Rip11 is a Rab11- and AS160-RabGAP-binding protein required for insulin-stimulated glucose uptake in adipocytes

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Summary

The translocation of GLUT4 to the plasma membrane underlies the ability of insulin to stimulate glucose uptake, an event that involves the activation of protein kinase B, several members of the Rab family of GTP-binding proteins and the phosphorylation of the Rab GTPase-activating protein AS160. Here, we explored the regulation by insulin of the class I Rab11-interacting proteins Rip11, RCP and FIP2. We show that Rip11, but not RCP or FIP2, translocates to the plasma membrane of 3T3-L1 adipocytes in response to insulin. This unique response of Rip11 prompted us to explore the role of this protein in more detail. We found that Rip11 partially colocalises with GLUT4 in intracellular compartments. siRNA-mediated knockdown of Rip11 inhibits insulin-stimulated uptake of 2-deoxyglucose, and overexpression of Rip11 blocks insulin-stimulated insertion of translocated GLUT4 vesicles into the plasma membrane. We additionally show that Rip11 forms a complex with AS160 in a Rab11-independent manner and that insulin induces dissociation of AS160 from Rip11. We propose that Rip11 is an AS160- and Rab-binding protein that coordinates the protein kinase signalling and trafficking machinery required to stimulate glucose uptake in response to insulin.

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Introduction

Insulin regulates uptake of glucose into adipose tissue and muscle by promoting the rapid translocation of the insulin-responsive isoform of the glucose transporter, GLUT4, from intracellular vesicles to the plasma membrane (reviewed in Bryant et al., 2002; Holman and Sandoval, 2001; Watson and Pessin, 2006). In adipocytes, there is a low level of GLUT4 resident at the plasma membrane in the basal state, with the bulk of the protein being sequestered both in the recycling endosomal system, together with GLUT1 and transferrin receptors, and in a specialised intracellular pool termed 'GLUT4 storage vesicles' (GSVs). In the presence of insulin, there is a 10-20-fold increase in the level of GLUT4 at the plasma membrane. This results primarily from the insulin-stimulated release of GLUT4 from the GSV pool, although insulin also increases the translocation of GLUT4 from recycling endosomes and decreases the rate of GLUT4 endocytosis.

Protein kinase B (PKB/Akt) is known to play a crucial role in mediating the effect of insulin on glucose transport (reviewed in Watson and Pessin, 2006; Welsh et al., 2005). Recently, a novel Rab GTPase-activating protein (GAP), called AS160, has been shown to be a PKB substrate and to regulate GLUT4 translocation in response to insulin (Kane et al., 2002; Sano et al., 2003). AS160 associates with GLUT4 vesicles in the basal state, and this is reported to occur through its interaction with the cytosolic tail of insulin-responsive aminopeptidase (IRAP), a known component of these vesicles (Larance et al., 2005; Peck et al., 2006). Phosphorylation of AS160 is increased by insulin on six sites in vivo, five of which conform to the PKB substrate consensus sequence {RxRxx[S(*P*)/T(*P*)]}. A mutant AS160, which lacks these PKB sites, blocks insulin-stimulated GLUT4 translocation, without affecting endocytosis (Kane et al., 2002; Larance et al., 2005; Sano et al., 2003; Zeigerer et al., 2004). Furthermore, ablation of AS160 using siRNA (small interfering RNA) leads to an increased level of GLUT4 at the plasma membrane in the absence of insulin (Eguez et al., 2005; Larance et al., 2005). These data suggest that, in the basal state, AS160 binds to GLUT4 vesicles, negatively regulating its target Rab(s). In response to insulin, AS160 has been reported to dissociate from the GLUT4 vesicles (Larance et al., 2005), which might lead to the activation of the target Rab(s) necessary for the translocation to proceed.

RabGTPases are known to be key players in many vesicle formation, fusion and trafficking events (Grosshans et al., 2006; Jordens et al., 2005). At present, the physiologically relevant target Rab(s) for AS160 has not been identified. It has

been shown that a recombinant GAP domain of AS160 acts as an active GAP for Rab 2a, 8a, 10 and 14, and that Rab proteins 2a, 8a, 10, 11a, 11b and 14 are present on purified GLUT4 vesicles (Larance et al., 2005; Miinea et al., 2005). Of these, Rab10 and Rab11 have been implicated previously in GLUT4 trafficking (Kessler et al., 2000; Kong et al., 2006; Sano et al., 2007; Uhlig et al., 2005). Artificially increasing the amount of plasma-membrane-localised phosphatidylinositol 3-phosphate [PtdIns(3)*P*] promotes GLUT4 translocation to the plasma membrane, and this also appears to occur through a Rab11-dependent pathway (Kong et al., 2006). Rab4 has been proposed to play a role in GLUT4 trafficking, most likely by modulating trafficking through early endosomes (Mari et al., 2006). Rab4 is not thought to be a substrate for AS160 (Miinea et al., 2005). Finally, Rab31 has also been implicated in insulin-stimulated GLUT4 translocation, although the trafficking step involved is not yet fully understood (Lodhi et al., 2007).

Several Rab11-interacting proteins, termed FIPs, have been identified that share a highly homologous Rab11-binding domain [RBD (Hales et al., 2001; Lindsay et al., 2002; Prekeris, 2003)] at their C-termini. The FIP family is split into two main classes depending on their domain organisation (Hales et al., 2001; Lindsay et al., 2002; Prekeris, 2003; Prekeris et al., 2000; Wallace et al., 2002). The class I Rab11-FIPs, which consists of Rab coupling protein (RCP), Rip11 (Gaf-1/pp75) and Rab11-FIP2, all have an N-terminal C2 domain that binds to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] and phosphatidic acid (Lindsay and McCaffrey, 2004b). Class I FIPs have been implicated in regulating the transport of cargo from recycling endosomes to the plasma membrane and/or apical membrane (Hales et al., 2001; Lindsay et al., 2002; Lindsay and McCaffrey, 2002). Indeed, Rip11, RCP and Rab11-FIP2 all translocate to the plasma membrane of A431 cells in response to stimulation by epidermal growth factor (EGF) (Lindsay and McCaffrey, 2004b). Class II FIPs possess a proline-rich region and two EF hands, and include FIP3 and FIP4, which are important in Arf-directed membrane trafficking events during cytokinesis (Fielding et al., 2005; Horgan et al., 2004; Prekeris, 2003) and for structural integrity of the endosomal recycling compartment (Horgan et al., 2007). Here, we show that one member of the class I family of FIPs, Rip11, binds to the AS160 RabGAP and translocates to the plasma membrane in response to insulin in a PKB- and phosphoinositide 3-kinase (PI 3-kinase)-dependent manner. Furthermore, we demonstrate that Rip11 regulates the fusion of GLUT4 vesicles with the plasma membrane, suggesting that this protein plays an important role in the regulation of glucose transport by insulin.

Results

Rip11 translocates to the plasma membrane in response to insulin and colocalises with GLUT4

We have previously reported that the class I Rab11-FIPs translocate to the plasma membrane in response to EGF or phorbol ester in A431 cells (Lindsay and McCaffrey, 2004b). Pretreating the cells with the PI 3-kinase inhibitor wortmannin blocked this affect. To determine whether the class I Rab11-FIPs translocate in response to insulin in 3T3-L1 adipocytes, GFP-tagged versions of RCP, Rip11 and Rab11-FIP2 were expressed by electroporation and their localisation analysed during stimulation with insulin.

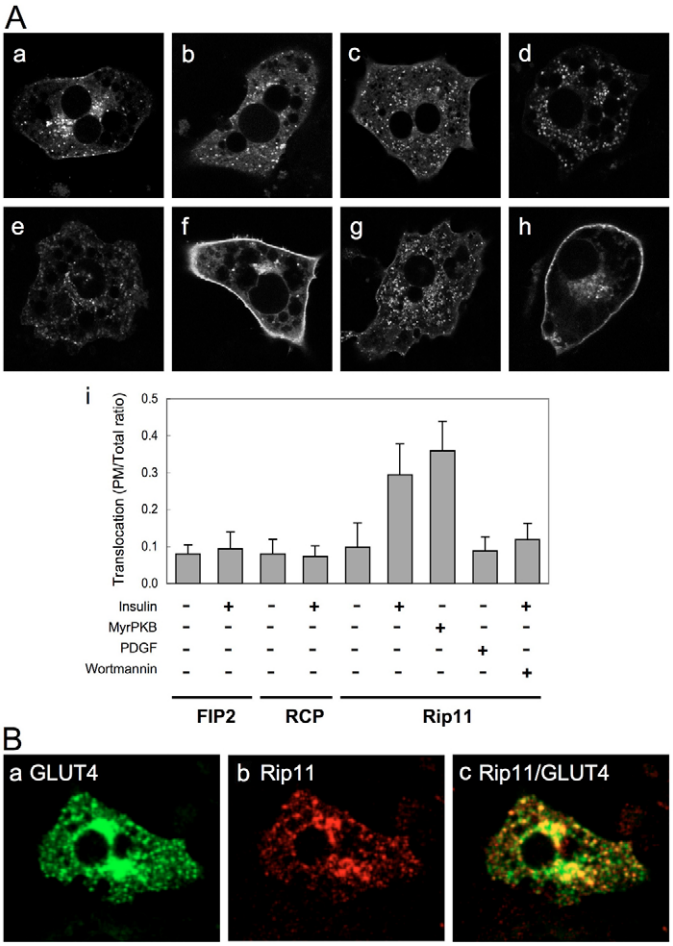

Fig. 1. Insulin and constitutively active PKB promote Rip11 translocation to the plasma membrane. (A) 3T3-L1 adipocytes were electroporated with a plasmid encoding either GFP-FIP2 (a,b), GFP-RCP (c,d) or GFP-Rip11 (e-h), and in the absence (a-g) or presence (h) of plasmid directing the expression of a constitutively active PKB (MyrPKB). The cells were imaged 24 hours later after incubation in the absence (a,c,e,h) or presence (b,d,f) of 100 nM insulin for 30 minutes. In panel (g), the cells were pre-treated with 100 nM wortmannin for 30 minutes before the addition of 100 nM insulin for 30 minutes. The cells were fixed, and visualisation of GFP fluorescence performed by laser-scanning confocal microscopy. Selected representative cells are shown. In (i) the data are expressed as the intensity of GFP fluorescence in the plasma membrane as a fraction of the total cellular GFP fluorescence (note that images for treatment with 50 ng/ml PDGF for 30 minutes are not shown). The data are expressed as means ± s.e.m., with each bar representing data from a minimum of 25 cells. (B) 3T3-L1 adipocytes were electroporated with plasmids encoding HA-GLUT4-GFP (a) and mRFP-Rip11 (b) and imaged 24 hours later. The figure shows representative laser-scanning confocal micrographs of the distribution of each protein, with a merged image provided in panel c (green is GLUT4, red is Rip11) in the absence of insulin. Both panels are representative of at least three separate preparations of adipocytes.

In the basal state, all three Rab11-FIPs localise both to the perinuclear region and to peripheral intracellular vesicles, but

apparently they are excluded from the plasma membrane (Fig. 1Aa,c,e). There was no apparent affect of insulin on the distribution of either Rab11-FIP2 or RCP (Fig. 1Ab,d,i). However, insulin induced a significant translocation of intracellular Rip11 to the plasma membrane within 30 minutes (Fig. 1Af,i). This effect of insulin was blocked by wortmannin (Fig. 1Ag,i), but it was not mimicked by platelet-derived growth factor (Fig. 1Ai), which stimulates PI 3-kinase in these cells without promoting GLUT4 translocation (Heller-Harrison et al., 1996; Navé et al., 1996; Ricort et al., 1996). Rip11 translocation was induced in the absence of insulin through the coexpression of a constitutively active mutant of PKB (Fig. 1Ah,i). These are all properties highly reminiscent of the behaviour observed for insulin-stimulated GLUT4 translocation, suggesting that Rip11 and GLUT4 traffic to the plasma membrane in response to insulin via the same sorting pathway(s). Indeed, the time course of Rip11 translocation to the plasma membrane closely paralleled that of GLUT4 itself (data not shown). In further support of a link between Rip11 and GLUT4, we found that these proteins exhibit a significant degree of colocalisation in the basal state in the perinuclear region and also in some more-peripheral vesicles distributed throughout the cytoplasm (Fig. 1Ba-c).

A more detailed analysis of the localisation of Rip11, shown in Fig. 2, failed to reveal any significant colocalisation of Rip11 with markers of recycling endosomes (transferrin receptors), early endosomes (EEA1), late endosomes (mannose 6-phosphate receptor), lysosomes (LAMP1) or trans-Golgi network (TGN38). Rip11 vesicles in the perinuclear region were found juxtapositioned to Golgi elements stained by antibodies against giantin, but there was otherwise no convincing colocalisation. This lack of colocalisation with several well-characterised intracellular membrane markers makes the colocalisation of Rip11 with GLUT4, while not complete, all the more significant.

Overexpression of Rip11 inhibits insulin-stimulated fusion of GLUT4 with the plasma membrane

To explore a potential role of Rip11 in GLUT4 translocation, we examined its effect on the translocation of a haemagglutinin (HA)-tagged GLUT4-GFP to the plasma membrane. This GLUT4 fusion protein is tagged with an HA epitope in the first exofacial loop of GLUT4, so allowing the exposure of GLUT4 to the extracellular milieu to be quantitatively determined by staining fixed, but not permeabilised, 3T3-L1 adipocytes with antibodies against HA. It is tagged at the cytosolic C-terminus with GFP, which allows the translocation of GLUT4 to be monitored independently by determining the distribution of the GFP moiety between the plasma membrane and cytosolic compartments. In addition, the expression level of GLUT4 in each cell can be determined by measuring the total cellular GFP fluorescence; this allows the surface level of GLUT4 to be normalised to

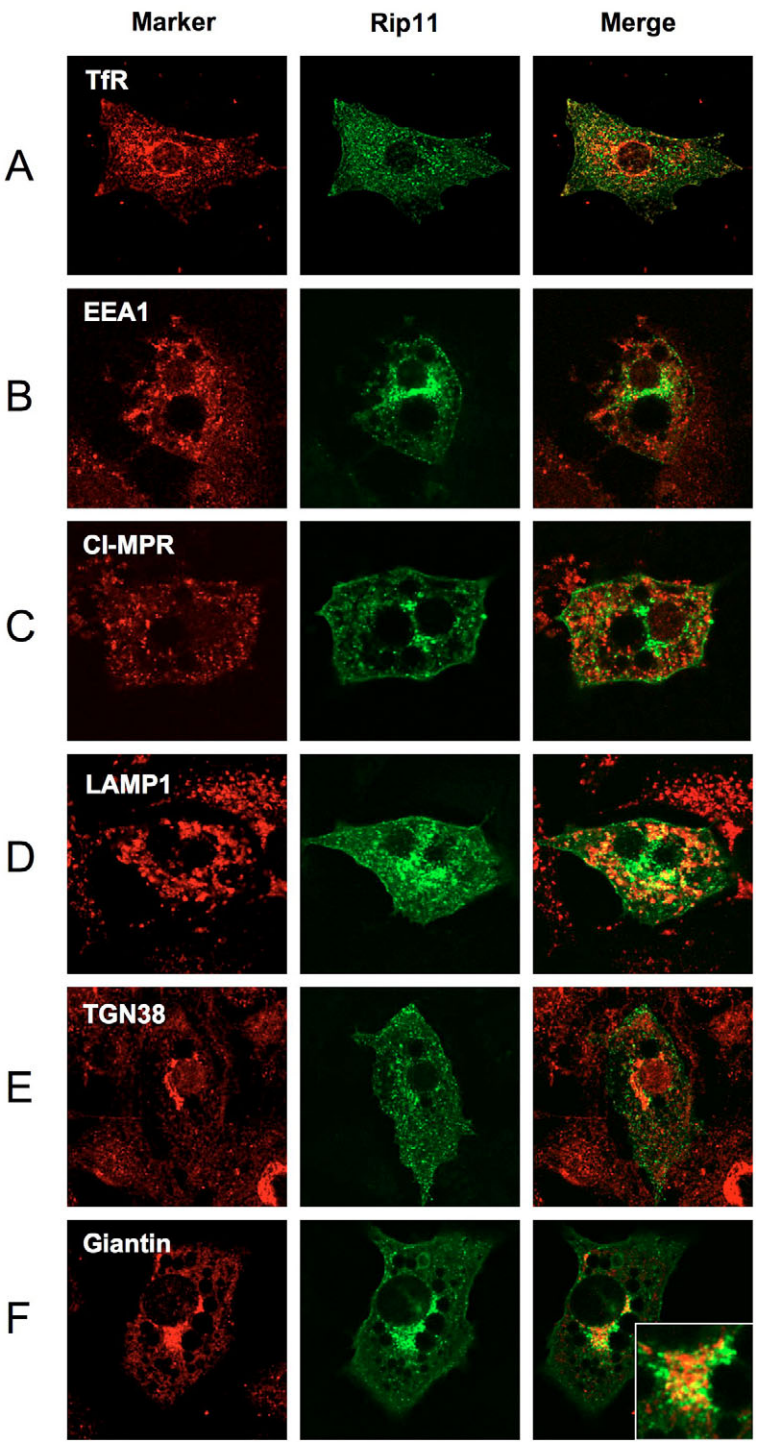

Fig. 2. Lack of apparent colocalisation of Rip11 with markers of early and recycling endosomes, lysosomes and Golgi elements. 3T3-L1 adipocytes were electroporated with plasmids encoding GFP-Rip11 and transferrin receptor (row A), or GFP-Rip11 alone (rows B-F). In row A, the cells were incubated 24 hours later for 30 minutes with transferrin-Alexa Fluor 633 and then fixed and imaged for the localisation of recycling transferrin receptors (red) or GFP-Rip11 (green). In rows B-F, the cells were fixed 24 hours after transfection, permeabilised and stained with antibodies against EEA1 (row B), the mannose 6-phosphate receptor (CI-MPR; row C), LAMP-1 (row D), TGN38 (row E) and giantin (row F). Each row comprises a single cell illustrating the distribution of the relevant marker (red) or GFP-Rip11 (green), with a merged image shown in the right-hand column.

the total cellular expression of the protein (Zeigerer et al., 2002).

HA-GLUT4-GFP was coexpressed in 3T3-L1 adipocytes with either mRFP-Rip11 or an mRFP vector control, and the insulin-stimulated exposure of the exofacial HA tag at the cell surface was measured. As can be seen in Fig. 3A, insulin promoted a tenfold increase in the exposure of GLUT4 at the cell surface, and this was completely blocked when mRFP-Rip11 was coexpressed. We investigated the underlying reason for this phenomenon by analysing the images in more detail, as the inhibitory effect of Rip11 in this assay could have been caused by the prevention of any GLUT4 vesicle recruitment (translocation), docking with the plasma membrane or the final fusion of docked vesicles with the plasma membrane. As shown in Fig. 3B, GLUT4 translocation to the plasma membrane was inhibited modestly by overexpressed mRFP-Rip11, but the major effect was to prevent those vesicles that had translocated from undergoing full incorporation into the plasma membrane such that the exofacial HA epitope on GLUT4 became exposed to the extracellular milieu. Thus the HA:GFP fluorescence ratio measured in the plasma membrane itself was substantially reduced in the presence of overexpressed Rip11 (Fig. 3Bf).

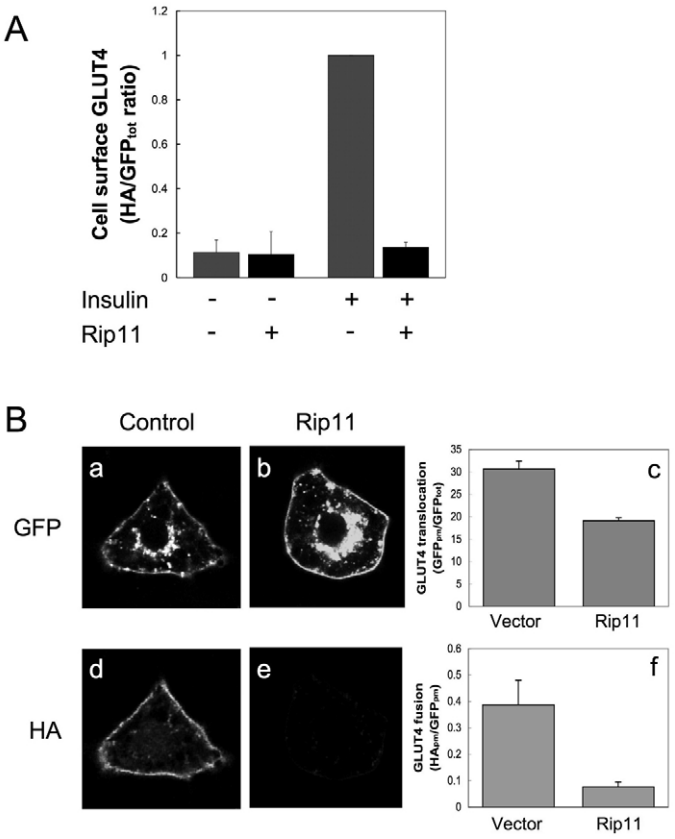

Fig. 3. Overexpression of Rip11 inhibits insulin-stimulated GLUT4 translocation to, and fusion with, the plasma membrane. (A) 3T3-L1 adipocytes were electroporated with plasmids encoding HA-GLUT4-GFP in the presence of either mRFP-Rip11 or mRFP vector, as indicated. 24 hours later, the cells were treated in the absence or presence of 100 nM insulin for 30 minutes and were then fixed and stained with an antibody against HA to detect surface-localised GLUT4. The intensity of HA antibody staining was divided by the intensity of total cellular GFP fluorescence. This provides a ratio that represents the degree of fusion of GLUT4 with the plasma membrane, corrected for the expression level of GLUT4. (B) Representative images are provided of insulin-stimulated cells from the experiment in panel A (images of additional cells can be found in supplementary material Fig. S1). The cells were analysed for the effect of Rip11 overexpression (panels b and e; where panels a and d are from cells expressing an mRFP vector control) on insulin-stimulated GLUT4 translocation, as determined by the distribution of GFP (panels a-c, where panel c is expressed as the amount of GFP fluorescence in the plasma membrane divided by total cellular GFP fluorescence) and the extent of fusion of translocated GLUT4 with the plasma membrane (panels d-f; where panel f is expressed as the intensity of HA fluorescence in the plasma membrane divided by the intensity of GFP fluorescence found at the plasma membrane) in the presence of insulin. Both panels of the figure are representative of three separate preparations of adipocytes, with the data expressed as the mean ± s.e.m.

siRNA knockdown of Rip11 inhibits insulin-stimulated glucose uptake

To examine whether there was an obligate requirement for Rip11 in insulin-stimulated glucose uptake, we used siRNA technology to ablate the protein from 3T3-L1 adipocytes. We used two separate siRNA duplexes (Rip11A and Rip11B) targeted to distinct regions of the mRNA encoding Rip11 to deplete Rip11 from these cells using electroporation. In addition, we used either a control duplex that lacks homology with any known mammalian protein (Rutherford et al., 2006) or a scrambled siRNA duplex in which the A, T, C, G content was identical to that found in each of the two Rip11 siRNA duplexes A and B.

None of the antibodies against Rip11 available to us was found to be suitable for western blotting of Rip11 in 3T3-L1 adipocytes. As a result, we examined the effect of these siRNAs on Rip11 mRNA expression by real-time quantitative RT-PCR. As shown in Fig. 4A, 48 hours of treatment with siRNA against either Rip11A or Rip11B substantially depleted the levels of Rip11 mRNA but had no significant effect on the related class I FIP-family members FIP2 or RCP. This demonstrates that the siRNA duplexes used were selective for ablation of Rip11. siRNAs against both Rip11A and Rip11B reduced insulin-stimulated, but not basal, uptake of 2-deoxyglucose (Fig. 4B). Furthermore, neither Rip11A nor Rip11B siRNAs had any effect on the expression of two different control mRNAs (encoding β2-microglobulin and cyclophilin A; data not shown) or on the apparent expression or subcellular distribution of endogenous GLUT4, as assessed by immunofluorescence (supplementary material Fig. S2). When added in combination, Rip11A and Rip11B siRNAs were significantly more effective at depleting Rip11 mRNA expression and insulin-stimulated, but again not basal, 2-deoxyglucose uptake, the effect being dose dependent (Fig. 5A,B).

Taken together, the data suggest that there is an obligate requirement for Rip11 for insulin-stimulated glucose uptake into adipocytes. Approximately 40% of intracellular GLUT4 colocalises with transferrin receptors in the recycling endosomal compartment (Martin et al., 1996). Insulin also stimulates translocation of the transferrin receptor to the plasma membrane, although to a much lesser extent than observed with GLUT4. Thus, as GLUT4 translocation to the plasma

membrane could also involve GLUT4 trafficking from or through the recycling endosomal system, we examined the effect of Rip11 knockdown on insulin-stimulated transferrin receptor translocation to the cell surface. To do this, we measured cell-surface binding of ^{125}I-labelled transferrin in 3T3-L1 adipocytes previously electroporated with either scrambled or Rip11 siRNA duplexes (Fig. 5C). The effect of insulin on translocation of the transferrin receptor was reduced by the electroporation process to 1.2-fold, from 1.7-fold, in non-electroporated cells (data not shown). Despite this, there was no apparent effect of Rip11 knockdown on insulin-stimulated ^{125}I-labelled transferrin binding. The data suggest that Rip11 regulates GLUT4 trafficking directly to the plasma membrane from the GSV compartment, rather than from or through recycling endosomes. GLUT1 colocalises with transferrin receptors in recycling endosomes and translocates to the plasma membrane in response to insulin. Our data, therefore, further suggest that any reduction observed in 2-deoxyglucose uptake is unlikely to be due to a reduction in GLUT1 translocation to the plasma membrane. This is consistent with the lack of any apparent effect of Rip11 knockdown on basal 2-deoxyglucose uptake, which is predominantly due to GLUT1 localised to the plasma membrane.

Rip11 interacts with AS160 in a Rab-independent manner

The fact that Rip11 overexpression and depletion both appear to inhibit the fusion of docked GLUT4 vesicles with the plasma

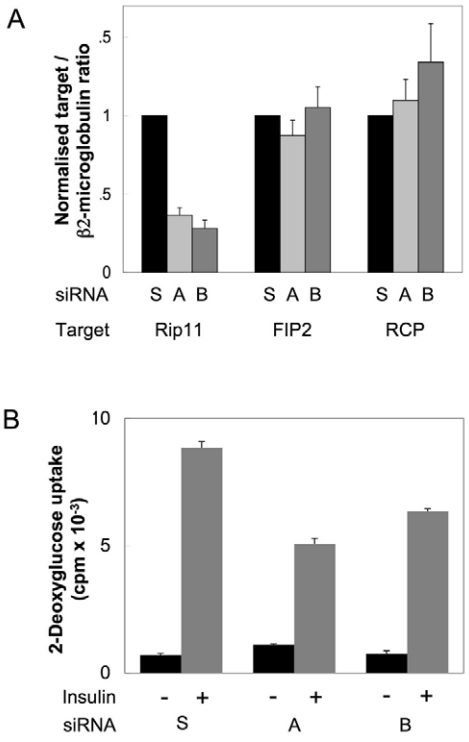

Fig. 4. siRNA-mediated knockdown of Rip11 reduces Rip11 but not FIP2 or RCP mRNA expression and inhibits insulin-stimulated uptake of 2-deoxyglucose. 3T3-L1 adipocytes were electroporated with scrambled siRNA duplex (S) or two different siRNA duplexes directed towards distinct regions of the Rip11 mRNA sequence (A and B, respectively). (A) The level of expression of Rip11, FIP2 and RCP mRNA was measured 48 hours later using quantitative real-time PCR. The level of expression of each mRNA was determined relative to the level of expression of β2-microglobulin mRNA, and, for each mRNA, this was normalised to the level of expression in the presence of the scrambled mRNA (therefore, equal to 1 in each case). Each bar is representative of determinations arising from five independent preparations of adipocytes, with the data expressed as the mean ± s.e.m. (B) In a parallel experiment, the adipocytes were treated in the absence or presence of 100 nM insulin, as indicated, and 2-deoxyglucose uptake measured in triplicate, as described in Materials and Methods. The panel is representative of two separate experiments, with each bar indicating the mean ± s.d.

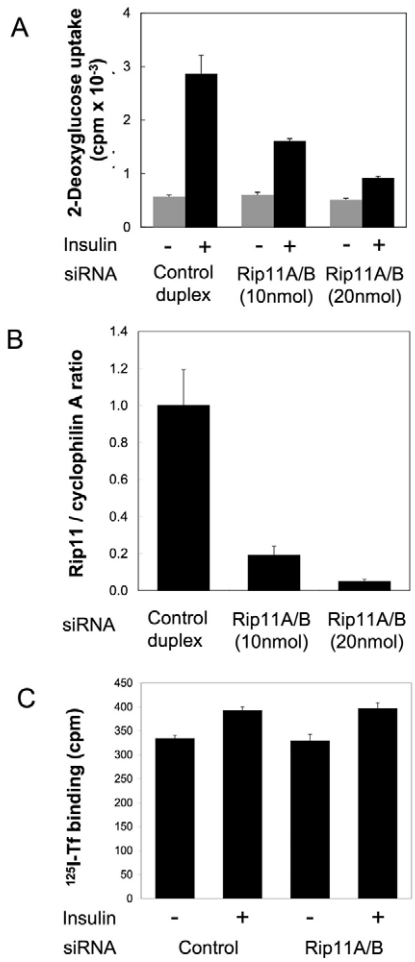

Fig. 5. A combination of Rip11 siRNA duplexes blocks insulin-stimulated uptake of 2-deoxyglucose but not transferrin binding by 3T3-L1 adipocytes. 3T3-L1 adipocytes were electroporated with control siRNA duplex or a combination of Rip11A and Rip11B siRNA duplexes (Rip11A/B) at two different concentrations directed to deplete Rip11. The cells were left for 48 hours before measuring: (A): uptake of 2-deoxyglucose in the absence or presence of 100 nM insulin. (B) The level of expression of Rip11 mRNA, which was measured using quantitative real-time PCR with the data shown relative to the level of expression of mRNA encoding cyclophilin A, which was itself unchanged by the Rip11 siRNA. (C) ^{125}I-labelled transferrin binding in the absence or presence of 100 nM insulin. Each panel is representative of three separate preparations of adipocytes, with the data expressed as the mean ± s.e.m.

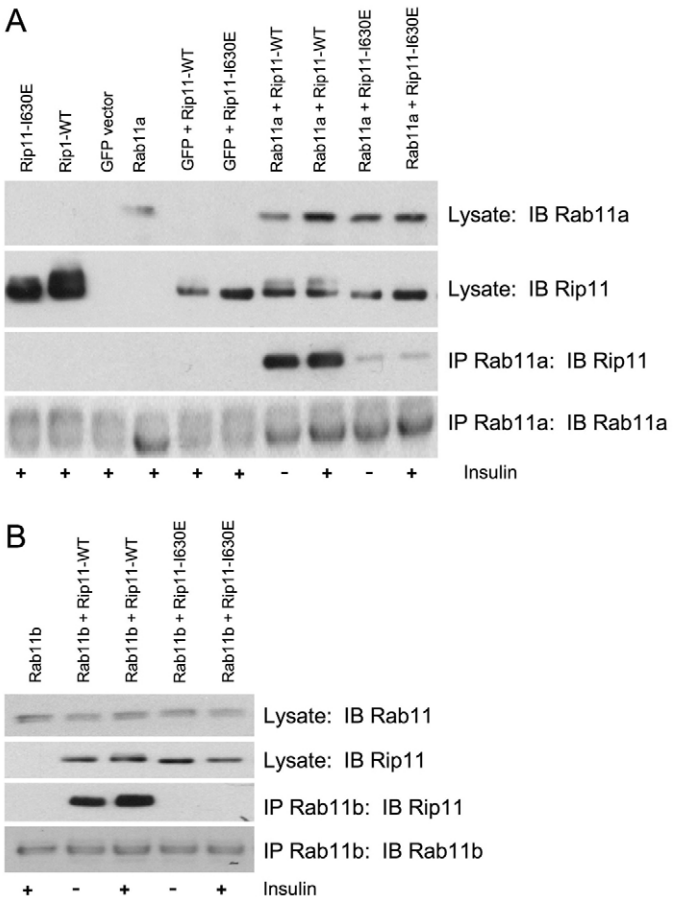

Fig. 6. The I630E mutation in Rip11 blocks Rab11a and Rab11b binding. (A) CHO.T cells were transfected with plasmids encoding GFP-tagged Rab11a or GFP-vector, along with plasmids encoding either Xpress-tagged wild-type Rip11 (Rip11-WT) or Rip11-I630E, as indicated. The cells were incubated in the absence or presence of 100 nM insulin for 10 minutes, as shown, before lysis. Cell lysates were blotted with antibodies against GFP (lysate: IB Rab11a) or antibodies against Xpress (lysate: IB Rip11) to assess the level of expression of these proteins. Alternatively, Rab11a was immunoprecipitated using antibody against GFP, and the complexes were blotted with antibody against Xpress (IP Rab11a: IB Rip11) or against GFP (IP Rab11a: IB Rab11a) to detect the presence of Xpress-tagged Rip11 and GFP-tagged Rab11a in the precipitates, respectively. (B) A similar experiment to panel A was performed, except that the Rab11a expression plasmid was replaced with an equivalent plasmid expressing Rab11b.

membrane and glucose uptake, respectively, is consistent with Rip11 acting as a scaffolding protein that complexes multiple signalling and/or trafficking intermediates that control the fusion event. Overexpression of Rip11 would be expected to titrate out these intermediates into separate complexes, thus inhibiting translocation. Conversely, depletion of Rip11 would limit the amount of the complex available for fusion to proceed. Membrane fusion events frequently require the presence of members of the Rab family of GTP-binding proteins. For example, Rab11 has been demonstrated to be important for insulin-stimulated glucose uptake in cardiomyocytes (Kessler et al., 2000; Uhlig et al., 2005) and adipocytes (Kong et al., 2006; Zeigerer et al., 2002). We confirmed that Rip11 interacted with both Rab11a and Rab11b, two highly related Rab proteins that differ mainly at their C-terminal 20 amino acids, and that the extent of the interaction with both was not affected by insulin (Fig. 6). Consistent with previous observations (Junutula et al., 2004; Lindsay and McCaffrey, 2004a), a single I630E point mutation in the Rab-binding domain (RBD) of Rip11 substantially blocked the interaction of Rip11 with Rab11a (Fig. 6A) and completely prevented its interaction with Rab11b (Fig. 6B).

Rab proteins exist in active GTP-bound or inactive GDP-bound states. The rate of hydrolysis of the GTP in the active complex, by the GTPase activity intrinsic to the Rab, is substantially enhanced by the binding of Rab protein GAPs. One such RabGAP reported to be important for insulin-stimulated GLUT4 translocation is AS160, and so we next explored whether Rip11 interacts with the AS160 RabGAP. To do this, we coexpressed an Xpress-tagged Rip11 (wild-type or I630E mutant) with a FLAG-tagged AS160. The transfection efficiency was too low to undertake these experiments in 3T3-L1 adipocytes, so we used insulin-responsive CHO.T cells instead. As shown in Fig. 7A, wild-type AS160 did indeed selectively co-immunoprecipitate with Rip11. Insulin has been reported to increase the amount of Rab11 associated with intracellular GLUT4 vesicles (Kessler et al., 2000). By contrast, while the interaction between Rip11 and Rab11 apparently was unaffected by insulin, we found that the interaction of AS160 with Rip11 was reduced by insulin (Fig. 7). Also, while the I630E mutation in the Rip11 RBD that essentially abrogated Rab11 binding caused a small reduction in the amount of AS160 associated with Rip11, the ability of insulin to promote dissociation of AS160 and Rip11 was largely unaffected (Fig. 7B). This suggests that the interaction of AS160 with Rip11 is predominantly independent of Rab11 (or any other Rab protein that binds to Rip11 by means of Ile630) but reduced by insulin.

AS160 is phosphorylated on up to six serine residues in response to insulin stimulation by means of the activation of PKB/Akt (Sano et al., 2003). One or more of these sites can be recognised by an antibody (PAS) that binds to AS160, but only when phosphorylated in response to insulin. Use of this antibody allowed us to show that, despite the reduction in the amount of AS160 that was associated with Rip11, the AS160 remaining present in the complex was still phosphorylated in response to insulin (Fig. 8). As expected, this phosphorylation was absent in a mutant AS160 lacking four of the six PKB phosphorylation sites (data not shown).

Consistent with previous reports (Prekeris et al., 2001), we found that the I630E mutation reduced the localisation of Rip11 in vesicular structures (S.E.L. and J.M.T., unpublished), although it did not completely obliterate it perhaps because of the presence of the lipid-binding C2 domain. In a minority of cells, the I630E mutation caused a small increase in Rip11 binding to the plasma membrane in the basal state. However, the Rip11[I630E] mutant still exhibited a pronounced translocation to the plasma membrane in response to insulin, suggesting that the translocation event was largely independent of Rab11 (S.E.L. and J.M.T., unpublished). Interestingly, the Rip11[I630E] mutant inhibited the translocation of GLUT4 to the plasma membrane, albeit to a slightly lesser extent than wild-type Rip11 (Fig. 9). Taken together, the data suggest that

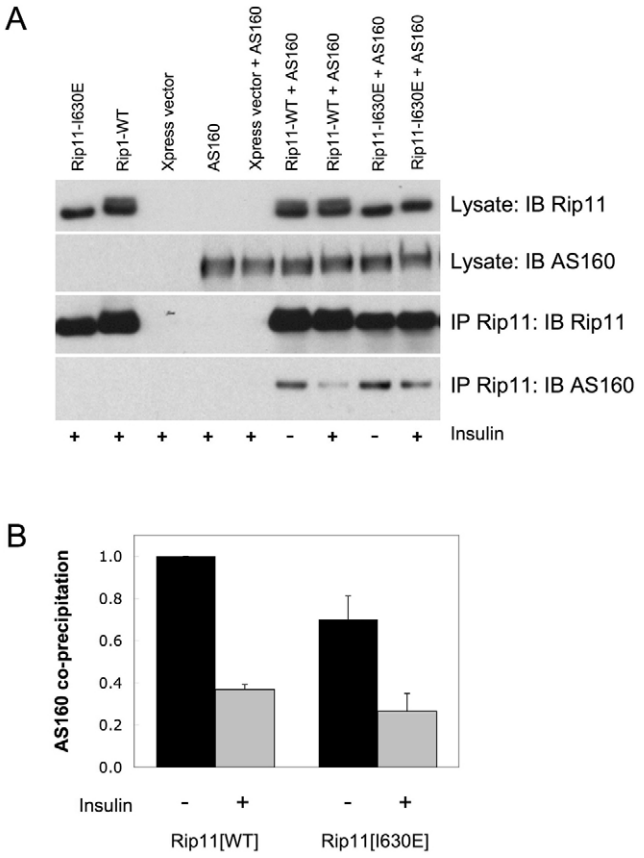

Fig. 7. AS160 interacts with Rip11. (A) CHO.T cells were transfected with plasmids encoding FLAG-tagged wild-type AS160 (AS160-WT), along with either Xpress-tagged wild-type Rip11 (Rip11-WT) or Rip11-I630E, or the Xpress vector, as indicated. The cells were incubated in the absence or presence of 100 nM insulin for 10 minutes, as shown, before lysis. Cell lysates were blotted with antibodies against Xpress (lysate: IB Rip11) or against FLAG (lysate: IB AS160) to assess the level of expression of these proteins. Alternatively, Rip11 complexes were immunoprecipitated using the antibody against the Xpress tag, and these complexes were blotted with antibodies against Xpress (IP Rip11: IB Rip11) or FLAG (IP Rip11: IB AS160) to detect the presence of Xpress-tagged Rip11 and FLAG-tagged AS160 in the precipitates, respectively. (B) The extent of the interaction of the wild-type (WT) and I630E mutant Rip11 with AS160 was quantitated by densitometry and is plotted as the mean ± s.e.m. for four separate preparations of adipocytes.

Rab11 is not important in the ability of Rip11 to translocate to the plasma membrane and that the ability of overexpressed Rip11 to inhibit GLUT4 translocation is independent of its ability to sequester Rab11a or Rab11b.

The data suggest that Rip11 is a central component of a complex containing Rab11a or Rab11b and the AS160 RabGAP, although our data suggest that Rab11 is probably not a key component of the complex that regulates the incorporation of GLUT4 vesicles into the plasma membrane. In addition, the AS160 within the complex can be phosphorylated in a regulated manner by an insulin-dependent protein kinase, which is highly likely to be PKB/Akt. Further studies are required to confirm that Rip11, Rab11 (or any other associated Rab) and AS160 form a trimeric complex.

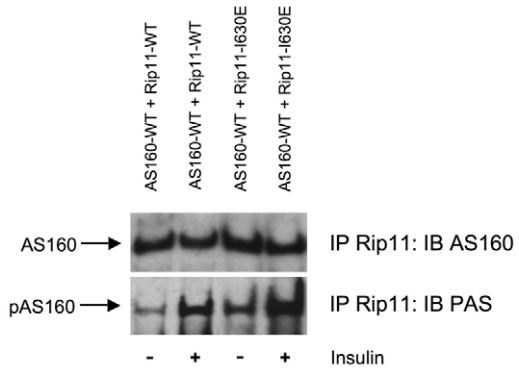

Fig. 8. AS160 becomes phosphorylated in the Rip11 complex in response to insulin. CHO.T cells were cotransfected with plasmids encoding FLAG-tagged wild-type AS160 (AS160-WT) together with either Xpress-tagged wild-type Rip11 (Rip11-WT) or Rip11-I630E, as shown. The cells were incubated in the absence or presence of 100 nM insulin for 10 minutes, as indicated, before lysis and immunoprecipitation of Rip11 complexes with antibodies against Xpress. These complexes were blotted with the antibody against FLAG (top panel) or the antibody against PAS, which recognises the PKB phosphorylation consensus sequence(s) present on AS160 (lower panel).

Discussion

In this study, we propose that Rip11 is an important component of the signalling and trafficking machinery that mediates insulin-stimulated glucose uptake. Overexpression or ablation of Rip11 in 3T3-L1 adipocytes substantially inhibits insulin-stimulated GLUT4 translocation and glucose uptake, respectively. The major site of action of Rip11 appears to be at the level of the fusion of GLUT4 vesicles with the plasma membrane, although an additional role in the recruitment of GLUT4 storage vesicles to the membrane cannot be discounted as this was partially inhibited by overexpression of Rip11. The

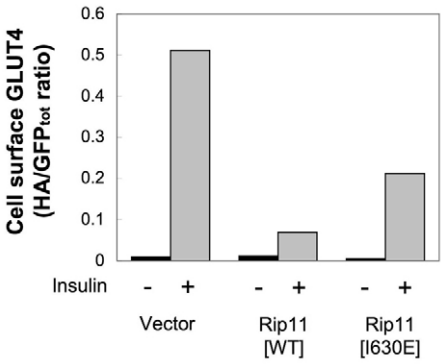

Fig. 9. A Rip11[I630E] mutant retains the ability to inhibit the insulin-stimulated appearance of GLUT4 at the cell surface. 3T3-L1 adipocytes were electroporated with plasmids encoding HA-GLUT4-GFP in the presence of either mRFP vector, mRFP-Rip11 or mRFP-Rip11[I630E], as indicated. 24 hours later, the cells were treated in the absence or presence of 100 nM insulin for 30 minutes and were then fixed and stained with an antibody against HA to detect surface-localised GLUT4. The intensity of HA antibody staining was divided by the intensity of total cellular GFP fluorescence, giving an estimate of the surface appearance of the HA-tagged GLUT4. The data shown are representative of two independent experiments.

fact that translocation of the transferrin receptor was unaffected by Rip11 knockdown is suggestive of only the GLUT4 resident in the highly insulin-responsive GSV pool being regulated by Rip11. This would be consistent with previous studies that suggested that class I Rab11-FIPs function along a pathway distinct from the recycling pathway containing the transferrin receptor (Lindsay and McCaffrey, 2004b).

Our data suggest that Rip11 must be expressed within a narrow window of levels to ensure an optimal insulin response, a characteristic expected of scaffolding proteins. Consistent with this hypothesis, we subsequently found that Rip11 not only bound to Rab11a and Rab11b, as expected, but also to the AS160 RabGAP in a Rab11-independent manner, although whether these proteins form a trimeric complex remains to be established. Furthermore, we found that the AS160 within the complex could be phosphorylated in response to insulin, a process that is thought to inhibit AS160 RabGAP activity (Kane et al., 2002; Sano et al., 2003). The study provides a significant advance in our understanding of the regulation of GLUT4 trafficking and raises a number of important questions that require further investigation.

Rip11 exhibits a partial colocalisation with GLUT4 in the basal state in both the perinuclear region and in some more-peripheral intracellular vesicles and translocates to the plasma membrane in response to insulin. Rip11 translocation, like that of GLUT4, is promoted by a constitutively active mutant of PKB, is dependent on PI 3-kinase activity and does not occur in response to PDGF. These characteristics are very similar to those exhibited by GLUT4 itself. It is possible, therefore, that the compartment in which GLUT4 and Rip11 colocalise specifically represents the insulin-responsive GSV pool that has, to date, not been isolated in a homogeneous form. Rip11 might, therefore, be a potential marker by which such vesicles could be isolated and characterised. However, Rip11 is also expressed in compartments devoid of GLUT4. This suggests that Rip11 might have an important role in other trafficking events, perhaps in combination with RCP or FIP2 that do not translocate to the plasma membrane in response to insulin. Of further interest is the recent demonstration that the class II Rab11-FIP, Rab11-FIP3, is important in maintaining the structural integrity of the endosomal recycling compartment (Horgan et al., 2007). Thus, the possibility that other members of the class I and class II FIP proteins play an additional role(s) in other GLUT4 trafficking steps, while beyond the scope of this current work, requires very detailed future examination.

The insulin-stimulated appearance of GLUT4 at the plasma membrane from intracellular stores involves at least three main processes: (1) recruitment of GLUT4 vesicles to the plasma membrane (‘translocation’); (2) docking/tethering of translocated vesicles with the plasma membrane; and (3) direct and complete fusion of the GLUT4 vesicles with the plasma membrane (at which point the HA tag on the HA-GLUT4-GFP construct used in our study finally becomes visible to the extracellular milieu). At which trafficking step(s) the incoming insulin signal regulates the overall process is not yet fully understood.

Overexpression of Rip11 blocks the appearance of GLUT4 at the cell surface, as determined by staining fixed, but not permeabilised, cells with antibodies against HA to detect the exofacial HA tag on GLUT4. This suggests that Rip11 overexpression perturbs the fusion of docked/tethered GLUT4 vesicles with the plasma membrane (Fig. 3Bf), although an additional inhibitory effect on the initiation of translocation cannot be ruled out (as observed in Fig. 3Bc). Absolute confirmation that the main effect of Rip11 overexpression is on the fusion of docked vesicles with the plasma membrane would require further analysis, perhaps by using total internal reflection fluorescence (TIRF) microscopy imaging. However, the phenotype we observe is similar to that found when the exocyst complex is inhibited by overexpressing a mutant Exo70, which also translocates to the plasma membrane in response to insulin and forms a complex containing Sec6 and Sec8 (Inoue et al., 2003). Interestingly, FIP3 and FIP4, members of the class II Rab11-FIP proteins, interact with Exo70 within the exocyst complex, and this is proposed to couple Rab11-positive vesicles to the cleavage furrow during cytokinesis (Fielding et al., 2005). Furthermore, Rip11 has been reported to interact with γSNAP, a component of the SNARE complex (Chen et al., 2001). These observations are of significant interest because Rab proteins are well established to play an important modulatory role in the SNARE-mediated fusion complex that controls several homotypic and heterotypic membrane fusion events (Stamnes et al., 1998). As a result, the relationship between Rip11 complexes and the exocyst and SNARE complexes certainly warrants further investigation.

Of the 65 or more Rab proteins, the only currently known Rab binding partners for Rip11 are the Rab11-family isoforms (Prekeris et al., 2000). Rip11 does not appear to bind to any other Rab GTPases so far tested, including Rab2a, Rab8a and Rab10 (B.L.-L., F. Barr and J.M.T., unpublished) that are found on GLUT4 vesicles (Larance et al., 2005; Miinea et al., 2005). Rab11a colocalises with GLUT4 in the perinuclear region of 3T3-L1 adipocytes but does not visibly translocate to the plasma membrane in response to insulin (G.I.W., S.E.L. and J.M.T., unpublished). Furthermore, overexpression of the Rip11[I630E] mutant, which binds to the Rab11a and Rab11b isoforms poorly, inhibits the appearance of GLUT4 at the cell surface in response to insulin almost as well as wild-type Rip11 (Fig. 9B). This suggests that Rab11 is not an important component of the regulatory complex; however, we cannot discount the possibility that Rab11 plays a role in initiation of translocation because we do observe a small reduction in GLUT4 movement towards the plasma membrane upon overexpression of Rip11 (Fig. 3Bc). This would be consistent with the reported role for Rab11 in GLUT4 translocation in cardiomyocytes (Kessler et al., 2000; Uhlig et al., 2005) and fat cells (Kong et al., 2006; Zeigerer et al., 2002). It remains possible, therefore, that Rip11 binds to an as-yet-unidentified Rab protein independently of Ile630, and which is key to the fusion of GLUT4 vesicles with the plasma membrane. Clearly, future work must focus on identifying and confirming the relevant Rab(s) complexed to Rip11 within the various GLUT4 compartments.

Our data demonstrate that Rip11 interacts with the AS160 RabGAP and that this interaction is unaffected by an I630E mutation in Rip11 that blocks the binding of Rab11a or Rab11b. The identity of the Rab(s) that is a substrate for AS160 and which is relevant for GLUT4 translocation is not yet known. Our data do not prove that Rab11 is necessarily important as the I630E mutation in Rip11 might be expected to block the interaction of any other Rab with Rip11, although

this remains to be proven. Furthermore, Rab11a and Rab11b are apparently not substrates for a recombinant truncated GAP domain derived from AS160 in in vitro GAP assays, under conditions where GTP hydrolysis on Rab2a, Rab8a, Rab10 and Rab14 was accelerated (Miinea et al., 2005). However, we would not exclude Rab11a/Rab11b being substrates for the AS160 RabGAP in intact cells. Indeed, Rab11a/Rab11b might only become efficient substrates for AS160 when in complex with Rip11, perhaps because Rip11 induces a conformational change in either AS160 or Rab11a/Rab11b, or because they simply become juxtapositioned and so the effective available concentration of Rab11a/Rab11b is increased. This important possibility requires further exploration.

Insulin stimulates the phosphorylation of AS160 that is complexed with Rip11 and at the same time inhibits the association of AS160 with Rip11. Such a reduction in association is consistent with the fact that insulin reduces the amount of AS160 associated with GLUT4 vesicles (Larance et al., 2005) and that AS160 does not translocate to the plasma membrane with Rip11. This suggests, therefore, that PKB/Akt-directed AS160 phosphorylation in the Rip11 complex might inhibit the GAP activity of AS160 towards the bound Rab and that dissociation of AS160 could additionally contribute to the reduced activity towards the Rab-GTP found in the complex.

An important emerging concept in Rab biology is that these proteins target to specific subcellular compartments through protein-protein and protein-lipid interactions. AS160 has been reported to bind to the cytosolic C-terminus of IRAP (Larance et al., 2005; Peck et al., 2006). The importance of this interaction in targeting AS160 to GLUT4 vesicles remains to be established, especially as mice that lack IRAP through homozygous deletion still retain normal glucose tolerance and insulin-stimulated glucose uptake into fat cells, despite significantly lower expression levels of GLUT4 (Keller et al., 2002). Our data suggest that AS160 might target to GLUT4 vesicles and juxtaposition with its cognate Rab, through an interaction with Rip11, perhaps in combination with IRAP (see Fig. 10 for a model).

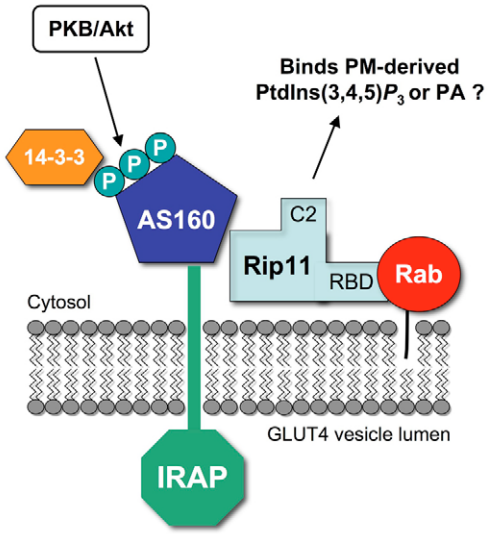

Fig. 10. Model for the role of the Rip11-AS160 complex in insulin-stimulated GLUT4 translocation and fusion with the plasma membrane. See text for further details. IRAP, insulin-responsive aminopeptidase; PA, phosphatidic acid; PM, plasma membrane; RBD, Rab-binding domain.

Rip11 contains a C2 domain that has been shown in vitro to bind preferentially to 3-phosphorylated phosphoinositide lipids, including PtdIns(3,4,5)P_3, as well as to phosphatidic acid that is produced in response to the activation of phospholipase D (PLD), and to assist in targeting the protein to membranes (Lindsay and McCaffrey, 2004b; Prekeris et al., 2000). PtdIns(3,4,5)P_3 is a key phosphoinositide lipid that is generated exclusively in the plasma membrane in insulin-stimulated cells by PI 3-kinase (Oatey et al., 1999), and PLD has been reported to be important for insulin-stimulated glucose uptake (Chen et al., 2002; Sajan et al., 2002). It is tempting to speculate that insulin-induced plasma-membrane-derived PtdIns(3,4,5)P_3 acts as a receptor for the Rip11 C2 domain and so is, in part, central to the mechanism by which GLUT4 vesicles recognise and fuse specifically with the plasma membrane. Intriguingly, this would imply that PtdIns(3,4,5)P_3 acts as both a receptor for the incoming GLUT4 vesicle as well as a mediator of the activation of PKB/Akt, a central player in GLUT4 translocation.

Insulin-stimulated translocation of GLUT4 requires one or more PI-3-kinase-dependent steps that might, in turn, involve the targeting of PI 3-kinase (and PKB/Akt) to the GLUT4 vesicle. This is an event that is not recapitulated by PDGF, which is reported to activate PI 3-kinase to the same extent as insulin but not to stimulate glucose transport (Chen et al., 2003; Ducluzeau et al., 2002; Heller-Harrison et al., 1996; Navé et al., 1996; Ricort et al., 1996). Furthermore, insulin stimulates the production of plasma membrane PtdIns(3,4,5)P_3 to a much greater extent than PDGF (Oatey et al., 1999). That Rip11 does not translocate in response to PDGF stimulation is consistent, therefore, with a potential role for Rip11 in insulin-stimulated glucose uptake and in a role for the C2 domain in binding to PtdIns(3,4,5)P_3 in the plasma membrane.

Neither RCP nor FIP2 translocates to the plasma membrane in response to insulin despite being highly related to Rip11, although all three class I FIP proteins translocate in A431 cells in response to EGF (Lindsay and McCaffrey, 2004b). This suggests that other, as-yet-unidentified, determinants for membrane targeting specificity are important. Of particular interest is the presence of a discrete 23-residue insert present in the C2 domain of Rip11, but not that of RCP or FIP2. The possibility that this confers plasma membrane binding selectivity upon Rip11 in 3T3-L1 adipocytes requires further investigation.

In conclusion, our work suggests a key scaffolding role for Rip11 in insulin-stimulated glucose uptake. The work not only enhances our understanding of insulin action but also adds to the growing field showing the importance of the Rab proteins and their effectors in regulating many disparate trafficking events, including, for example, cargo sorting in polarised epithelial cells. The recent identification of the gene encoding the AS160-related TBC1D1 protein as a candidate gene associated with severe obesity (Stone et al., 2006), and the possibility that small-molecule inhibitors of the interaction between Rip11 and AS160 might be expected to stimulate Rab-GTP loading and so promote GLUT4 translocation in the absence of insulin, means that furthering our understanding of the role and regulation of the Rip11 complex might be vital if

we are to unravel the molecular basis that underlies the pathology of diabetes, a major metabolic disease of unmet medical need.

Materials and Methods

Cells and reagents

The murine 3T3-L1 fibroblast clone was obtained from ATCC (CL-173; Manassas, VA). The antibody against FLAG was from Invitrogen (Paisley, UK), the antibody against HA was from Covance (Berkeley, USA) and the antibody against GFP was from Roche Applied Science (Burgess Hill, UK). Except where otherwise stated, all other chemicals, biochemicals and tissue-culture reagents were from Sigma (Poole, Dorset, UK) or GIBCO BRL (Paisley, UK). siRNA was obtained from Dharmacon (Lafayette, CO).

Plasmids

The GFP-Rip11 plasmid and method of construction of the I630E mutant has been described previously by us (Lindsay and McCaffrey, 2004a). In short, it comprises an *Eco*RI fragment of the Rip11 cDNA cloned into the *Eco*RI site of pEGFP-C1 (Clontech, Mountain View, CA). In order to tag Rip11 at the N-terminus with mRFP, the Rip11 cDNA was excised from GFP-Rip11 using *Eco*RI and then cloned in frame into the *Eco*RI site of pmRFP-C1. The latter plasmid was kindly provided by Jez Carlton (University of Bristol, UK) using an mRFP plasmid provided by Roger Tsien (University of California, CA), and essentially replaces EGFP in the pEGFP-C1 plasmid with mRFP.

Cell culture, adipocyte differentiation and electroporation

3T3-L1 fibroblasts were grown and differentiated into adipocytes as described previously (Oatey et al., 1997). Five days after starting differentiation, cells were electroporated with 45 μg of plasmid DNA at 180 V and 950 μF, as described previously (Zeigerer et al., 2002), and then incubated in DMEM containing 10% (v/v) myoclone-plus foetal calf serum for 16-24 hours. The cells were serum starved for 2 hours before any further manipulations. Where appropriate, 100 nM insulin or 50 ng/ml PDGF was added for 30 minutes before fixation. For the inhibition of PI 3-kinase, 100 nM wortmannin was added to the cells for 30 minutes at 37°C before stimulation with insulin.

For siRNA-mediated knockdown experiments, differentiated 3T3-L1 adipocytes were electroporated, as described previously (Mitra et al., 2004), and then plated in either six-well plates (glucose-uptake assays) or 6 cm dishes (RNA extraction). Electroporation was with either 10 nmol or 20 nmol of Rip11 siRNA [a combination of equal amounts of Rip11A and Rip11B oligonucleotides, TCATCGGCGTGGACAAGTT (bp 456-474) and TGAGCGCCAGCATGTTTGA (bp 624-643), respectively] or with 20 nmol of a control siRNA duplex (GACAAGAACCAGAACGCCA) that has no homology to any known sequence (Rutherford et al., 2006). Alternatively, 20 nmol of either Rip11A or Rip11B were used individually along with a control scrambled siRNA duplex (GTCAGTTCGGAGATCGCTA) that comprised an A, T, C, G composition identical to that of the Rip11A and Rip11B oligonucleotides. RNA was extracted on the same day as the glucose-uptake assays using TRI reagent (Sigma, Poole, UK) according to the manufacturer's protocol. Freshly isolated RNA was reverse transcribed using M-MLV RT enzyme and oligo dT (both from Promega, Southampton, UK), according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed using SYBR Green (Finnzymes, distributed by NEB) as the method of detection in an Opticon 2.0 Real-time PCR machine (BioRad, Hemel Hempstead, UK). A 250 bp amplicon for Rip11 was generated using forward primer TGAGGGTCCTGAAAGGGGGCT and reverse primer CCTGCTTCCAGACAAACCCAC. Amplicons were similarly amplified for RCP (forward GCTGCCTCCGCTGCTGTCCTC and reverse TGCTGGCCGTCATGTTATTCCTCA) and FIP2 (forward TTTAGGGCAGGTGGCAATCAATC and reverse AACACCCCGTCGCTTTTCCTC). Each sample was run in triplicate using the target primers, together with primers against cyclophilin A (forward TATCTGCACTGCCAAGACTGA and reverse CCACAATGCTCATGCCTTCTTTCA) or β2-microglobulin (forward ATTCACCCCCACTGAGACTG and reverse TGCTATTTCTTTCTGCGTGC) as control housekeeping mRNA sequences, to which the data were normalised. The amount of the target mRNA was determined using the comparative CT method, which uses the formula $2^{-\Delta\Delta CT}$ (where C_T is the threshold cycle). 2-deoxyglucose uptake was assayed as described previously (Fletcher et al., 2000).

Cell transfection and co-immunoprecipitations

CHO.T cells were transfected using Genejuice (Merck Biosciences, Nottingham, UK), according to the manufacturer's instructions. After 24 hours, the cells were serum-starved for 16 hours, treated with insulin (87 nM) for the time period indicated in the figure legends, and washed twice in ice-cold phosphate-buffered saline before extraction by scraping into 500 μl of ice-cold Nonidet P-40 extraction buffer (50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 120 mM NaCl, 50 mM NaF, 1 mM benzamidine, 1% NP40, 1 μM microcystin, 7.2 mM 2-mercaptoethanol, 5 mM orthovanadate and 1 μg/ml each of pepstatin, leupeptin and antipain). Cell extracts were centrifuged at 10,000 ***g*** for 10 minutes at 4°C and the supernatant was taken for immunoprecipitation.

Proteins were immunoprecipitated by rotating 400 μl of total cell extract with the anti-Xpress antibody and 20 μl of protein-G-sepharose (50%, w/v) at 4°C. The protein-G-sepharose beads were isolated by centrifugation and washed three times with NP40 extraction buffer. Sepharose-bound proteins were resuspended in 40 μl of SDS sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose.

Confocal microscopy, immunofluorescence staining, image and statistical analysis

Laser-scanning confocal microscopy of fixed cells was performed as described previously (Fletcher et al., 2000). Visualisation of GFP was achieved by excitation with a 488 nm laser and collection of fluorescence using a 500-530 nm emission window. Calculation of the amount of the Rab11-FIP proteins expressed at the plasma membrane was achieved using Metamorph (Universal Imaging, West Chester, PA) by drawing a region of interest around the 'outer' and 'inner' faces of the plasma membrane and determining the total integrated fluorescence intensities enclosed by each of these regions (I_{outer} and I_{inner}, respectively). The amount of GFP-Rab11-FIP in the plasma membrane was taken as $100(I_{outer}–I_{inner})/I_{outer}$, as previously described by us (Foran et al., 1999).

In some immunofluorescence staining experiments, GFP-tagged Rip11 was expressed in 3T3-L1 adipocytes by electroporation, as described above. 24 hours later, the cells were fixed, blocked for 1 hour in PBS, 0.1% Triton X-100 and 3% BSA (PTB buffer) and incubated for 45 minutes with antibodies as follows: Anti-TGN38 (a gift of G. Banting, University of Bristol, UK) at 1:250 in PTB buffer; anti-mannose-6-phosphate receptor (CI-MPR; Serotec) at 1:100 in PTB buffer; anti-EEA1 (BD Biosciences) at 1:50 in PTB buffer; anti-LAMP-1 (a gift of A. Toye, University of Bristol, UK) at 1:200 in PBS, 0.1% saponin and 3% BSA. The cells were washed three times in PBS and then incubated for 45 minutes in a 1:1000 dilution of goat anti-mouse IgG labelled with Alexa Fluor 568 nm (Molecular Probes) in PBS with 3% BSA. The cells were washed three times in PBS and mounted in mowiol. For the localisation of transferrin receptors, 3T3-L1 adipocytes were electroporated with 45 μg of GFP-Rip11 and 45 μg of human transferrin receptor. The following day, the cells were incubated for 1 hour in serum-free DMEM supplemented with 0.1% BSA. 50 μg/ml of transferrin-Alexa Fluor 633 (Molecular Probes) was then added for 30 minutes, the cells washed with PBS, fixed and imaged for the presence of GFP and Alexa Fluor 633.

Trafficking assays

The insulin-stimulated redistribution of GLUT4 to the plasma membrane was examined using the HA-GLUT4-GFP fusion protein (kindly provided by T.E. McGraw). Adipocytes were electroporated as above. 24 hours after electroporation, cells were incubated in serum-free DMEM for 4 hours. Insulin (83 nM) was added to the required dishes for 30 minutes. Cells were fixed in 4% paraformaldehyde and the appearance of the HA epitope was detected in non-permeabilised cells by immunostaining with 10 μg/ml antibody against HA followed by incubation with Alexa Fluor 633 goat anti-mouse IgG (Molecular Probes, Oregon). Confocal microscopy of the fixed cells was performed using a Leica SP2 confocal microscope. All images were acquired using a 60× numerical aperture oil-immersion objective. Cells expressing the HA-GLUT4-GFP construct were identified based on their GFP fluorescence. Images of the HA-GLUT4-GFP-expressing cells were collected for both the 633 nm and GFP channels. The laser intensity settings remained constant throughout the experiment. Approximately 30-50 cells per condition were collected in each experiment. Metamorph image-processing software was used for quantification. The average 633 nm and GFP intensity per pixel were determined within each cell expressing HA-GLUT4-GFP. Each cell was corrected for background by subtracting the average 633 nm and GFP fluorescence in cells that did not express HA-GLUT4-GFP.

To determine the surface-to-total distribution of GLUT4, that is the relative amount of GLUT4 incorporated into the plasma membrane, the 633:GFP ratio was calculated for each cell to normalise the expression level of the HA-GLUT4-GFP construct, and the average 633:GFP ratio was determined for each condition. To determine the efficiency with which GLUT4 present at the plasma membrane becomes fused with the plasma membrane, the 633:GFP ratio was calculated for the plasma membrane region only, by defining the plasma membrane as the region of interest. To determine the amount of GLUT4 translocation based on GFP fluorescence alone, the same region of interest used to define the fusion efficiency of GLUT4 was used to calculate the ratio of plasma membrane GFP fluorescence to total GFP fluorescence.

Insulin-stimulated transferrin receptor translocation was measured by determining ^{125}I-labelled transferrin binding to the cell surface of adipocytes. In brief, this involved electroporating 3T3-L1 adipocytes with siRNA duplexes A and B, as described above, which were then seeded in six-well tissue-culture plates. The cells were allowed to attach for 30 hours and were then serum starved for 8 hours before stimulation with or without 87 nM insulin for 15 minutes. The cells were washed once with ice-cold working buffer (DMEM supplemented with 20 mM HEPES, pH 7.5), and then incubated with 83 ng/ml ^{125}I-labelled transferrin (1.85 kBq/well) in working buffer for 20 minutes on ice to allow binding to transferrin

receptors at the plasma membrane. The cells were washed three times with ice-cold working buffer and then lysed in 1 M NaOH. Radioactivity associated with the cell lysates was determined using a gamma-counter.

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