# Heterologous expression of rab4 reduces glucose transport and GLUT4 abundance at the cell surface in oocytes

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To evaluate the role of the small rab GTP-binding proteins in glucose transporter trafficking, we have heterologously co-expressed rab4 or rab5 and GLUT4 or GLUT1 glucose transporters in *Xenopus* oocytes. Co-injection of rab4 and GLUT4 cRNAs resulted in a dose-dependent decrease in glucose transport; this effect was specific for rab4, since co-injection of an inactive rab4 mutant or rab5 cRNA did not have any effect on glucose transport. The effect of rab4 was selective for GLUT4, since no effect was detected in GLUT1-expressing oocytes. The inhibitory effect of rab4 on GLUT4-induced glucose transport was not the result of a change in overall cellular levels of GLUT4 glucose transporters. However, rab4 expression caused a marked decrease in the abundance of GLUT4 transporters present at the cell surface. Finally, rab4 and inhibitors of PtdIns 3-kinase showed additive effects in decreasing glucose transport in GLUT4-expressing oocytes. We conclude that rab4 plays an important role in the regulation of the intracellular GLUT4 trafficking pathway, by contributing to the intracellular retention of GLUT4 through a PtdIns 3-kinase-independent mechanism.

## INTRODUCTION

Insulin stimulates glucose uptake in adipose tissue and skeletal muscle by promoting glucose transporter translocation from an intracellular locus to the plasma membrane. Muscle and fat express two isoforms of glucose transporters, named GLUT1 and GLUT4. Under basal conditions GLUT1 is found mainly in the plasma membrane, but is also found in the interior of the cell, and insulin causes its redistribution to the plasma membrane in adipocytes and cardiomyocytes [1–4]. In contrast, GLUT4 is almost completely excluded from the plasma membrane and is localized to an intracellular storage pool in the basal state; insulin causes the recruitment of GLUT4-containing vesicles to the cell surface [1–3]. Based on the fact that the translocation of GLUT4 is quantitatively more important than that of GLUT1, GLUT4 accounts for most of the insulin-stimulated glucose transport in adipose and muscle cells [1–3].

The molecular mechanisms responsible for the translocation of these glucose transporters to the plasma membrane in response to insulin remain poorly understood. It is known that low-molecular-mass GTP-binding proteins participate in the regulation of vesicular traffic events [5–7], and several lines of evidence suggest that members of the ras-related small GTPase family might be involved in the translocation of GLUT4. First, guanosine 5′-[γ-thio]triphosphate stimulates GLUT4 translocation in permeabilized adipocytes [8–10]. Secondly, a 24 kDa GTP-binding protein has been found to associate with the intracellular GLUT4 compartment in isolated rat cardiomyocytes [11]. Finally, rab4, which is thought to be localized in the early endosome compartment and which has been suggested to play a role in the early endosome/plasma membrane recycling pathway [12,13], has been found in GLUT4-containing vesicles in rat adipocytes [14] and skeletal muscle [15,16]. However, Uphues et al. [11] failed to detect rab4 associated with GLUT4 in rat cardiomyocytes. In adipocytes, rab4 moves from intracellular membranes to the cytosol upon insulin stimulation [14]. Moreover, 3T3-L1 adipocytes made insulin-resistant by chronic treatment with insulin show a reduced intracellular pool of GLUT4 and no recruitment of the glucose transporter to the plasma membrane upon insulin stimulation; under these conditions, a parallel alteration in rab4 movement from intracellular membranes to the cytosol was observed in response to insulin [17]. Furthermore, a synthetic peptide corresponding to the hypervariable C-terminal domain of rab4 inhibits insulin-induced GLUT4 translocation in rat adipocytes [18].

To obtain further insight into the possible involvement of rab4 protein in the trafficking mechanism of GLUT4, we have heterologously co-expressed two rab proteins (rab4 and rab5) with GLUT1 and GLUT4 glucose transporter isoforms in *Xenopus laevis* oocytes. In this cellular system, GLUT1 is targeted to the plasma membrane more efficiently than GLUT4, as previously reported [19–23]. We have compared the effects of rab4 and rab5 expression in GLUT4-expressing oocytes with those in oocytes expressing the GLUT1 isoform. Our results indicate that rab4, but not rab5, decreases glucose transport and GLUT4 abundance at the cell surface of *Xenopus* oocytes.

## MATERIALS AND METHODS

### Oocytes and injections

*Xenopus laevis* females were obtained from African Xenopus Facility C. C. (Knysna, Republic of South Africa). Small clumps of oocytes were treated with collagenase type I (Sigma) at 2 mg/ml in a calcium-free modified Barth's medium as previously reported [19]. Healthy looking stage VI oocytes were selected and kept in modified Barth's solution overnight at 18 °C. Then oocytes were injected with various amounts of cRNA corresponding to human GLUT1 or rat GLUT4.

### *In vitro* cRNA synthesis

GLUT1 and GLUT4 cDNAs were kindly provided by Dr. Graeme I. Bell (University of Chicago, IL, U.S.A.), Dr. Morris

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Birnbaum (University of Pennsylvania, Philadelphia, PA, U.S.A.) and Dr. Mike Mueckler (Washington University, St Louis, MO, DC, U.S.A.). Wild-type rab4b and rab5 and inactive [$^{121}$Ile]rab4 and [$^{133}$Ile]rab5 mutants were gifts from Dr. Marino Zerial (EMBL, Heidelberg, Germany) [24,25]. cDNAs were linearized and transcribed *in vitro* using an AMBION kit as previously reported [19].

### Glucose uptake measurements

At 3 or 4 days after the injection of the cRNAs, glucose transport measurements were carried out using $^{3}$H-labelled 2-deoxy-D-glucose (50 $\mu$M; 1 $\mu$Ci/250 $\mu$l, for 45 min) or 3-*O*-methylglucose (1 mM; 2.5 $\mu$Ci/250 $\mu$l; for 5 min) in modified Bath's medium. After incubation, oocytes were washed four times with ice-cold PBS containing 100 mM glucose and 0.1 mM phloretin. Then oocytes were disrupted in 1 % SDS and the radioactivity was measured.

### Membrane isolation

#### Isolation of total membranes

Whole membranes of oocytes were isolated as previously described [22]. Briefly, groups of 40 oocytes were homogenized with a P-200 pipette and a glass/glass homogenizer in a buffer containing 83 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes (pH 7.9), 200 $\mu$M PMSF, 1 $\mu$M leupeptin and 1 $\mu$M pepstatin. Homogenates were centrifuged twice at 1000 ***g*** for 10 min at 4 °C to pellet all yolk protein, and the supernatant was further centrifuged at 100000 ***g*** for 1 h. Proteins were determined by the BCA method (Pierce) with BSA as standard, and samples were prepared in Laemmli buffer.

#### Isolation of plasma membranes

Isolation of plasma membranes from groups of 40 oocytes was performed by the method of Keller et al. [19]. Briefly, oocytes were disrupted using a P-10 pipette and plasma membranes were separated manually under the microscope. Plasma membrane sheets were then washed and pelleted by centrifugation at 100000 ***g*** for 1 h at 4 °C.

### Electrophoresis and immunoblot analysis

SDS/PAGE was performed and proteins were transferred to Immobilon membranes as previously reported [26]. After transfer, filters were blocked with 5 % non-fat dry milk and 0.02 % $NaN_3$ in PBS for 1 h at room temperature, and then incubated with antibodies overnight at 4 °C. The polyclonal antibody OSCRX, raised against the 15-amino-acid C-terminal peptide of GLUT4, and a polyclonal antibody against the C-terminal domain of rab5 (a gift from Dr. Marino Zerial) were used. Detection of the immune complex was carried out with $^{125}$I-labelled rabbit IgG antibody, incubated at 100000 c.p.m./ml for 1 h at room temperature. The autoradiograms were quantified by scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

## RESULTS

In keeping with previous reports [19–23], injection of GLUT4 cRNA (20 ng) resulted in a 3–10-fold induction of 2-deoxyglucose uptake in oocytes (Table 1). Under these conditions, we did not observe any stimulatory effect of insulin on glucose transport (results not shown). This is in contrast with a previous report [23] in which we found that insulin stimulated glucose transport in GLUT4-expressing oocytes. This discrepancy regarding insulin responsiveness in *Xenopus* oocytes has been reported previously in the literature. Whereas some authors have found that insulin stimulates glucose transport in oocytes [27,28], others have failed to detect this [29]. Although the mechanistic basis for this difference is not known, it is important to note that the present experiments were performed in a different laboratory where housing conditions for *Xenopus* were different.

**Table 1 Uptake of 2-deoxyglucose in GLUT4-expressing oocytes injected with rab4 or rab5 cRNA**

(**a**) Oocytes were co-injected with 10 ng of GLUT4 cRNA and 40 ng of the corresponding rab cRNA per oocyte (rab4m and rab5m indicate the inactive mutant forms [$^{121}$Ile]rab4 and [$^{133}$Ile]rab5 respectively). At 3 days after injection of the cRNA, 2-deoxyglucose uptake was measured as indicated in the Materials and methods section. Values represent the means ± S.E.M. from a representative experiment. The numbers in parentheses indicate the numbers of oocytes per group. * indicates a significant difference compared with oocytes expressing GLUT4 alone ($P < 0.05$). (**b**) Oocytes were co-injected with 10 ng of GLUT4 cRNA and various amounts of rab4 or rab4m cRNA. Values are means ± S.E.M. ($n = 5$) from a representative experiment. * indicates a significant difference compared with oocytes expressing GLUT4 ($P < 0.05$).

(a)

| Group | 2-Deoxyglucose uptake (pmol/45 min per oocyte) |
|---|---|
| Sham | 4.44 ± 0.55 (30) |
| Rab4 | 3.98 ± 0.58 (15) |
| Rab5 | 4.44 ± 0.98 (15) |
| GLUT4 | 11.36 ± 0.45 (16) |
| GLUT4 + rab4 | 5.52 ± 1.47 (11)* |
| GLUT4 + rab4m | 10.02 ± 0.45 (24) |
| GLUT4 + rab5 | 12.76 ± 0.85 (12) |
| GLUT4 + rab5m | 9.72 ± 1 (5) |

(b)

| Group | RNA (ng/oocyte) | 2-Deoxyglucose uptake (pmol/45min per oocyte) |
|---|---|---|
| Sham | – | 1.18 ± 0.08 |
| Rab4 | 15 | 1.01 ± 0.09 |
| GLUT4 | 10 | 10.64 ± 0.79 |
| GLUT4 + rab 4 | 10 + 20 | 5.16 ± 0.34* |
| GLUT4 + rab4 | 10 + 40 | 2.34 ± 0.36* |
| GLUT4 + rab4 | 10 + 50 | 2.17 ± 0.16* |
| GLUT4 + rab4m | 10 + 40 | 9.76 ± 0.64 |

Co-injection of rab4 and GLUT4 cRNAs into the oocyte resulted in a statistically significant decrease in 2-deoxyglucose uptake; in some experiments, rab4 totally blocked GLUT4-induced 2-deoxyglucose uptake (Table 1a). This effect was specific for rab4, since the mutated form (Ile-121)rab4 did not significantly modify 2-deoxyglucose uptake in the oocyte. Additionally, co-injection of rab5 and GLUT4 cRNAs had no effect on 2-deoxyglucose uptake (Table 1a), in spite of abundant expression of the rab5 protein (results not shown). The inhibitory effect of rab4 was dose-dependent, and we found a significant decrease in 2-deoxyglucose uptake ranging from 35 to 48 % (in four independent experiments) after injection of 20 ng of rab4 cRNA per oocyte, and a maximal inhibitory effect of 52–87 % (in five independent experiments) after injection of 40 ng of rab4 cRNA per oocyte (Table 1b).

To test whether the inhibitory effect of rab4 was selective for GLUT4, we next examined the co-injection of rab4 or rab5

**Table 2 Rab4 inhibits glucose transport in GLUT4-expressing oocytes**

Portions of 20 ng of GLUT4 cRNA and 30 ng of rab4 cRNA were sequentially injected into oocytes (rab4m is the inactive mutant form [$^{121}$Ile]rab4). In group A, GLUT4 was injected first, and 24 h later the cRNA of rab4 was transfected. In group B, rab4 cRNA was injected 24 h prior to the injection of GLUT4 cRNA. At 2 days after the second injection, 2-deoxyglucose uptake was measured. Values represent the means ± S.E.M. from a representative experiment. The numbers in parentheses indicate the numbers of oocytes per group. * indicates a significant difference compared with oocytes expressing GLUT4 ($P < 0.05$).

| Group | RNAs injected | 2-Deoxyglucose uptake (pmol/45 min per oocyte) |
|---|---|---|
| Controls | Sham | 0.85 ± 0.02 (29) |
| | Rab4 | 0.72 ± 0.02 (16) |
| | Rab4m | 1.09 ± 0.05 (17) |
| Group A | GLUT4 | 10.88 ± 0.92 (17) |
| | GLUT4 + rab4 | 6.20 ± 0.56 (17)* |
| | GLUT4 + rab4m | 11.10 ± 0.67 (5) |
| Group B | GLUT4 | 7.31 ± 0.81 (12) |
| | GLUT4 + rab4 | 2.92 ± 0.10 (32)* |
| | GLUT4 + rab4m | 6.96 ± 0.36 (31) |

**Table 3 Rab4 inhibits 3-*O*-methylglucose uptake in GLUT4-expressing oocytes**

Portions of 10 ng of GLUT4 cRNA and 30 ng of rab4 cRNA were co-injected per oocyte; 3 days later, 3-*O*-methylglucose uptake was measured as described in the Materials and methods section. Values represent means ± S.E.M. from a representative experiment. The numbers in parentheses indicate the numbers of oocytes per group. * indicates a significant difference compared with oocytes expressing GLUT4 alone ($P < 0.05$).

| Group | 3-*O*-Methylglucose uptake (pmol/5 min per oocyte) |
|---|---|
| Sham | 26.48 ± 1.22 (14) |
| Rab4 | 25.32 ± 0.89 (14) |
| GLUT4 | 57.28 ± 3.4 (9)* |
| GLUT4 + rab4 | 30.76 ± 1.58 (14) |

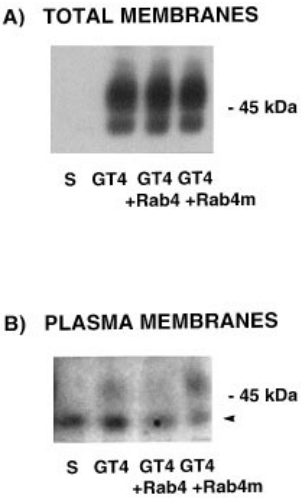


**Figure 1 Effect of rab4 on the abundance of GLUT4 in total membranes and plasma membranes**

Oocytes were co-injected with 20 ng of GLUT4 (GT4) cRNA and 30 ng of rab4 cRNA [rab4 or rab4 mutant (rab4m)]. At 4 days after injection, the isolation of total membranes (**A**) and plasma membranes (**B**) was performed as described in Materials and methods section. GLUT4 protein was detected by immunoblot analysis using a specific antibody directed against the C-terminus. Densitometric analysis was performed with a GT-8500 EPSON Scan and the data were processed using Phoretix 1D (Microsoft). The arrowhead in (**B**) indicates a non-specific band which is detected in both sham-injected (S) and GLUT4-expressing oocytes.

**Table 4 Effects of PtdIns 3-kinase inhibitors on oocytes co-injected with GLUT4 and rab4 cRNAs**

Oocytes were co-injected with 10 ng of GLUT4 cRNA and 30 ng of rab4 cRNA, and 2-deoxyglucose uptake was measured as described in the Materials and methods section. Wortmannin (1 $\mu$M) and LY294002 (10 and 100 $\mu$M) were added 30 min prior to the uptake assay. * indicates a significant difference compared with GLUT4-expressing oocytes ($P < 0.05$); † indicates a significant difference compared with oocytes expressing GLUT4 + rab4 ($P < 0.05$).

| | 2-Deoxyglucose uptake (pmol/45 min per oocyte) | | | |
|---|---|---|---|---|
| | | + LY294002 | | |
| Group | No treatment | 10 $\mu$M | 100 $\mu$M | + Wortmannin (1 $\mu$M) |
| Sham | 2.82 ± 0.42 | 3.13 ± 0.60 | 2.14 ± 0.41 | 3.67 ± 0.48 |
| GLUT4 | 22.1 ± 1.57 | 15.51 ± 0.56 | 6.12 ± 0.57* | 6.80 ± 0.59* |
| GLUT4 + rab4 | 6.09 ± 0.69 | 6.64 ± 0.39 | 2.77 ± 0.35† | 1.95 ± 0.18† |

cRNA with the GLUT1 cRNA. Injection of GLUT1 cRNA alone (15 ng) induced a 2-deoxyglucose transport activity of 109.7 ± 18.2 pmol/45 min per oocyte (mean ± S.E.M.). No effect of rab4 or rab5 expression on 2-deoxyglucose uptake was detected in oocytes expressing GLUT1 (values of 103.0 ± 18.6 and 99.0 ± 21.8 pmol of 2-deoxyglucose/45 min per oocyte in rab4- and rab5-expressing oocytes respectively).

To test that the inhibitory effect on 2-deoxyglucose uptake induced by the injection of rab4 cRNA was not dependent on the protocol of co-injection itself, we injected GLUT4 and rab4 cRNAs in a sequential manner (Table 2). We found similar results as in the co-injected group described above. Thus rab4 inhibited 2-deoxyglucose uptake induced by GLUT4 expression. In fact, the percentage inhibition caused by rab4 expression was similar when rab4 cRNA was injected either before or after GLUT4 cRNA (Table 2).

In addition, to rule out any possible effect of rab4 on hexokinase activity, we measured 3-*O*-methylglucose uptake in GLUT4-expressing oocytes. Expression of GLUT4 resulted in a 2.5-fold increase in 3-*O*-methylglucose uptake compared with water-injected oocytes (Table 3). As also shown in Table 3, co-injection of rab4 cRNA with GLUT4 cRNA decreased 3-*O*-methylglucose uptake in a significant manner, reducing GLUT4 transport activity nearly to the level detected in water-injected oocytes.

In order to rule out the possibility that the decrease in glucose transport found in GLUT4 + rab4-expressing oocytes was due to a lower expression of the transporter (i.e. competition between the cRNAs), we next examined the level of GLUT4 expression by Western blot analysis. To this end, we isolated whole membrane or plasma membrane fractions of oocytes expressing GLUT4 and GLUT4 plus rab4 or rab4 mutant. To isolate plasma membranes we used two different procedures in a total of

five independent experiments: the methods described by Thomas et al. [22] and by Keller et al. [19]. Both techniques yielded the same result: co-injection of rab4 cRNA (30 ng/oocyte) with GLUT4 (20 ng/oocyte) did not modify the abundance of GLUT4 in total membranes (Figure 1A), but caused a marked decrease in the GLUT4 content of the plasma membrane (Figure 1B). Thus the GLUT4 present at the plasma membrane after rab4 expression accounted for $47.1 \pm 6.3\,\%$ (mean $\pm$ S.E.M. of three independent observations) of the level found in the GLUT4 control group. This effect was specific for rab4, and was not mimicked by the presence of an inactive mutant form of rab4 (Figure 1B).

We previously reported that wortmannin, a specific inhibitor of PtdIns 3-kinase, inhibits insulin-stimulated glucose transport in GLUT4-expressing oocytes [23]. In addition, Holman and colleagues have recently documented that wortmannin blocks the exocytosis of GLUT4 in 3T3-L1 adipocytes [30,31], suggesting that PtdIns 3-kinase regulates GLUT4 exocytosis. In addition, Tanti et al. [32] showed that overexpression of a constitutively activated form of PtdIns 3-kinase was sufficient to activate GLUT4 translocation in adipocytes. For these reasons we raised the question of whether the PtdIns 3-kinase inhibitors LY294002 and wortmannin would alter the inhibitory effect of rab4 on glucose transport observed in oocytes. To this end, oocytes were preincubated with 10 or 100 $\mu$M LY294002 or 1 $\mu$M wortmannin for 30 min at room temperature, and then 2-deoxyglucose uptake was determined. Both wortmannin and LY294002 decreased 2-deoxyglucose uptake in GLUT4-expressing oocytes (Table 4). Furthermore, these inhibitors and rab4 showed additive inhibitory effects on 2-deoxyglucose uptake in oocytes (Table 4). These results suggest that the mechanism by which rab4 diminishes glucose transport in GLUT4-expressing oocytes is independent of PtdIns 3-kinase.

## DISCUSSION

The heterologous expression of rab4 caused the inhibition of glucose transport and a decreased abundance of GLUT4 glucose transporters at the cell surface in *Xenopus* oocytes. A number of observations allow us to conclude that we are dealing with a specific effect of rab4: (a) injection of a cRNA encoding a mutant inactive form of rab4 did not have any effect on GLUT4-induced glucose transport or on the abundance of GLUT4 at the cell surface; (b) co-injection of cRNAs encoding for rab5 or a mutant inactive form of rab5 did not modify glucose transport in GLUT4-expressing oocytes; (c) the effect of rab4 on GLUT4-induced glucose transport was detected regardless of whether the experimental protocol involved co-injection or sequential injection of the two cRNAs; and (d) the effect was dependent on the dose of rab4 cRNA injected. Furthermore, the effect of rab4 on GLUT4-induced glucose transport was not a consequence of alterations in the expression of GLUT4, but rather of changes in the abundance of GLUT4 protein at the cell surface. In consequence, our studies in oocytes support the conclusions that (a) rab4 contributes to the sequestration of GLUT4 intracellularly, and (b) this capacity of rab4 is observed in a cell system that does not natively express GLUT4. Regarding the mechanisms activated by rab4 that lead to a decreased abundance of GLUT4 at the cell surface, and based on prior studies indicating that rab4 regulates recycling through the early endosomal compartment [33,34], a mechanism could be hypothesized by which rab4 activates endocytosis or inhibits exocytosis of GLUT4. In our study we have not directly assessed endocytosis or exocytosis of GLUT4. However, we have found that rab4 exerts its effects on GLUT4-induced glucose transport in an additive fashion to the effects of PtdIns 3-kinase inhibitors. In adipocytes, wortmannin, a PtdIns 3-kinase inhibitor, has been found to inhibit the exocytosis of GLUT4 [30,31]. Based on this, we favour the idea that rab4 might be activating the endocytosis of GLUT4 in oocytes. Further direct analysis of GLUT4 endocytosis and exocytosis should be carried out in rab4-expressing oocytes.

In the present study we have also found that rab4 did not modify GLUT1-induced glucose transport in oocytes. Based on the fact that GLUT1 is more efficient than GLUT4 at reaching the plasma membrane in the *Xenopus* oocyte [35], it is likely that GLUT1 undergoes exocytosis from the oocyte much more efficiently than GLUT4; in consequence, GLUT1, once internalized, is rapidly recycled to the plasma membrane. Based on this scenario, it is feasible that rab4 should have no impact on the glucose transport activity induced by GLUT1.

We have reported previously that GLUT4-expressing oocytes have the ability to respond to insulin by increasing glucose transport [23]. Furthermore, insulin-stimulated oocytes, but not oocytes under basal conditions, have been shown to respond to the PtdIns 3-kinase inhibitor wortmannin by reducing glucose transport [23]. In the present study we have worked with GLUT4-expressing oocytes that were in a clearly different physiological condition, i.e. they were insensitive to insulin and sensitive to wortmannin in a basal non-stimulated state. We postulate that, due to unknown reasons (e.g. environmental and seasonal conditions), *Xenopus* oocytes can be in different states with more or less sensitivity to insulin and insulinomimetic agents. The discrepancy between our previous report [23] and the present one is in line with the literature regarding the varying insulin-responsiveness of *Xenopus* oocytes [27–29].

In conclusion, our results demonstrate that rab4 regulates the cellular trafficking of GLUT4 in oocytes so that it enhances the intracellular retention of GLUT4 in this cell type. Based on the fact that *Xenopus* oocytes do not express GLUT4 naturally, it is likely that the capacity of rab4 to regulate GLUT4 trafficking in the oocyte is conferred through the endosomal recycling machinery.

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