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The Rab4 effector Rabip4 plays a role in the endocytotic trafficking of Glut 4 in 3T3-L1 adipocytes

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Summary
Insulin regulates glucose uptake in the adipocytes by modulating Glut 4 localization, a traffic pathway involving the endocytic small GTPases Rab4, Rab5, and Rab11. The expression of the Rab4 effector Rabip4 leads to a 30% increase in glucose uptake and Glut 4 translocation in the presence of insulin, without modifications in the basal condition. This effect was not due to modifications of Glut 4 expression or insulin signaling, suggesting that Rabip4 controls Glut 4 trafficking. We present evidence that Rabip4 defines a subdomain of early endosomes and that Rabip4 is redistributed to the plasma membrane by insulin. Rabip4 is mostly absent from structures positive for early endosome antigen 1, Rab11 or transferrin receptors and from Glut 4 sequestration compartments. However, Rabip4 vesicles can be reached by internalized transferrin and Glut 4. Thus, Rabip4 probably defines an endocytic sorting platform for Glut 4 towards its sequestration pool. The expression of a form of Rabip4 unable to bind Rab4 does not modify basal and insulin-induced glucose transport. However, it induces an increase in the amount of Glut 4 at the plasma membrane and perturbs Glut 4 traffic from endosomes towards its sequestration compartments. These observations suggest that the uncoupling between Rabip4 and Rab4 induces the insertion of Glut 4 molecules that are unable to transport glucose into the plasma membrane.

Key words: Endocytosis, Rab, Glut recycling, Glucose transporter

Introduction
Glucose transporter 4 (Glut 4) plays an important role in glucose homeostasis. Glut 4 is mainly expressed in cells that exhibit regulated glucose uptake such as adipocytes, skeletal muscle cells and cardiomyocytes. The intracellular trafficking is a major determinant of the acute regulation of glucose transport. Glut 4 continuously recycles between the plasma membrane and intracellular organelles. In the basal state, this cycling favors an intracellular location for Glut 4, from where it undergoes stimulus-responsive movement to the cell surface. In adipocytes, insulin is the major physiological stimulus able to evoke Glut 4 translocation to the plasma membrane whereas both insulin and exercise act on Glut 4 location in skeletal muscle (Bryant et al., 2002; Rea and James, 1997).

In non-stimulated adipocytes, Glut 4 is mainly present in three defined intracellular compartments: the endosomal recycling system containing transferrin receptors (TfR), a perinuclear region which possibly represents a sub-compartment of the trans-Golgi network (TGN), and small vesicles characterized by the presence of VAMP2, a v-SNARE of the secretory apparatus of neuronal cells (Bryant et al., 2002). The two latter locations, which are devoid of transferrin receptors, form specialized Glut 4 sequestration pools. The ability to segregate Glut 4 from TfR occurs alongside adipocyte differentiation and the ability of insulin to induce full Glut 4 translocation (Bryant et al., 2002; El-Jack et al., 1999; Govers et al., 2004). Endosomes appear to play an important role in insulin effects. First, the recycling endosomes represent one of the sorting steps needed to trigger Glut 4 into its sequestration pools, an event which could be more efficient in the presence of insulin (Foster et al., 2001; Zeigerer et al., 2002). Second, in adipocytes insulin efficiently induces the recruitment to the plasma membrane of proteins from the endosomal recycling system such as the TfR and the ubiquitous glucose transporter Glut 1. Third, Glut 4 molecules present in endosomes are also recruited to the plasma membrane in response to insulin (Millar et al., 1999; Zeigerer et al., 2002).

In accordance with the importance of the endosomal recycling system for insulin-induced Glut 4 translocation, three of the endosomal Rab proteins (Rab5, Rab4 and Rab11) have been involved in the Glut 4 trafficking pathway. Rab proteins form a family of more than 60 small GTPase proteins that have key roles in the control of membrane-transport steps (Takai et al., 2001). Rab5, Rab4 and Rab11 have been localized on single endosomes, in which they probably define specialized functional membrane domains (Sonnichsen et al., 2000). Rab5 is involved in the formation and function of sorting endosomes (Somsel Rodman and Wandinger-Ness, 2000; Zerial and McBride, 2001) and microinjection of anti Rab5 antibodies into adipocytes induces an inhibition of Glut 4 internalization (Huang et al., 2001). Rab11 has a role in trafficking from the recycling endosomes both back to the plasma membrane (Ren et al., 1998; Ulrich et al., 1996) and to the TGN (Wilcke et al., 2000). When a dominant inhibitor of Rab11 function is
used in adipocytes, the trafficking of Glut 4 from endosomes to the sequestration compartment is inhibited (Zeigerer et al., 2002). Several studies indicate that Rab4 is also an important player in Glut 4 trafficking in adipocytes, skeletal muscle and cardiomyocytes. However, its exact function is still unknown (reviewed by Cormont and Le Marchand-Brustel, 2001; Mohrmann and van der Sluijs, 1999).

In the present study, we investigate the role of Rabip4, a Rab4 effector (Cormont et al., 2001b; Mari et al., 2001; Monzo et al., 2005b), in the control of Glut 4 subcellular localization and trafficking in 3T3-L1 adipocytes, a cell type in which Glut 4 translocation is stimulated by insulin. We studied the effect of expression of wild-type Rabip4 and a mutated form unable to bind Rab4, on glucose transport and Glut 4 translocation. We also determined the localization of endogenous Rabip4. Altogether our observations suggest that Rabip4 is involved in Glut 4 sorting from a subdomain of early endosomes towards its sequestration compartments.

**Results**

Insulin-induced glucose transport and Glut 4 translocation are increased in 3T3-L1 adipocytes stably expressing Rabip4

To determine whether Rabip4 might be involved in Glut 4 trafficking processes, we studied basal and insulin-stimulated glucose transport in adipocytes overexpressing Rabip4. We used a tetracycline-inducible system (Tet-off) to evoke the expression of Rabip4 only when cells were fully differentiated into adipocytes. Problems linked to a possible effect of the protein in the differentiation process were thus eliminated. When Rabip4 was overexpressed, insulin-induced glucose transport was significantly increased by 30% at maximal and submaximal concentrations of insulin, but not under basal conditions (Fig. 1A). The expression of Rabip4 was increased five- to eightfold when 3T3-L1 Rabip4 Tet-off adipocytes were cultured without tetracycline for 2 days without any change in Rab4 levels (Fig. 1B). The increase in insulin-induced glucose transport was accompanied by an increase in the amount of Glut 4 in plasma membrane sheets of adipocytes expressing Rabip4 compared with control cells (Fig. 1C). This effect was observed without modification of Glut 4 expression (Fig. 1B). Moreover, insulin signaling, i.e. tyrosine phosphorylation of the insulin receptor (IR) and insulin receptor substrates (IRS), activation of the protein kinase B (PKB) and activation of extracellular regulated kinases (ERK) were similar in both conditions (Fig. 1D). All these results suggest that the expression of Rabip4 favors insulin-induced Glut 4 translocation and glucose transport by modifying Glut 4 trafficking.

Rabip4 and Glut 4 define different intracellular compartments

To understand the effect of Rabip4 overexpression, we first looked for a potential colocalization between endogenous Glut 4 and Rabip4 under basal conditions. 3T3-L1 adipocytes were co-labeled with antibodies directed against Rabip4 (Fig. 2a-b) and Glut 4 (Fig. 2c-d) for detection of the two proteins. Either close to the bottom of the cell (Fig. 2a,c,e,g) or in the middle (Fig. 2b,d,f,h), the majority of Rabip4 and Glut 4 did not colocalize as shown in the merged image (Fig. 2e-h). When overexpressed, Rabip4 and Glut 4 also defined neighboring, but not overlapping, compartments (data not shown). Thus, Rabip4 and Glut 4 were not colocalized in steady-state conditions although the two proteins were enriched in a perinuclear region.

Glut 4 traffics through Rabip4 containing vesicles

We next determined whether internalized Glut 4 vesicles could reach Rabip4-containing compartments. To this aim, adipocytes

**Fig. 1.** Rabip4 expression induces an increase in glucose uptake and Glut 4 translocation to the plasma membrane. 3T3-L1 Rabip4 Tet-off adipocytes were cultivated for 3 days with or without tetracycline (+/-tetra) to induce the expression of Rabip4. Cells were serum starved overnight, and then treated with insulin for 20 minutes. (A) Deoxyglucose uptake was then determined as described in the Materials and Methods. The results were expressed as the mean ± s.e.m. of five experiments. (B) Glut 4 translocation to the plasma membrane. 3T3-L1 Rabip4 Tet-off adipocytes cultivated with or without tetracycline for 3 days were separated by SDS-PAGE and analyzed by western blot for the presence of Rabip4, Glut 4 and Rab4. (C) Glut 4 translocation was determined by the amount of Glut 4 present in plasma membrane sheet preparations as described in the Materials and Methods. Data are the mean ± s.e.m. of three experiments of the fold increase in insulin stimulation over the basal condition of cells cultivated with tetracycline (no Rabip4 expression). (D) YP-IR, tyrosine-phosphorylated β subunits of the insulin receptor; YP-IRS, tyrosine-phosphorylated insulin receptor substrates. Activation of protein kinase B (PKB) and extracellular-regulated kinases (ERK) was estimated using anti-phospho-PKB and anti-phospho-ERK. *P<0.001 compared with levels in the +tetra control.
expressing both GFP-Rabip4 and Glut 4-myc-DsRed (a fluorescent Glut 4 molecule with an extracellular myc epitope) were treated with insulin to induce the translocation of the transporters to the plasma membrane. Myc antibodies were allowed to bind to cell surface Glut 4-myc-DsRed at 4°C for 2 hours before extensive washing (Fig. 3A). Following this step, the cells were warmed to induce the internalization of the anti-myc antibodies/Glut 4 complexes (Fig. 3B,C,D). We took advantage of the temperature sensitivity of various endocytotic steps to emphasize the presence of internalized anti-myc antibodies into GFP-Rabip4 structures. When cells were incubated for 1 hour at 15°C, a temperature that slows down the rate of entry of internalized molecules into early endosomes (Lim et al., 2001) (our unpublished observations), anti-myc antibodies were found in vesicular structures negative for Rabip4 (Fig. 3B). When endocytosis is performed at 20°C, many sorting steps from early/recycling endosomes are either blocked or slowed down (Czekay et al., 1997; Mallard et al., 1998; Sipe et al., 1991). In particular, Shiga toxin B fragment is blocked into early/recycling endosomes on route to the Golgi (Mallard et al., 1998) and Glut 4 traffic between the endosomes and the TGN seems to be altered (van Dam et al., 2005). In those conditions (20°C for 1 hour), many GFP-Rabip4-positive structures were also positive for anti-myc antibodies (Fig. 3C), indicating that some internalized Glut 4 is present in GFP-Rabip4-containing vesicles. DsRed-Glut 4 myc was also detected in GFP-Rabip4-containing vesicles. However, anti-myc antibodies were not found into the perinuclear compartment containing DsRed-Glut 4 myc (Fig. 3C). When the cells were warmed to 37°C for 1 hour, anti-myc antibodies were mainly colocalized with the perinuclear compartment containing DsRed-Glut 4 myc, indicating that internalized Glut 4 returned to their major location as previously described by Shewan et al. (Shewan et al., 2003).

Rabip4 defines a specific subdomain of early endosomes

To identify the nature of the vesicles containing Rabip4, we studied its localization along the endocytic pathway compared with various endocytic markers (Fig. 4). No obvious colocalization was observed between endogenous Rabip4 and the early endosomal marker EEA1 (Fig. 4Aa-c). However, as evidenced in an enlarged view of the labeled vesicles (Fig. 4Aa’-c’), Rabip4- and EEA1-positive structures that are present in the periphery of the cell were often close to each other. This suggests that the two proteins are associated with successive microdomains of the same early endosomes as described for Rab5 and Rab4 (Sonnichsen et al., 2000) and for overexpressed Rabip4 and endogenous EEA1 (Mari et al., 2001). The Rabip4-containing structures enriched in the perinuclear region did not colocalize with TIR that define the recycling endosomes (Fig. 4Ad-f). Moreover, they did not colocalize with TGN38, a marker of the TGN (Fig. 4Ag-i). Next, we studied the extent of colocalization between various Rab proteins fused to GFP and myc-Rabip4 as well as GFP-Rabip4 and myc-Rab11 (Fig. 4B). Rabip4 and Rab4 mainly colocalized in enlarged circular structures (Fig. 4Ba-c) that are evoked by the coexpression of the two proteins (data not shown), similarly to our observations in CHO cells (Cormont et al., 2001b; Mari et al., 2001; Monzo et al., 2005b). Few Rab5-containing vesicles were positive for Rabip4 (Fig. 4Bd-f). Although localized in the perinuclear region, Rabip4 and Rab11 were mainly not colocalized, however, some vesicles containing Rab11 appeared to be connected to Rabip4-containing structures (Fig. 4Bj,k,l and enlarged views). By contrast, Rabip4 and Rab7 defined distinct compartments (Fig. 4Bg-i). To determine the nature of Rabip4-containing vesicles, we investigated whether internalized transferrin could reach Rabip4-containing vesicles. To this aim, 3T3-L1 adipocytes

![Fig. 2. Rabip4 and Glut 4 define distinct intracellular compartments.](image-url)
were incubated with Texas-Red-coupled mouse transferrin for 1 hour at 20°C to inhibit sorting events at the level of the early endosomes (Fig. 5a-c) (Mallard et al., 1998) or at 4°C to block internalization (Fig. 5d-f). Then, cells were labeled for endogenous Rabip4. When incubated at 20°C, transferrin was found in some Rabip4-positive vesicles essentially in the periphery of the adipocyte (Fig. 5a-c). By contrast, transferrin remained at the plasma membrane when incubated at 4°C, as indicated by a corresponding rim in a confocal section (Fig. 5d-f). The same experiment was performed in HeLa cells, a cell type that expresses a larger number of TfR. We observed that transferrin internalized at 20°C was also detected in Rabip4-positive compartments (Fig. 5g-l), whereas it was not clearly associated with EEA1-positive compartments (data not shown). This series of experiments suggested that Rabip4 was an early endosomal protein because the compartment labeled by Rabip4 was accessible to transferrin. However, Rabip4 was associated with a specific subdomain of early endosomes, because it did not colocalize with EEA1.

**Insulin modifies Rabip4 localization**

To determine whether Rabip4 localization, similarly to Glut 4, was subjected to modification following insulin treatment, Rabip4 and Glut 4 immunoreactivities were quantified in plasma membrane sheets obtained from 3T3-L1 adipocytes treated or not with insulin (Fig. 6A,B). In basal conditions, Rabip4 was already detected at the plasma membrane whereas Glut 4 was almost undetectable. Insulin increased the amount of Rabip4 present in the plasma membrane sheets threefold, whereas the amount of Glut 4 was increased eightfold. Fractionation experiments indicated that there was a concomitant decrease in the amount of intracellular Rabip4 (Fig. 6C), suggesting that insulin could recruit Rabip4-containing structures to the plasma membrane. The
translocation of Rabip4 was parallel to that of Glut 4 and TfR.

Expression of a mutated form of Rabip4 unable to bind Rab4 triggers Glut 4 translocation in 3T3-L1 adipocytes but does not modify insulin-induced glucose transport

to determine whether the increase in insulin-induced glucose transport evoked by the expression of Rabip4 involved its interaction with Rab4, we studied the effect of the expression of a form of Rabip4 unable to bind Rab4, Rabip4Δ507-517 (Mari et al., 2001). The inducible Tet-off system was used as in Fig. 1. We did not observe any change in insulin-induced glucose uptake when adipocytes expressed Rabip4Δ507-517 (Fig. 7A). This indicated that Rabip4 might need to interact with Rab4 to affect glucose transport. However, we detected a four- to fivefold increase in the amount of Glut 4 present in the plasma membrane sheets when the expression of Rabip4Δ507-517 was induced (Fig. 7B). We also observed 34% of cells with an endogenous Glut 4 rim when 3T3-L1 Tet-off Rabip4Δ507-517 adipocytes were cultured in the absence of tetracycline compared with only 5% in the presence of tetracycline (data not shown). Interestingly, the plasma membrane level of TfR was not modified by the expression of Rabip4Δ507-517 (Fig. 7B).

To confirm this unexpected result, we used a different experimental approach to study the effect of Rabip4Δ507-517 expression on Glut 4 translocation (Fig. 7C,D). 3T3-L1 adipocytes were transiently transfected with Glut 4 myc-DsRed together with GFP or GFP-Rabip4Δ507-517. We then determined the number of adipocytes expressing Glut 4-myc-
DsRed at the cell surface, i.e. in which the myc epitope present in its first extracellular loop was externally accessible to myc mAb, revealed by anti mouse Cy5-coupled immunoglobulins (Fig. 7C). GFP-Rabip4 unable to bind Rab4 increased the number of adipocytes that possess the glucose transporter at the plasma membrane in basal conditions compared with GFP-expressing cells, whereas no changes were observed in the presence of insulin (Fig. 7D). This second experimental approach further indicated that Glut 4 was incorporated into the plasma membrane because the extracellular myc epitope was recognized by the anti-myc antibodies on non-permeabilized adipocytes. In conclusion, the expression of a form of Rabip4 unable to bind Rab4 yielded to an increase in the amount of Glut 4 at the plasma membrane without a parallel increase in glucose transport.

Expression of a mutated form of Rabip4 unable to bind Rab4 affects Glut 4 trafficking between early endosomes and its storage compartment

We searched for a possible explanation of the Glut 4 increase at the plasma membrane. We postulated that this could be due to...

Fig. 5. Transferrin internalized at 20°C reaches Rabip4-positive vesicles. The same experiment was performed using 3T3-L1 adipocytes (a-f) and HeLa cells (g-i). Cells were incubated for 1 hour at 20°C (a-c, g-i) or 4°C (d-f, j-l) with Texas-Red-coupled transferrin (500 μg/ml). Cells were washed in PBS, fixed with paraformaldehyde and permeabilized. Endogenous Rabip4 was detected by using specific antibodies followed by FITC-coupled anti-rabbit antibodies. Arrowheads indicate Rabip4-positive vesicles containing internalized transferrin. The inserts are enlargements of the boxed regions. Bar, 1 μm.

Fig. 6. Rabip4 translocates to the plasma membrane in response to insulin. (A) Plasma membrane sheets were prepared from 3T3-L1 adipocytes, untreated or treated with 100 nM insulin, for 20 minutes. Rabip4 and Glut 4 were detected by immunofluorescence using rabbit anti-Rabip4 antibodies and goat anti-Glut 4 antibodies followed by incubation with FITC-coupled anti-rabbit antibodies and Texas-Red-coupled anti-goat antibodies, respectively. Bar, 10 μm. (B) Quantification of fluorescence levels from A, performed using MetaMorph software as described in the Materials and Methods. (C) 3T3-L1 adipocytes were untreated or treated with 100 nM insulin for 20 minutes. Plasma membranes (PM), intracellular microsomes (IM), and cytosol (Cyt) were then prepared and 40 μg proteins from each fraction were separated by SDS-PAGE and transferred to PVDF membrane. Antibodies against Rabip4, Glut4 and Rab4 were then used to probe the blot.
Expression of Rabip4 unable to bind Rab4 does not affect insulin-induced glucose transport, but induces the translocation of Glut 4-myc-DsRed to the plasma membrane. Fig. 7. (A-B) Serum-deprived 3T3-L1 Rabip4Δ507-517 Tet-off adipocytes were cultivated for 3 days with or without tetracycline (tetra) to induce the expression of Rabip4Δ507-517. (A) Serum-deprived 3T3-L1 Rabip4Δ507-517 Tet-off adipocytes were untreated or treated with the indicated concentrations of insulin for 20 minutes. Deoxyglucose uptake was then measured and the results of three independent experiments are shown, expressed, as the fold increase compared with basal levels in tetracycline-treated cells. The inset shows the expression of Rabip4Δ507-517 in adipocytes cultivated with or without tetracycline by western blot using anti-Rabip4 antibodies. (B) Serum-deprived cells were untreated or treated with 100 nM insulin for 20 minutes and used to prepare plasma membrane sheets as described in the Materials and Methods. The graph represents the mean ± s.e.m. of three experiments of the fold increase in insulin stimulation over the basal condition of cells cultivated with tetracycline (no Rabip4 expression). The amounts of Glut 4 and of transferrin receptors were quantified. (C-D) 3T3-L1 adipocytes expressing Glut 4-myc-DsRed in combination with GFP or GFP-Rabip4Δ507-517 were untreated or treated with 100 nM insulin for 20 minutes. Fixed cells were incubated with anti-myc mAb that binds the extracellular myc epitope of Glut 4-myc-DsRed incorporated into the plasma membrane. The anti-myc antibody is then detected using Cy5-coupled anti mouse antibodies to visualize the plasma membrane Glut 4 (mGlut 4) (in blue), Glut 4-myc-DsRed (Glut 4, in red) and membrane Glut 4-myc-DsRed (mGlut 4) (in green). (C) Typical labeling for GFP and GFP-Rabip4Δ507-517 (in green), Glut 4-myc-DsRed (Glut 4, in red) and membrane Glut 4-myc-DsRed (mGlut 4) (in blue) are shown for basal and insulin-stimulated conditions. (D) The number of adipocytes with Glut 4-myc-DsRed detected at the plasma membrane was counted. 50-100 cotransfected cells were counted in each experimental condition and the figure shows the mean ± s.e.m. of the numbers obtained in six independent experiments. *P<0.001 compared with levels in the +tetra control.

Discussion

In the present study, we aimed to determine the involvement of Rabip4, a Rab4 effector we previously identified (Cormont et al., 2001b), in the trafficking of the glucose transporter Glut 4 in the 3T3-L1 adipocyte cell line. The overexpression of Rabip4 allowed for a higher insulin-stimulated glucose transport correlated with an increase in Glut 4 translocation without modification of the total amount of Glut 4 and the insulin signaling pathways. By contrast, basal glucose uptake is not modified by Rabip4 expression suggesting that the plasma membrane level of the ubiquitous glucose transporter Glut 1 is unchanged. The increase in Glut 4 translocation could result from either change in the rate of Glut 4 externalization/internalization or from an increase in the size of the Glut 4 sequestration pools. The recent model from Govers et al. (Govers et al., 2004) and Coster et al. (Coster et al., 2004) proposes that insulin-induced Glut 4 recruitment to the plasma membrane is due to a graded or quantal release of Glut 4 from its sequestration compartments towards the endocytic recycling pathway rather than in kinetic changes of Glut 4 recycling between plasma membrane and its sequestration compartments. Thus, the simplest explanation for the increased Glut 4 translocation by Rabip4 expression is that more Glut 4 transporters are sequestered in the sequestration compartments. This implies that an increased level of Rabip4 favors the targeting of Glut 4 to this sequestration pool, a similar function also proposed for Rab4 in adipocytes and cardiomyocytes (Cormont et al., 1996a; Cormont et al., 2001a; Dransfeld et al., 2000). It would be logical that Rab4 and its effector Rabip4 could play a similar function.

The subcellular localization of Rabip4 favors a role in a Glut-4-sorting event at the level of the early endosomes, rather than in the recruitment from its intracellular, vesicle-associated...
However, it induces the incorporation of Glut 4 molecules into membranes and does not affect basal nor insulin-stimulated glucose transport. Indeed, the expression of a form of Rabip4 unable to bind Rab4 result from a Glut 4 traffic defect from endosomes to its sequestration compartment. Rabip4-positive endosomes would constitute a post early endosome Glut 4 sorting compartment. Uncoupling between Rabip4 and Rab4 would lead to a specific recruitment of inactive Glut 4 (in blue) towards the plasma membrane. Insulin recruits Glut 4 from its sequestration compartments as well as from the endosomes and is necessary to allow plasma membrane Glut 4 to transport glucose (active Glut 4 in green). TfR, transferrin receptor; Rabip4\DeltaRab4BD, Rabip4 without Rab4 binding domain or Rabip4\Delta507-517.

The increase in insulin-induced glucose transport when Rabip4 is overexpressed required its interaction with Rab4. Indeed, the expression of a form of Rabip4 unable to bind Rab4 does not affect basal nor insulin-stimulated glucose transport. However, it induces the incorporation of Glut 4 molecules into the plasma membrane. We can exclude the fact that the defect in 2-deoxy-D-[3H]glucose transport was due to a decrease in hexokinase function induced by the overexpression of the mutated form of Rabip4 rather than a defect in glucose transport, because no differences were observed when 2-deoxy-D-[3H]glucose uptake was performed in the presence of insulin. The inconsistency between Glut 4 translocation and glucose transport reported here is reminiscent of some previous studies. Selective inhibitors of p38 (Somwar et al., 2002) or low concentrations of the PI3-kinase inhibitor wortmannin (Hausdorff et al., 1999) partially inhibit insulin-stimulated glucose transport without affecting the amount of Glut 4 at the plasma membrane. Some selective inhibitors of p38 as well as intracellular delivery of phosphatidylinositol (3,4,5)-trisphosphate causes incorporation of Glut 4 into the plasma membrane without increasing glucose uptake (Ishiki and Klip, 2005; Somwar et al., 2001; Sweeney et al., 2004). A cell-permeable phosphoinositide-binding peptide also leads to Glut 4 translocation without any increase in glucose transport (Funaki et al., 2004). All these observations indicate two insulin-sensitive steps, one leading to Glut 4 translocation, the other allowing glucose transport by Glut 4.

We propose that the increased plasma membrane levels of Glut 4 induced by the expression of the form of Rabip4 unable to bind Rab4 result from a Glut 4 traffic defect from endosomes to its sequestration compartments. Indeed, 1 hour after insulin withdrawal Glut 4 was mainly detected with TfR and absent from VAMP2-positive vesicles in cells that expressed Rabip4\Delta507-517, whereas Glut 4 was found in VAMP2-positive vesicles and was segregated from TfR-containing compartments in control cells. Surprisingly, the expression of Rabip4 unable to bind Rab4 also led to a decreased hexokinase function, which suggests that Rabip4-dependent glucose transport is dependent on the integrity of the Rab4 pathway.
bind Rab4 yields to a redistribution of Glut 4, but not TIR, to the plasma membrane. TIR recycling has been described as a Rab4-sensitive (Cormont et al., 2003; Deneka et al., 2003; McCaffrey et al., 2001; Mohrmann et al., 2002; van der Sluijs et al., 1992) and Rabip4-sensitive process (Foureaux et al., 2004). But, TIR and Glut 4 could use different vesicles to traffic through endosomes (Lampson et al., 2001; Lim et al., 2001; Xu and Kandror, 2002) and thus would need different molecular mechanisms. Thus, when retrograde traffic from the plasma membrane towards the sequestration compartment was impaired, more Glut 4 transporters would recycle through a TIR-independent endosomal recycling pathway, yielding to an increased amount of inactive Glut 4 at the plasma membrane.

We propose a model in which insulin, once it recruits Glut 4 from its intracellular compartment, would uncouple Rabip4 from Rab4, allowing newly internalized Glut 4 transporters to return to the plasma membrane by intermediate vesicles budded from early endosomes (Fig. 9). It will be interesting to determine whether insulin modifies the association between Rab4 and Rabip4, an hypothesis which cannot yet be tested owing to the difficulty to detect the interaction between Rab proteins and their effectors by classical approaches (Nizak et al., 2003). A direct recruitment of an endosomal-trafficking molecule, the αvβ3 integrin, from early endosomes to the plasma membrane has also been observed in fibroblasts treated with PDGF (Roberts et al., 2001). Interestingly, this effect requires Rab4 and thus presents some similarities with insulin-induced Glut 4 translocation. Future studies will aim at determining how extracellular signals could control Rab4-dependent intracellular trafficking pathways.

Materials and Methods
Antibodies and reagents
Goat polyclonal anti Glut 4, mAb antibodies directed against phosphotyrosine (clone 4G10) were from Sigma (St Louis, MO). Anti-EEA1 and Rab4 mAbs were from Cell Signaling Technology (Beverly, MA). Anti-EEA1 and Rab4 mAbs were from BD Biosciences Pharmingen (San Diego, CA). Rabbit polyclonal anti-Rabip4 were produced as described (Mari et al., 2001) and were affinity purified. mAb against TIR was from Zymed Laboratories (San Francisco, CA). Anti-VAMP2 mAb was from Synaptic System (Göttingen, Germany). Horseradish peroxidase-conjugated, fluorochrome-conjugated secondary anti-species antibodies and mouse Texas-Red-coupled transferrin were from Jackson ImmunoResearch Laboratories (West Grove, PA). Human Texas-Red-coupled transferrin was from Molecular Probes (Invitrogen). Rabbit anti-TGN38 antibodies were a gift from Matthew Seaman (University of Cambridge, Cambridge, UK). Restriction and DNA-modifying enzymes were from New England BioLabs (Beverly, MA). Chemicals were from Sigma (St Louis, MO).

Plasmids
A plasmid encoding a C-terminal fusion of the fluorescent protein DsRed with Glut 4-myc (Glut 4-myc-DsRed) was obtained by subcloning a PCR amplification of Glut 4-myc into the Smul restriction site of pDsRed-N vector (BD Biosciences Clontech, Palo Alto, CA). Glut 4-myc is a Glut 4 transporter with a myc epitope in its first extracellular loop (Cormont et al., 1996a). The behavior of Glut 4-myc-DsRed is similar to that of Glut 4-GFP (Dobson et al., 2002). The coding sequences of Rabip4 and Rabip4 Δ507-517 were subcloned into pRevTRE to permit the inducible expression of the proteins in cells expressing a tetracycline-controlled transactivator (Tet-off system from BD Clontech, Palo Alto, CA).

Cells
3T3-L1 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) and induced to differentiate into adipocytes as described previously (Cormont et al., 1996b). 3T3-L1 fibroblasts were treated with 10% FCS supplemented with isobutylmethylxanthine (250 μM), dexamethasone (250 μM) and insulin (800 nM), then for 2 further days with DMEM, 10% FCS and insulin (800 nM). The cells were then fed every 2 days with DMEM, 10% FCS and used between days 2 and 7 after the end of the differentiation protocol, when more than 90% of the cells presented adipocyte phenotype.

To establish 3T3-L1 fibroblast cell lines expressing Rabip4 and Rabip4 Δ507-517 in an inducible way, 3T3-L1 fibroblasts were first infected with retrovirus produced in BOSC cells after transfection with pRevTRE. The cells were then selected in geneticin-containing medium (250 μg/ml) and then infected with the retrovirus produced by BOSC cells transfected with pRevTRE-Rabip4 or pRevTRE-Rabip4 Δ507-517. After selection with hygromycin B (250 μg/ml), cells were grown in the presence of tetracycline (0.1 μg/ml) and induced to differentiate into adipocytes, as described above. 2 days after differentiation, Rabip4 or Rabip4 Δ507-517 expression was induced by culturing cells in the absence of tetracycline for 2-3 days.

3T3-L1 adipocytes were transfected by electroporation (Thurmond et al., 1998). 3T3-L1 adipocytes were trypsinized and resuspended in DMEM supplemented with 10% FCS with a 0.4 cm gap cuvette with 150-300 μg plasmid DNA (final volume of 500 μl). Electroporation was performed with an electric shock (180 V, 1050 μF) using an Easyjet electroporator system (Equibio, Ashford, UK). After electroporation, cells were plated on collagen-IV-coated glass coverslips and allowed to recover in complete medium. HeLa cells were cultured in DMEM containing 10% FCS, as described previously (Monzo et al., 2005a).

Measurement of 2-deoxy-D-H3glucose uptake
After serum starvation, 3T3-L1 adipocytes were incubated with various insulin concentrations as indicated in the figure legends. Glucose transport was determined by the addition of 2-deoxy-D-[3H]glucose (0.1 mM, 0.5 μCi/ml) (Gual et al., 2002). The reaction was stopped after 3 minutes by removing the labeled medium and washing four times with ice-cold PBS. Cells were lysed in 20 mM Tris-HCl pH 7.4 containing 1% Triton X-100 and 1% SDS, protease inhibitors (Complete™, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (10 mM NaF, 10 mM PPi, and 200 μM orthovanadate). Aliquots of homogenates were used to assay glucose uptake by scintillation counting. Results were normalized for protein measured by BCA assay. Remaining aliquots were used for the quantification of Glut 4 expression and the analysis of phosphoproteins by western blot.

Preparation of plasma membrane sheets and subcellular fractionation
3T3-L1 cells were grown on glass coverslips and differentiated as described above. Cells were incubated overnight in DMEM 0.5% BSA. They were then treated with or without insulin. After rapid washes with PBS, cells were fixed with 0.55 mg/ml Poly-L-lysine for 1 minute and then swollen by three successive rinses with an hypertonic buffer (30 mM HEPES pH 7.5, 70 mM KCl, 5 mM MgCl2, 3 mM EGTA). The swollen cells were sonicated and the bound membrane sheets were fixed with 4% paraformaldehyde and blocked with 4% BSA (Robinson et al., 1992). Plasma membrane sheets were then processed for immunofluorescence. Subcellular fractionation of 3T3-L1 adipocytes was performed as described previously by using differential centrifugations (Cormont et al., 1996b).

Immunofluorescence
Indirect immunofluorescence experiments were performed on intact adipocytes (either permeabilized or not) and on plasma membrane sheets (Cormont et al., 2003). Cells were then analyzed by scanning confocal fluorescence microscopy with a PL-ALPO 63× 1.4 oil objective (TSP SP, Leica, Deerfield, IL). The images were combined and merged using PhotoShop software (Adobe system, Mountain View, CA). The plasma membrane sheets were analyzed with an Axiovert 200 microscope using a Plan-Neofluar 40× 1.3 numerical aperture oil objective (Carl Zeiss, Göttingen, Germany). Images were acquired using a cooled digital camera (CoolSnap HQ, Roper Scientific Princeton Instruments, Eqty, France) and quantification was made using Metamorph image analysis software with auto-threshold detection of pixels (Universal Imaging Corporation, Downingtown, PA). For the determination of Glut 4-myc-DsRed internalization, transfected adipocytes were first incubated for 20 minutes with 100 nM insulin, then for 2 hours with anti-myc mAb at 4°C. They were then extensively washed at 4°C to remove the unbound mAb and incubated in DMEM medium containing 0.5% BSA for 1 hour at 15°C, 20°C or 37°C. Adipocytes were then fixed by incubating the cells for 2 minutes in 4% paraformaldehyde before neutralization in 10 mM NH4Cl for 10 minutes. After washes in PBS, cells were permeabilized in PBS containing 0.1% Triton X-100 and 5% BSA, and then incubated with Cy5-coupled anti-mouse antibodies. After washes in permeabilization buffer, cells were mounted as described (Cormont et al., 2003).

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