90 min at 4 °C with 2  $\mu$ g of antibodies to Myc (9E10) adsorbed on Protein G–Sepharose beads. Immune pellets were successively washed twice with each of the three following buffers: (i) PBS containing 1% Nonidet P40 and 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>; (ii) 100 mM Tris/HCl, pH 7.4, 0.5 M LiCl and 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub> and (iii) 10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA and 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>. PI3-kinase activity was measured on the immune pellets as described previously [32].

The kinase activity of the Myc-PKB was measured as follows. Transfected adipocytes were treated or not with insulin (100 nM), solubilized for 40 min at 4 °C in 20 mM Tris/HCl, pH 7.4/5 mM EDTA/10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>/100 mM NaF/2 mM Na<sub>3</sub>VO<sub>4</sub> containing 1% Nonidet P40, 10  $\mu$ g/ml aprotinin and 1 mM PMSF. Lysates were centrifuged for 10 min at 12 000 g and the supernatants were immunoprecipitated for 4 h at 4 °C as described above. Immune pellets were washed three times with solubilization buffer containing 1% Nonidet P40 and twice with 50 mM Tris/HCl, pH 7.5/10 mM MgCl<sub>2</sub>/1 mM dithiothreitol. Kinase assay was performed using Crosstide (Gly-Arg-Pro-Arg-Thr-Ser-Ser-Phe-Ala-Glu-Gly) as a substrate [33]. Activation of PKB was also visualized by a mobility-shift assay of the protein in SDS/PAGE.

## RESULTS

### Rab4 $\Delta$ CT inhibition of the insulin-induced translocation of GLUT4-Myc is GTP-dependent

We reported previously that the overexpression of Rab4  $\Delta$ CT, a



**Figure 1** Characterization of the inhibitory effects of Rab4  $\Delta$ CT on the translocation of GLUT4 induced by insulin and insulinomimetic agents

(A) The Rab4  $\Delta$ CT effect is GTP-dependent. Isolated adipocytes were transiently co-transfected with pCIS GLUT4-Myc alone (2  $\mu$ g; control) or together with 1  $\mu$ g of pCIS Rab4 WT (wild-type), pCIS Rab4  $\Delta$ CT or pCIS Rab4 S22N  $\Delta$ CT as indicated. The total amounts of plasmids were adjusted to 10  $\mu$ g with pCIS. After 16–24 h, cells were treated or not with 100 nM insulin for 20 min before determining the amount of GLUT4-Myc at the cell surface, as described in the Experimental procedures section. (B) Rab4  $\Delta$ CT inhibits the effect of hyperosmotic shock but not of zinc ions on GLUT4-Myc translocation. Isolated adipocytes were transiently co-transfected, as described in (A) with pCIS GLUT4-Myc without (control) or with 1  $\mu$ g of Rab4  $\Delta$ CT. After 16–24 h, adipocytes were treated or not for 20 min with insulin (Ins; 100 nM), sorbitol (600 mM) or zinc sulphate (2 mM) alone or in combination, before determination of the amount of GLUT4-Myc at the cell surface. The results represent the means  $\pm$  S.E.M. from three to four (A) and four (B) independent experiments. The units on the y-axis are percentage of insulin effect in control conditions.

Rab4 molecule with the geranylgeranylation consensus motif Cys-Gly-Cys deleted, and thus cytosolic, is able to inhibit insulin-induced GLUT4 Myc translocation in adipocytes [21]. A similar observation has been made more recently in 3T3-L1 adipocytes [25]. We first wanted to determine whether the GTP-binding function of Rab4 was necessary for this effect. Adipocytes were co-transfected with pCIS GLUT4-Myc alone (control condition) or various forms of pCIS Rab4 (wild-type,  $\Delta$ CT or S22N  $\Delta$ CT, a cytosolic form of Rab4 without GTP-binding activity), the total amount of plasmid DNA being kept constant in each condition by addition of empty pCIS plasmid. After transfection (18–24 h), cells were stimulated for 30 min with 100 nM insulin, before quantification of the amount of GLUT4-Myc at the cell surface, as described in the Experimental procedures section. Under control conditions (adipocytes transfected with GLUT4-Myc only), insulin induced a 2–2.5-fold increase in the amount of GLUT4 at the cell surface (Figure 1A). As described previously [21], wild-type Rab4 overexpression mainly induced a decrease in the basal amount of GLUT4-Myc at the cell surface, whereas the

expression of Rab4  $\Delta$ CT markedly hampered the insulin-induced translocation of GLUT4. More importantly, the effect of insulin on GLUT4 translocation was not affected when Rab4 S22N  $\Delta$ CT, the cytosolic mutant Rab4 unable to bind GTP, was expressed at the same level as Rab4  $\Delta$ CT. The total expression of GLUT4-Myc was similar under all conditions tested (results not shown). This result indicates that the cytosolic form of Rab4 was able to inhibit insulin-induced GLUT4 translocation only when its ability to bind GTP was preserved.

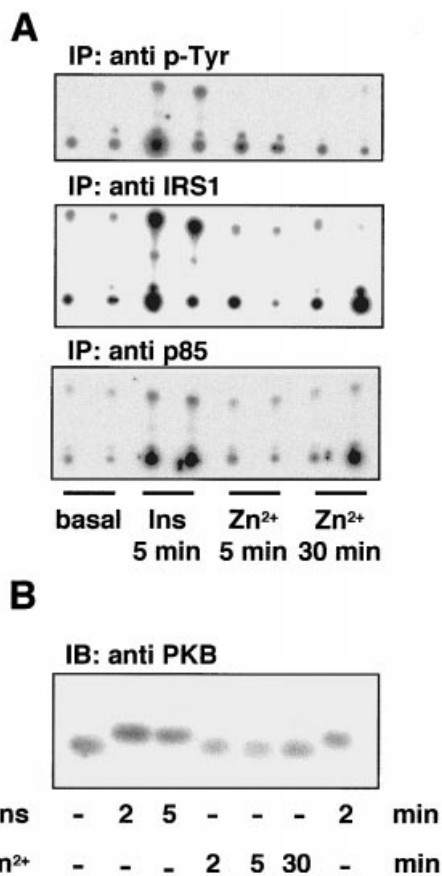
#### Rab4 $\Delta$ CT inhibits GLUT4-Myc translocation induced by osmotic shock but not by zinc

We next determined whether overexpression of Rab4  $\Delta$ CT was able to inhibit GLUT4 translocation induced by two insulinomimetic agents in rat adipocytes. Zinc ions and osmotic shock were shown previously to mimic insulin action on glucose transport by post-receptor/kinase mechanisms [34–36]. Following co-transfection with pCIS GLUT4-Myc and pCIS Rab4  $\Delta$ CT (both 2  $\mu$ g), adipocytes were treated for 20 min with 100 nM insulin, 2 mM zinc sulphate and 600 mM sorbitol, alone or in combination (Figure 1B). In control cells, insulin and zinc sulphate stimulated the translocation of GLUT4 to the same level, while sorbitol was slightly less efficient. Further, the effects of zinc sulphate and sorbitol were not additive to that of insulin. Interestingly, while the overexpression of Rab4  $\Delta$ CT profoundly altered the efficiency of insulin and osmotic shock to promote GLUT4-Myc translocation, it did not prevent the effect of zinc sulphate, even in the presence of insulin. This observation was unexpected since zinc sulphate was recently reported to stimulate the activities of PI3-kinase and PKB in COS and Swiss 3T3 cells [37], enzymes known to trigger GLUT4 translocation in adipocytes [11,13,14].

This led us to study whether zinc sulphate activates this signalling pathway in adipocytes, under the same experimental conditions as used to study GLUT4 translocation. Adipocytes were treated with zinc sulphate (2 mM) for 5 and 30 min or with insulin (100 nM) for 5 min. Lysates were prepared as described in the Experimental procedures section, and were used to measure the total lipid kinase activity of PI3-kinase (in anti-p85 immunoprecipitates) or its activity associated with proteins phosphorylated on tyrosine or IRS1. By contrast with insulin, zinc sulphate did not stimulate PI3-kinase activity, whatever the immunoprecipitation conditions (Figure 2A). Further, a zinc sulphate treatment for 2, 5 or 30 min did not induce any shift in the electrophoretic mobility of PKB, whereas a short treatment with insulin (2 and 5 min) efficiently increased the apparent molecular mass of PKB (Figure 2B). It should also be noted that zinc did not increase insulin-receptor phosphorylation after short-term incubation, although it did it after 2 h (results not shown). These results suggest strongly that neither PI3-kinase nor PKB was activated after 30 min incubation with zinc sulphate in isolated adipocytes.

#### Rab4 $\Delta$ CT inhibits GLUT4 translocation induced by active PI3-kinase and PKB

To dissociate between an early effect of Rab4  $\Delta$ CT in a preliminary step in the insulin cascade [25] and a more distal effect, we tested whether the prenylation-deficient Rab4 protein was able to interfere with the GLUT4 translocation induced by an active form of PI3-kinase or PKB. Adipocytes were transfected with pCIS GLUT4-Myc (2  $\mu$ g) with and without Rab4  $\Delta$ CT and pCG-p110\* (both 2  $\mu$ g), which codes for a constitutively active form of PI3-kinase [11,38]. After 18–24 h, adipocytes were treated or not with 100 nM insulin before measuring the amount of

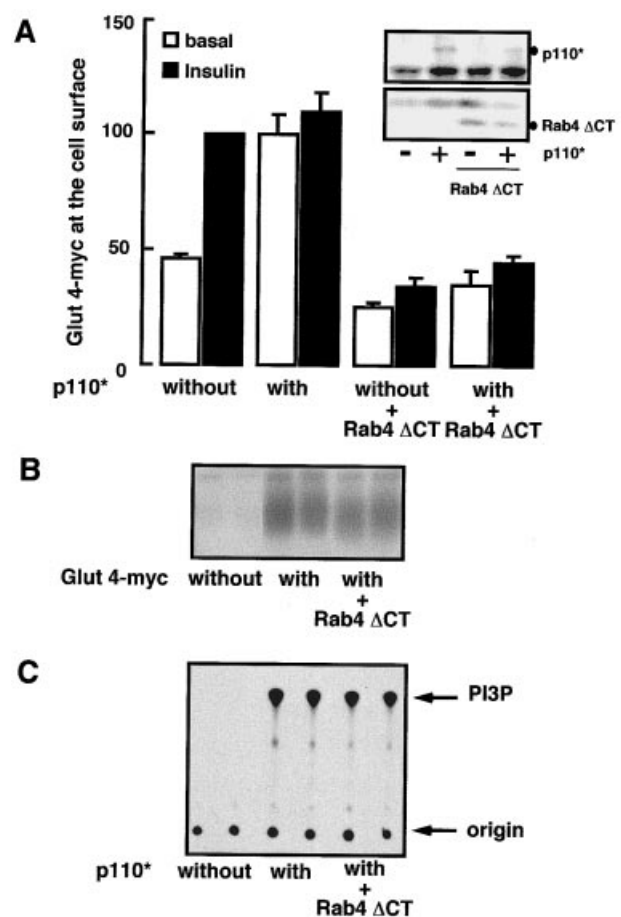


**Figure 2** Zinc does not activate PI3-kinase and PKB in isolated adipocytes

Isolated adipocytes were treated or not with insulin (Ins; 100 nM) or zinc sulphate (2 mM) for the indicated durations. PI3-kinase activity associated with phosphoproteins (p-Tyr), IRS1 or the p85 regulatory subunit of PI3-kinase was measured as described in the Experimental procedures section. Typical autoradiograms from TLC are shown in (A). In (B), cell lysates were analysed by SDS/PAGE and submitted to immunodetection with anti-PKB antibodies. Abbreviations: IB, immunoblot; IP, immunoprecipitation.

GLUT4-Myc at the cell surface. As shown in Figure 3(A), the expression of Rab4  $\Delta$ CT totally abolished the stimulatory effect of p110\* on the translocation of GLUT4-Myc to the cell surface. This inhibitory effect of Rab4  $\Delta$ CT was not due to a change in the expression of p110\* (Figure 3A, inset) or of GLUT4-Myc (Figure 3B). To exclude the possibility that Rab4  $\Delta$ CT could alter the activity of p110\* itself, cell lysates were prepared and the Myc-tagged p110\* was immunoprecipitated using anti-Myc antibody. PI3-kinase activity was then measured as described in the Experimental procedures section. As shown in Figure 3(C), the activity of p110\* was not changed by the expression of Rab4  $\Delta$ CT. Taken together, these results indicate that Rab4  $\Delta$ CT does not alter PI3-kinase activity but might inhibit GLUT4-Myc translocation at a step induced following PI3-kinase activation.

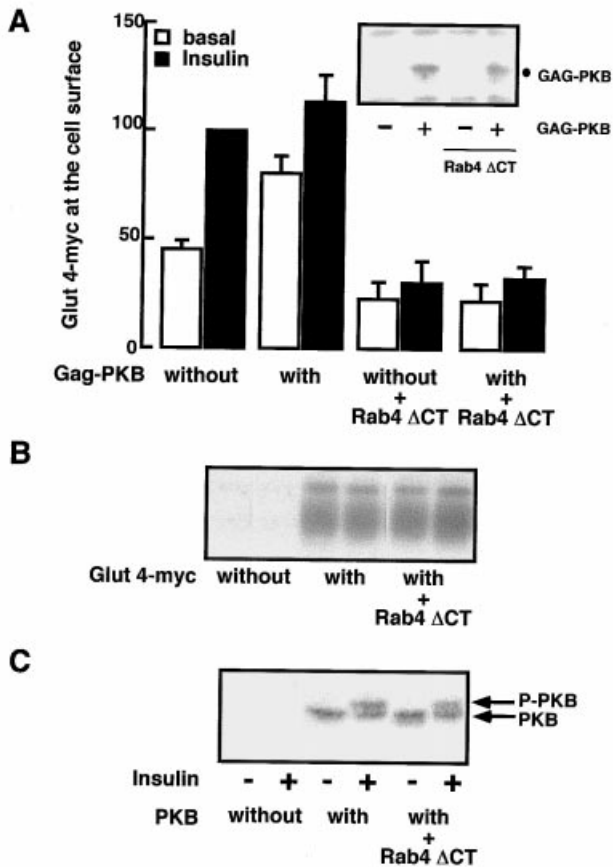
Further down the PI3-kinase activation pathway is PKB, which appears to be important in insulin action on glucose transport [13,14,39–41]. The effect of Rab4  $\Delta$ CT overexpression was determined towards the translocation of GLUT4-Myc induced by the expression of Gag-PKB, a constitutively active form of PKB [14,42]. Adipocytes were transfected with pCIS GLUT4-Myc (2  $\mu$ g) in presence or absence of pSG Gag-PKB and pCIS Rab4  $\Delta$ CT (both 2  $\mu$ g; Figure 4). As described previously [14], the expression of Gag-PKB alone promoted the



**Figure 3** Rab4  $\Delta$ CT inhibits the GLUT4-Myc translocation induced by the expression of p110\*, a constitutively active PI3-kinase

(A) Adipocytes were transiently co-transfected with 2  $\mu$ g of pCIS GLUT4-Myc, without or with 2  $\mu$ g of pCG-p110\* (p110\*) in the presence or absence of 2  $\mu$ g of pCIS Rab4  $\Delta$ CT. After 16–24 h, adipocytes were treated or not for 20 min with 100 nM insulin before determining the amount of GLUT4-Myc present at the cell surface. The results are the means  $\pm$  S.E.M. from five independent experiments. In the inset, the immunodetection of p110\* and Rab4  $\Delta$ CT performed on the corresponding lysates is presented. The units on the  $y$ -axis are percentage of insulin effect without p110\*. (B) GLUT4-Myc was immunoprecipitated using anti-Myc antibodies from cells transfected with empty vector (6  $\mu$ g) or with 2  $\mu$ g of pCIS GLUT4-Myc and pCG-p110\* in the presence or absence of 2  $\mu$ g of pCIS Rab4  $\Delta$ CT. GLUT4-Myc was then immunodetected with anti-GLUT4 antibodies. (C) Adipocytes were transfected without or with 2  $\mu$ g of pCG-p110\* alone or together with 2  $\mu$ g of pCIS Rab4  $\Delta$ CT. After 16–24 h, cell lysates were prepared and p110\* was immunoprecipitated using anti-Myc antibodies before measurement of PI3-kinase activity. An autoradiogram from TLC, in which duplicate analysis has been performed under each experimental condition, is shown, characteristic of three independent experiments. PI3P, PtdIns<sub>3</sub>.

translocation of GLUT4-Myc to the cell surface to a level slightly lower than that seen with insulin. This effect of Gag-PKB was completely abolished by the expression of Rab4  $\Delta$ CT, whether or not insulin was present. The amounts of GLUT4-Myc were identical in each experimental group (Figure 4B), and the expression of Gag-PKB was similar in control and Rab4  $\Delta$ CT-transfected cells (Figure 4A, inset). Further, Rab4  $\Delta$ CT did not affect the ability of insulin to stimulate the activity of Myc-PKB detected either by its increased apparent molecular mass (Figure 4C) or quantification in anti-Myc immunoprecipitates using Crosstide as a substrate (results not shown). These results indicate that the expression of Rab4  $\Delta$ CT inhibited the translocation of GLUT4-Myc induced by the expression of



**Figure 4** Rab4  $\Delta$ CT inhibits GLUT4-Myc translocation induced by the expression of Gag-PKB

(A and B) Adipocytes were treated as described in Figure 3 except that Gag-PKB was used instead of p110<sup>γ</sup>. The units on the y-axis are percentage of insulin effect without PKB. (C) Cells were transiently transfected without or with 2  $\mu$ g of pcDNA3 Myc-PKB alone or in combination with 2  $\mu$ g of pCIS Rab4  $\Delta$ CT. After 16–24 h, adipocytes were treated or not for 20 min with 100 nM insulin. Lysates were prepared and submitted to immunoprecipitation using anti-Myc antibodies. Immune pellets were analysed by SDS/PAGE and immunodetected with anti-PKB antibodies. P-PKB, phosphorylated PKB.

Gag-PKB, without interfering with the PKB activity itself. Thus, Rab4  $\Delta$ CT did not inhibit GLUT4 translocation by hampering the activation of PI 3-kinase or PKB.

## DISCUSSION

In the present paper, we demonstrate clearly that overexpressed Rab4  $\Delta$ CT is unlikely to inhibit insulin-stimulated GLUT4 translocation by only altering tyrosine phosphorylation of IRS1, and thus decreasing PI3-kinase activation, as recently proposed [25]. Indeed, first, overexpressed Rab4  $\Delta$ CT not only inhibits the translocation of GLUT4 vesicles induced by insulin but also blocks the effect of hyperosmotic shock. The exact molecular mechanism of the effect of hyperosmolarity is not known, but it does not activate the same signalling pathway as insulin. Its effect is only partially blocked by wortmannin and could use the mediation of Grb2 associated binder-1 (Gab-1) to induce GLUT4 translocation [35,36]. Second, overexpressed Rab4  $\Delta$ CT also inhibits GLUT4 translocation induced by expression of constitutively active PI3-kinase and PKB. Since Rab4  $\Delta$ CT does not hamper their enzymic activities, those results suggest that the main effect of Rab4  $\Delta$ CT to inhibit GLUT4 translocation does

not result from an inhibition of the insulin activation of PI3-kinase and PKB. By contrast, Rab4  $\Delta$ CT did not modify the effect of zinc on GLUT4 translocation. This indicates that zinc ions act differently from insulin and/or osmotic shock to increase glucose transport. The observation that zinc inhibits transferrin internalization in rat hepatocytes [43] could suggest that zinc might inhibit GLUT4 endocytosis. Thus, GLUT4 molecules that continuously recycle would accumulate at the plasma membrane in the presence of zinc. Different hypotheses could explain the role of Rab4  $\Delta$ CT to inhibit insulin-induced GLUT4 translocation. Since the overexpression of wild-type Rab4 appears to increase the intracellular sequestration of GLUT4, but not that of GLUT1 or insulin receptors in adipocytes (results not shown), we proposed that Rab4 could be of use for sorting GLUT4 molecules in their specific intracellular retention compartment [21]. The first possibility is thus that Rab4  $\Delta$ CT could inhibit this process, preventing GLUT4 from being sorted in this compartment. The second possibility is that Rab4  $\Delta$ CT interferes with the insulin-induced translocation of the GLUT4-containing vesicles from their storage compartment and/or their fusion with the plasma membranes. A direct interaction of Rab4 with syntaxin-4, the specific t-SNARE (target soluble *N*-ethylmaleimide-sensitive fusion-protein attachment-protein receptor) which acts in relation with vesicle-associated membrane protein 2 (VAMP-2), the v-SNARE (vesicle SNARE) found on GLUT4-containing vesicles, has very recently been reported [44]. Rab4  $\Delta$ CT could interfere with this interaction of Rab4, thus specifically preventing the fusion of the GLUT4-containing vesicles with the plasma membrane.

It now remains to understand how overexpressed Rab4  $\Delta$ CT acts to decrease insulin-induced GLUT4 translocation. This inhibition was observed only if the cytosolic form of Rab4 was able to bind GTP, since the S22N mutant was without effect. This implies that GDP exchange to GTP occurs on Rab4  $\Delta$ CT, and that a GDP-exchange factor for Rab4 should be present and active in the cytosol. Rab proteins, in their active GTP-bound form, are believed to recruit cytosolic factors on to membranes to allow their function. The best-characterized example of this process is the recruitment of two effectors of Rab5, Rabaptin-5 and EEA1 (early endosome antigen 1), on to Rab5-GTP-bearing endosomes, a process that stimulates subsequent endosome fusion [45,46]. Since Rab4  $\Delta$ CT inhibits insulin-stimulated GLUT4 translocation only when it is active, it suggests that Rab4  $\Delta$ CT would sequester, into the cytosol, effector proteins that would normally be relocalized into membrane fractions. In agreement with this proposal is our recent observation that Rab4  $\Delta$ CT is able to bind to Rabip4, a Rab4 effector that we identified recently [47]. Although Rab4  $\Delta$ CT alters tyrosine phosphorylation of IRS1 and the following signalling cascade [25], another important effect of Rab4  $\Delta$ CT might be to inhibit GLUT4 sorting into its sequestration compartment or the translocation/fusion process itself, downstream PI 3-kinase activation. It should be noted that such an inhibitory effect of a prenylation-deficient mutant protein is not a common process, since it has not been described for other Rabs. Further, it is possible that Rab4 acts at other levels and that Rab4  $\Delta$ CT exerts a negative effect only on some of them. The negative mutant Rab4  $\Delta$ CT could thus be of further use for a better understanding of insulin-induced GLUT4 translocation.

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