Protein kinase B phosphorylation of PIKfyve regulates the trafficking of GLUT4 vesicles

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Summary

Insulin-stimulated glucose uptake involves the recruitment of the glucose transporter 4 isoform (GLUT4) from an intracellular location to the plasma membrane of fat and muscle cells. Although the activation of the PI3-kinase/protein kinase B (PKB) pathway is central to this effect of insulin, the key substrates for PKB that are involved require identification. Here we report that serine318 on the FYVE domain-containing PtdIns(3)*P* 5-kinase (PIKfyve) is a novel substrate for PKB, and show that phosphorylation stimulates the PtdIns(3)*P* 5-kinase activity of the enzyme. We also demonstrate that PIKfyve is phosphorylated on serine318 in intact cells in response to insulin, in a PI3-kinase-dependent manner, and that PIKfyve colocalises with a highly motile subpopulation of insulin-regulated aminopeptidase (IRAP)/GLUT4 vesicles. Finally, we demonstrate that overexpression of a PIKfyve[S318A] mutant in 3T3-L1 adipocytes enhances insulin-stimulated IRAP/GLUT4 vesicle translocation to the plasma membrane suggesting a role for PKB-dependent phosphorylation of PIKfyve in insulin-regulated IRAP/GLUT4 trafficking. The phosphorylation and activation of PIKfyve by PKB provides a novel signalling paradigm that may link plasma membrane-localised PtdIns(3,4,5)P_3 signals via a protein kinase cascade to regulated PtdIns(3,5)P_2 production, and thereby to the control of trafficking of other membrane cargos.

Supplementary material available online at
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Key words: Insulin, Signalling, Glucose transport, Protein kinase B, Phosphoinositide

Introduction

Regulated trafficking of cargo between intracellular compartments is central to the control of many cellular processes including receptor signalling, endocytosis, degradation and down-modulation, as well as hormone secretion and metabolite uptake.

Insulin, for example, stimulates the uptake of glucose into muscle and adipose cells by promoting the translocation of the insulin-responsive isoform of glucose transporter, GLUT4, from intracellular vesicles to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). In the basal state, a dynamic trafficking process ensures that the bulk of GLUT4 is sequestered into intracellular vesicles resulting in a low level of GLUT4 at the cell surface. Insulin stimulates GLUT4 translocation to the plasma membrane predominantly by releasing GLUT4 from this specialised intracellular pool resulting in a pronounced increase in glucose uptake (Bryant et al., 2002; Holman and Sandoval, 2001).

The signalling mechanism employed by insulin to mobilise intracellular GLUT4 has not been fully defined. Class Ia phosphoinositide 3-kinases (PI3-kinases) play a central role, as demonstrated by the ability of wortmannin and dominant-negative mutants of PI3-kinase to inhibit GLUT4 translocation and glucose uptake (Clarke et al., 1994; Hara et al., 1994; Okada et al., 1994). Additional PI3-kinase-independent signalling pathways may also be involved including the activation of the GTP-binding protein, TC10 (Saltiel and Pessin, 2002).

PI3-kinase almost certainly acts to promote GLUT4 translocation through the ability of its lipid product, PtdIns(3,4,5)P_3, to stimulate the activation of protein kinase B (PKB or Akt) (Cong et al., 1997; Foran et al., 1999; Kohn et al., 1996; Tanti et al., 1997; Wang et al., 1999). To identify the PKB substrates involved in insulin action on GLUT4 translocation in adipocytes, we have taken a proteomic approach using a commercially available antibody (PAS) raised against the minimal PKB consensus phosphorylation site found on almost all of its known substrates, RXRXX(pS/pT) (a so-called 'phospho-motif') (Alessi et al., 1996; Berwick and Tavare, 2004; Obata et al., 2000). This approach has been used previously using a variety of cell types and has led to the identification of ATP-citrate lyase (ACL) (Berwick et al., 2002), PRAS40 (Kovacina et al., 2003) and AS160 (a Rab-GAP) (Kane et al., 2002) as novel PKB substrates. Interestingly, a mutant AS160 lacking the PKB phosphorylation sites is reported to inhibit the ability of insulin to mobilise GLUT4 to the plasma membrane and so this

protein may be a key component of the signalling mechanism downstream of PKB (Sano et al., 2003).

In the current study we purified a protein with an apparent molecular weight of 230,000 Da that was recognised by the PAS antibody, the phosphorylation of which was stimulated by insulin. We identified this protein as the PtdIns(3)*P* 5-kinase, PIKfyve, and the phosphorylation site as serine318. We further demonstrate that the phosphorylation of this protein by PKB stimulates the lipid kinase activity of PIKfyve in vitro, and that serine318 phosphorylation plays an important role in insulin-regulated IRAP/GLUT4 vesicle trafficking.

Materials and Methods

Materials

Except where otherwise stated, all chemicals were from BDH (Lutterworth, Leicester, UK), and biochemicals from Sigma (Poole, Dorset, UK). Adenosine 5′-[γ-^{32}P]trisphosphate was from ICN Radiochemicals (Basingstoke, UK). Plasmids containing constitutively active myristoylated PKB (Myr-PKB) and constitutively active p110 subunit of PI3-kinase (p110.CAAX) were kindly provided by B. Hemmings (Friedrich Miescher Institute, Basel) and J. Downward (Cancer Research UK, London), respectively. Male Wistar rats (150-200 g) were fed ad libitum on a stock laboratory diet (CRM, Cambridge, UK). Adipocytes from the epididymal fat pad were prepared and treated with insulin as described (Berwick et al., 2002). The phosphospecific antibody (PAS) raised against the minimal PKB consensus phosphorylation site was from Cell Signaling Technology (Beverly, MA, USA).

Protein sub-fractionation

Lysates from primary adipocytes, prepared and clarified as described (Berwick et al., 2002), were loaded onto a Pharmacia SMART system Mono-Q column PC 1.6/5 previously equilibrated in buffer A (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM DTT, 0.5 mM mM phenylmethylsuphonyl fluoride and 1 μg/ml each of pepstatin, antipain, leupeptin). A linear gradient of buffer A up to 1 M NaCl was developed over 2.5 ml at a flow rate of 50 μl/minute and 100 μl fractions were collected. Following western blotting with the PAS antibody, Mono-Q fractions containing potential substrate proteins were applied to a Pharmacia SMART system Superose 12 column equilibrated in buffer A containing 200 mM NaCl. Samples were eluted in this buffer. From one of these fractions we noted a M_r 230,000 protein, reactive with the PAS antibody in an insulin-stimulated and wortmannin-sensitive manner, and which was well separated from other proteins as determined by Coomassie Blue staining. The protein was excised from the gel and digested using sequencing grade porcine modified trypsin (Promega). The peptides produced were analysed by MALDI-TOF using a PE Biosystems Voyager-DE STR MALDI-TOF mass spectrometer, and the peptide masses searched against the NCBInr database using the ProFound website (Zhang and Chait, 2000).

Cell culture and transfection

CHO.T cells were cultured in Ham's Nutrient Medium F-12 (Invitrogen, Paisley, UK) as previously described (Ebina et al., 1985). 3T3-L1 pre-adipocytes were purchased from the American Type Culture Collection, grown as fibroblasts in DMEM (Sigma, Poole, UK) and were differentiated into adipocytes as detailed previously (Oatey et al., 1997). CHO.T cells were transfected with 15 μg DNA per 10 cm culture dish) using Fugene-6 reagent (Roche, Lewes, UK) according to the manufacturer's instructions (cells were ~30% confluent at the time of transfection). The cells were left for 24 hours before serum-starvation for 24 hours and then treatment with insulin and/or inhibitors.

Plasmids

A plasmid encoding murine PIKfyve tagged at the N-terminus with GFP was a gift of Peter Cullen (University of Bristol). Two different point mutations were constructed using the wild-type PIKfyve cDNA as template, with the Quikchange system (Stratagene, Amsterdam, Holland) following the manufacturer's protocol: (i) PIKfyve[S318A] and (ii) PIKfyve[R192A] in which phosphoinositide binding to the FYVE domain was predicted to be eliminated.

Antibody production

Rabbit polyclonal antibodies were raised to the N-terminus of PIKfyve (MATDDKSSPYLC; antibody anti-PIKfyve) or to a peptide containing phosphoserine318 from PIKfyve (CRNRSApSIT; antibody anti-pS318). The cysteine residues were used to allow crosslinking to the antibody purification column. Peptide synthesis and antibody production were carried out by Graham Bloomberg (University of Bristol, Bristol, UK). Polyclonal antisera were affinity purified using columns containing the appropriate peptide immunogen (generated using a SulfoLink kit; Pierce, Rockford, IL, USA). Antisera were incubated batch-wise with the coupled resin and then purified according to the manufacturer's instructions. In the case of the phosphospecific antibody, the antiserum was first incubated overnight with the non-phosphorylated peptide, in order to 'block' the non-phosphospecific antibodies from interacting with the affinity resin.

Western blotting

Cells were harvested in ice-cold RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.2, 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) containing protease and phosphatase inhibitors (1 μM microcystin-LR, 7.2 mM β-mercaptoethanol, 2 mM sodium orthovanadate and 1 μg/ml each of pepstatin, antipain and leupeptin). The lysates were clarified by centrifugation and the supernatants were subjected to SDS-PAGE (5-10% gradient gels) prior to electrotransfer onto Immobilon-P membrane (Millipore, Watford, UK). Membranes were blocked using 10% bovine serum albumin in TBS-T [10 mM Tris pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20] before being probed with 1:250 dilution of anti-PIKfyve or anti-pS318 in 5% (w/v) BSA in TBS-T.

In vitro phosphorylation of GST-PIKfyve by PKB

GST-PIKfyve was expressed in *Saccharomyces cerevisiae* and purified as described previously (McEwen et al., 1999). Briefly, GST fusion proteins of either PIKfyve or PIKfyve S-A were expressed in a *Δmig1* strain under the control of the *GAL-10* UAS. Yeast cells were cultured in synthetic complete medium without uracil (SDUra-1) at either 24°C or 30°C. A log-phase culture of yeast was diluted to 2.5×10^6 cells/ml and galactose was added to a final concentration of 2% (w/v) and protein expression was induced for 5 hours. Yeast cells were then harvested by centrifugation. All subsequent steps were carried out at 4°C. The cells were washed once in lysis buffer (50 mM Tris pH 7.6 at 24°C, 120 mM NaCl, 10 mM EDTA, 10 mM β-glycerophosphate, 10 mM benzamidine, 1 mM PMSF, 1 mM DTT), resuspended in 10 ml lysis buffer and lysed using a GlasCol bionebulizer. After lysis, Triton X100 was added to a final concentration of 1% (v/v). The lysate was cleared by centrifugation and added to 250-500 μl packed volume of glutathione-Sepharose beads (Amersham). After a 30 minute incubation with agitation, the beads were washed three times in lysis buffer with 1% Triton X100, four times with lysis buffer, and finally twice with lysis buffer with 50% (v/v) glycerol. Beads were stored at –20°C until use.

GST-PIKfyve (30 μl of bead slurry) attached to glutathione-Sepharose beads were washed three times with 1 ml PKB assay buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 1 mM EDTA and 0.1% β-mercaptoethanol). The beads were then resuspended in 50 μl PKB assay buffer supplemented with 5 mM $MgCl_2$ and 780-1000 mU recombinant PKB (Upstate, Milton Keynes, UK). The kinase reaction was initiated by adding [γ-^{32}P]ATP (50 μM; 40 kBq/mol). After 1 hour at 30°C, the reaction was terminated by addition of 12.5 μl SDS-PAGE sample buffer [100 mM Tris, pH 6.8, 3% (w/v) SDS, 38.9% (v/v) glycerol, 0.022% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol]. Samples were then analysed by SDS-PAGE (7.5% acrylamide) followed by transfer to PVDF membrane. ^{32}P incorporation was detected by PhosphorImaging using a Typhoon Imager (Amersham Biosciences, Chalfont St Giles, UK) and quantitative image analysis using ImageQuant software.

Lipid kinase assays

In vitro phosphorylation of GST-PIKfyve by PKB was performed as above, with the following exceptions: 100 μM unlabelled ATP was used in the incubations, which lasted 15 minutes and the reactions were stopped by placing on ice and removing the supernatant. The GST-PIKfyve beads were then used directly in the lipid kinase assay. Lipid vesicles were produced by resuspending 25 μg PE (Sigma) and 5.6 μg PtdIns(3)*P* (Cell Signals, Columbus, Ohio, UK) in 30 μl lipid kinase buffer (LKB; 25 mM HEPES pH 7.5, 120 mM NaCl, 5 mM β-glycerophosphate, 0.2 mM EDTA, 1 mM DTT) using sonication in a water bath. To this, $MgCl_2$ and ATP were added to final concentrations of 1.5 mM and 500 μM respectively; 10 μCi [γ-^{32}P]ATP was used per assay in a total volume of 65 μl. Reactions were incubated at 30°C for 15 minutes and stopped by the addition of excess (243 μl) 2:1 methanol:chloroform and vortexing. The lipids were then extracted by the sequential addition of 58 μl 2.4 mM HCl, 5 mM tetrabutylammonium hydrogen sulphate (TBAS) and 240 μl chloroform. The lower phase was taken and added to 237 μl of 47:48:3 1 M HCl 5 mM TBAS:methanol:chloroform (v/v/v), mixed and the lower phase taken. The extracted lipids were either dried under nitrogen or in vacuo. Following resuspension in 50 μl 2:1 chloroform:methanol, the lipids were separated by TLC. Whatman K6 silica gel 60 (Labsales, Over, UK) plates were activated by soaking in 1% oxalic acid (potassium salt), 2 mM EDTA in methanol:water (1:1) and then baked for 2 hours at 120°C. Lipids were separated using a mobile solvent of chloroform:methanol:water:25% (v/v) ammonia [90:70:16:4 (v/v/v/v)]. Following exposure of the dried TLC plates to autoradiographic film, the position of the lipid spots was marked and the lipids were scraped off the plate, collected and the radioactivity present quantified by scintillation counting.

IRAP translocation assay and live cell imaging

A fragment encoding amino acids 1-154 of murine IRAP (mIRAP) was amplified by PCR from cDNA prepared from 3T3-L1 fibroblasts. The fragment was amplified to incorporate a C-terminal HA tag and flanking *Xho*I/*Bam*HI sites. EGFP was excised from the retroviral vector pL-EGFP-C1 (Clontech) using *Age*I/*Bgl*II and the vector blunted and re-ligated to generate 'pL-Empty'. The *Xho*I-*Bam*HI IRAP-HA fragment was ligated into pL-Empty digested with the same enzymes. Retroviral packaging cells were transfected with pL-IRAP-HA using Fugene-6 transfection reagent (Roche) in medium supplemented with 25 μM chloroquine. 48 hours later, the virus-containing medium from the cells was harvested, supplemented with 6 μg/ml polybrene (Sigma), filtered through a 0.45 μm filter and applied to low-passage 3T3-L1 fibroblasts. 24 hours after infection, the 3T3-L1 fibroblasts were subject to selection with 500 μg/ml G418. After 7 days of selection, 3T3-L1 fibroblasts were tested for expression of the IRAP-HA and differentiated into adipocytes as previously described.

IRAP-HA translocation was determined in adipocytes 24 hours post microinjection and after further serum starvation for 2 hours. Cells were fixed with 4% paraformaldehyde and the appearance of the HA epitope at the cell surface was detected by immunostaining with 10 μg/ml monoclonal anti HA antibody (Berkley Antibody Co, Richmond, USA) followed by incubation with Alexa568 goat anti-mouse IgG (Molecular Probes, Oregon, USA). Confocal microscopy of the fixed cells was performed with a Leica SP2 confocal microscope. Adipocytes were classified as exhibiting HA-IRAP translocation to the plasma membrane by the visual presence of a well-defined ring of HA fluorescence around the plasma membrane as previously described (Ducluzeau et al., 2002).

3T3-L1 fibroblasts were grown on coverslips, differentiated and microinjected as described previously (Oatey et al., 1997) and imaging performed using an UltraView confocal microscope as described (Fletcher et al., 2000).

Results

Identification of PIKfyve as a protein whose phosphorylation is stimulated by insulin

Primary adipocytes isolated from rat epididymal fat pads were treated with insulin in the absence or presence of the PI3-kinase inhibitor wortmannin. The cell lysates were fractionated by MonoQ chromatography and fractions subjected to western blotting with the PAS antibody. One fraction, which contained several insulin-stimulated phosphoproteins, was subjected to further purification by size exclusion chromatography. Western blotting of these fractions with the PAS antibody, revealed a phosphoprotein of apparent molecular weight 230,000 Da that was well separated from other proteins upon Coomassie Blue staining (data not shown). This protein was excised from the gel, eluted and digested with trypsin. Analysis of tryptic peptides by mass spectrometry, and interrogation of the ProFound database (Zhang and Chait, 2000), led to the probable identification of this protein as the PtdIns(3)*P* 5-kinase, PIKfyve.

An analysis of the amino acid sequence of PIKfyve using Scansite (Yaffe et al., 2001), suggested the presence of a high stringency putative PKB phosphorylation site at serine318 located midway between the FYVE domain, which binds PtdIns(3)P and the DEP domain, which currently has no known function.

PIKfyve is phosphorylated by PKB in vitro on serine318

To determine whether PIKfyve is a substrate for PKB in vitro, we prepared recombinant PIKfyve expressed as a GST-fusion protein in *Saccharomyces cerevisiae*. This protein was purified by affinity chromatography and incubated in the presence or absence of recombinant active PKB and [γ-^{32}P]ATP. PIKfyve phosphorylation was substantially increased in the presence of active recombinant PKB under conditions where we did not observe any detectable autophosphorylation (Fig. 1A). Western blotting with the anti-pS318 antibody demonstrated that serine318 was phosphorylated by recombinant PKB (Fig. 1A). As there are no other high stringency PKB consensus sequences within PIKfyve, the data suggest that most of the ^{32}P incorporation observed was due to the phosphorylation of serine318, although this requires further confirmation.

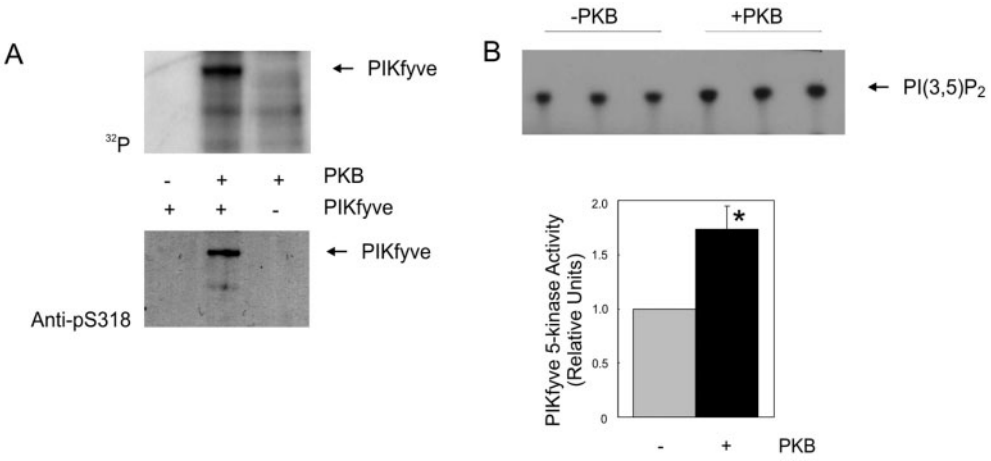

Fig. 1. Protein kinase B phosphorylates recombinant PIKfyve on serine318 and stimulates its 5-kinase activity. (A) Recombinant GST-PIKfyve fusion protein was incubated in the absence or presence of recombinant active PKB as indicated and with [γ-^{32}P]ATP. The upper panel shows an autoradiogram of the resulting gel. The lower panel shows a western blot of the same samples probed with anti-pS318 antibody. Data are representative of three separate experiments. (B) Recombinant GST-PIKfyve was incubated in the absence or presence of recombinant active [γ-^{32}P]ATP and PKB, as indicated. The activity of PIKfyve was determined using PtdIns(3)*P* as substrate according to Materials and Methods. The phosphorylated lipids were then separated by thin layer chromatography. The upper panel shows an autoradiogram of an experiment performed in triplicate. The lower panel shows data from three independent experiments. **P*<0.05 compared to activity in the control.

PIKfyve phosphorylation by PKB leads to stimulation of 5-kinase activity

To investigate whether phosphorylation of PIKfyve affected the PtdIns(3)*P* 5-kinase activity of the enzyme, we incubated recombinant GST-PIKfyve with active recombinant PKB in the presence of non-radioactive ATP. This reaction was then supplemented with PtdIns(3)*P*-containing liposomes in the presence of [γ-^{32}P]ATP and the production of PtdIns(3,5)P_2 was measured by subjecting the lipid products to thin layer chromatography. As shown in Fig. 1B, pre-incubation of PIKfyve with active PKB led to a ~1.8-fold increase in the PtdIns(3)*P* 5-kinase activity of PIKfyve.

PIKfyve serine318 is phosphorylated in intact cells in a PI3-kinase-dependent manner

Having shown that PIKfyve was phosphorylated by PKB in vitro on serine318, we next determined whether this site was phosphorylated in intact cells. In order to do this, cells transiently expressing a GFP-tagged PIKfyve fusion protein were incubated in the absence or presence of insulin and wortmannin to inhibit PI3-kinase activation. Lysates of the cells were western blotted with the anti-pS318 antibody (Fig. 2A, upper panel), or with an anti-PIKfyve antibody (Fig. 2A, lower panel) to confirm equal protein loading.

PIKfyve serine318 phosphorylation was stimulated ~2.5-fold by insulin, in a manner that was blocked by wortmannin suggesting an obligatory requirement for Class I isoforms of PI3-kinase (Fig. 2A,D). The mTOR and MEK inhibitors, rapamycin and U0126 respectively, did not affect serine318 phosphorylation (data not shown).

Mutagenesis of serine318 to an alanine completely abrogated the reactivity of the anti-pS318 antibody with PIKfyve (Fig. 2A). Coupled with the fact that the antibody exhibited no reactivity with PIKfyve prior to phosphorylation by PKB in vitro (Fig. 1A), the data confirm that the anti-pS318 antibody is highly specific for this site when it is phosphorylated. As a consequence, this means that the apparent basal phosphorylation state of serine318 is relatively high (Fig. 2A,C). Basal levels of PKB activity in cells are usually very low under serum-starved conditions, this suggests either that other kinases are also capable of phosphorylating this site or that the phosphatase(s) that operate on this site have limited access to PIKfyve resulting in a relatively low turnover of phosphate on serine318.

PIKfyve localises to PtdIns(3)*P*-containing intracellular vesicles via its N-terminal FYVE domain (Sbrissa et al., 2002). As a result, wortmannin may block PIKfyve phosphorylation by inhibiting Class II or Class III PI-kinases, which are responsible for the production of PtdIns(3)*P* on intracellular vesicles. This could promote PIKfyve dissociation from these structures making it inaccessible to the insulin-stimulated kinase responsible. To exclude this possibility we mutated arginine192 within the FYVE domain as this residue is predicted to contact the phosphoinositide head-group and so prevent localisation of PIKfyve to PtdIns(3)*P*-containing endosomes. Consistent with this, the mutant was expressed almost exclusively in the cytoplasm of the cells, as compared to the wild-type PIKfyve that was distributed between intracellular vesicles and the cytoplasm (Fig. 2B).

The GFP-PIKfyve[R192A] mutant was phosphorylated in an insulin- and wortmannin-sensitive manner, almost indistinguishable from that exhibited by the wild-type enzyme (Fig. 2C,D). This indicates that wortmannin inhibits PIKfyve phosphorylation by blocking insulin signalling, and not by causing the dissociation of PIKfyve from intracellular vesicles.

The effect of insulin on PIKfyve serine318 phosphorylation was mimicked by coexpression of constitutively active PKB (Myr.PKB) or constitutively active PI3-kinase (p110.CAAX), an upstream activator of endogenous PKB (Fig. 3). This suggests that PKB directly phosphorylates PIKfyve within cells.

PIKfyve colocalises with IRAP in a highly motile compartment

In order to examine the inter-relationship between GLUT4 and PIKfyve, we coexpressed an mRFP1-tagged insulin-responsive amino peptidase (IRAP) fusion protein with GFP-tagged PIKfyve in 3T3-L1 adipocytes. IRAP exhibits almost identical

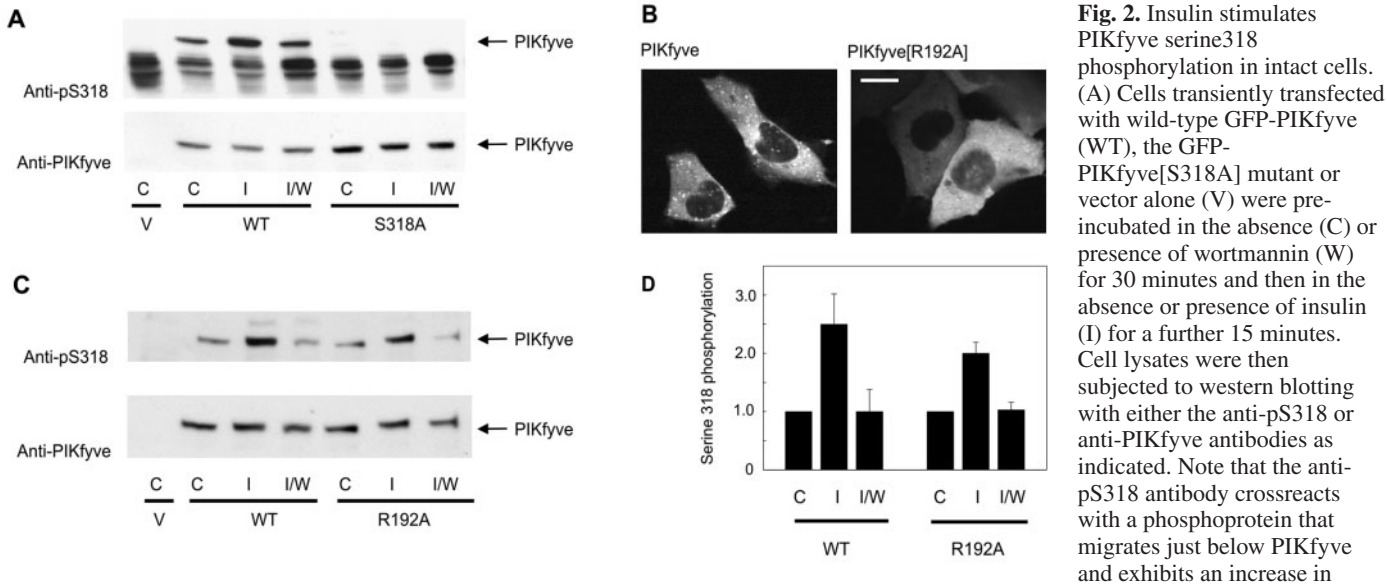

Fig. 2. Insulin stimulates PIKfyve serine318 phosphorylation in intact cells. (A) Cells transiently transfected with wild-type GFP-PIKfyve (WT), the GFP-PIKfyve[S318A] mutant or vector alone (V) were pre-incubated in the absence (C) or presence of wortmannin (W) for 30 minutes and then in the absence or presence of insulin (I) for a further 15 minutes. Cell lysates were then subjected to western blotting with either the anti-pS318 or anti-PIKfyve antibodies as indicated. Note that the anti-pS318 antibody crossreacts with a phosphoprotein that migrates just below PIKfyve and exhibits an increase in phosphorylation in the presence of wortmannin. The identity of this phosphoprotein is unknown but it is not endogenous PIKfyve as demonstrated by the lack of reactivity with an anti-PIKfyve antibody (lower panel). (B) Wild-type GFP-PIKfyve or a GFP-PIKfyve[R192A] mutant were transiently transfected into CHO.T cells. 24 hours later the cells were examined by confocal microscopy. Typical images of the distribution of these proteins are shown. Bar, 10 μm. (C) Cells were transfected with wild-type GFP-PIKfyve or the GFP-PIKfyve[R192A] mutant and treated as described in panel A for western blotting with either the anti-pS318 or anti-PIKfyve antibodies. (D) Data from at least three experiments performed according to panel C were quantified by densitometric scanning of the autoradiographs. The data are corrected for the amount of GFP-PIKfyve loaded onto the gels and are expressed relative to the appropriate control.

trafficking characteristics to GLUT4, and we chose mRFP1-IRAP because it can undertake both insulin-dependent translocation to the plasma membrane as well as re-internalisation after insulin withdrawal, unlike equivalent versions of GFP-tagged GLUT4 (Powell et al., 1999).

IRAP-mRFP1 was expressed in peripheral vesicles in 3T3-L1 adipocytes as well as in the perinuclear region that contains elements of recycling endosomes as well as the Golgi network (Fig. 4A, left panel). On the other hand, GFP-PIKfyve was expressed predominantly in peripheral vesicles (Fig. 4A, middle panel). There was little obvious colocalisation between IRAP-mRFP1 and GFP-PIKfyve (Fig. 4A, right panel), which is consistent with previous observations (Ikonomov et al., 2002). Remarkably, however, when we examined time-lapse animations of 3T3-L1 adipocytes we found that all of the highly motile IRAP-mRFP1-expressing vesicles that trafficked rapidly and in a linear fashion from the cell periphery to the perinuclear region, coexpressed GFP-PIKfyve (Fig. 4B; see Movie 1 in supplementary material). This suggests that IRAP-mRFP1 may internalise into PIKfyve-containing endosomes that are then delivered to the perinuclear region along microtubules for sorting. (Although it should be noted that some vesicles also track away from the perinuclear region.) Indeed, some IRAP-mRFP1 and GFP-PIKfyve coexpressing vesicles traffic into the heart of the perinuclear region before stalling and appearing to begin to fuse with surrounding membranous structures (see Movie 1 in supplementary material). The data suggest that these vesicles represent early endosomes that are undergoing delivery to the perinuclear region for sorting into the insulin-responsive Glut4 storage vesicle (GSV) pool, perhaps via the TGN.

PIKfyve regulates IRAP vesicle trafficking

We next examined whether the PIKfyve[S318A] mutant had any affect on the ability of insulin to stimulate IRAP translocation to the plasma membrane of 3T3-L1 adipocytes. To do this we used a 3T3-L1 adipocyte cell clone stably expressing IRAP with an exofacial HA tag. Insulin treatment of these cells caused an substantial increase in the appearance of IRAP on the cell surface, as judged by staining fixed but non-permeabilised cells with the anti-HA antibody (data not shown). Only IRAP exposed at the cell surface reacts with the anti-HA antibody.

Cells stably expressing the IRAP-HA chimera were

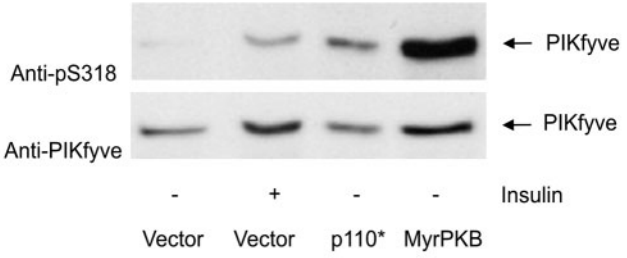

Fig. 3. PIKfyve serine318 phosphorylation is stimulated by constitutively active PKB and PtdIns 3-kinase mutants. Cells were transiently transfected with wild-type GFP-PIKfyve and vector alone, constitutively active PI3-kinase catalytic subunit (p110*) or constitutively active PKB (Myr-PKB). 48 hours later the cells were serum starved and then incubated in the absence or presence of insulin for 15 minutes, as indicated. Cell lysates were then subjected to western blotting with either the anti-pS318 or anti-PIKfyve antibodies as shown. The data are representative of three separate experiments.

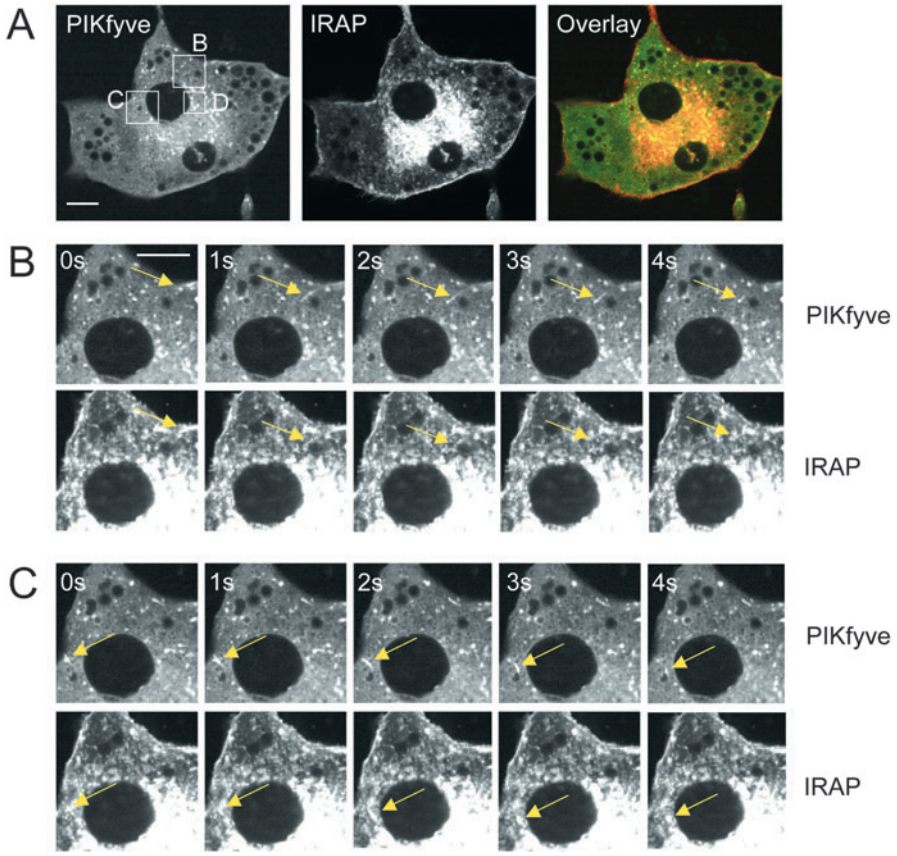

Fig. 4. IRAP and PIKfyve colocalise on highly dynamic vesicles. (A) A 3T3-L1 adipocyte, under basal conditions, coexpressing GFP-PIKfyve (left panel) and IRAP-mRFP1 (middle panel) was imaged by confocal microscopy. The right hand panel shows an overlay of the two images. Boxes B and C indicate two different GFP-PIKfyve and IRAP-mRFP1 coexpressing vesicles that are magnified and indicated with arrows in panels B and C, respectively. In Box D, another GFP-PIKfyve and IRAP-mRFP1 coexpressing vesicle enters the perinuclear region and exhibits behaviour suggestive of fusion with other membranous structures. For optimal viewing of this vesicle see the animation provided as supplementary material. (B,C) Sequential images of GFP-PIKfyve (upper panels) and IRAP-mRFP1 (lower panels) expression, 1 second apart were collected over two different time periods. This time-lapse sequence is best viewed in the accompanying supplementary material. Note that the IRAP-mRFP1 fluorescence has been enhanced to improve the resolution on peripheral vesicular structures; this results in the overexposure of fluorescence in the perinuclear region. Bars, 10 μm.

microinjected with a plasmid encoding GFP, wild-type GFP-PIKfyve or GFP-PIKfyve[S318A]. The cells were treated in the absence or presence of insulin, and the level of surface localised IRAP-HA was determined by staining fixed but non-permeabilised cells with anti-HA antibodies. The ability of insulin to stimulate the translocation of IRAP to the cell surface was similar in cells overexpressing GFP or wild-type PIKfyve, but was enhanced ~1.8-fold in the presence of the PIKfyve[S318A] mutant (Fig. 5A,B). In the absence of insulin, neither the wild type nor mutant PIKfyve had any affect on IRAP translocation (Fig. 5A).

As PIKfyve has been reported previously to play a role in trafficking of cargo from late endosomes to the lysosome, we next wanted to exclude the possibility that the mutant was increasing IRAP translocation simply because it blocked lysosomal targeting of the protein and thereby increased cellular IRAP expression levels. To do this we expressed wild-type PIKfyve or the PIKfyve[S318A] mutant in 3T3-L1 adipocytes stably expressing IRAP-HA, as shown in Fig. 5. These cells were then both fixed and permeabilised before immunostaining with anti-HA antibodies. The level of cellular fluorescence intensity was measured and found to be proportional to the level of expression of IRAP-HA. There was no difference between the level of expression of IRAP-HA in cells expressing either the wild-type PIKfyve or the PIKfyve[S318A] mutant (data not shown). Thus the PIKfyve mutant has no affect on IRAP expression levels and is, therefore, unlikely to increase IRAP cell surface expression as a result of inhibiting lysosomal targeting.

Discussion

In this study we have demonstrated that PIKfyve is a novel substrate for the insulin-stimulated protein kinase, PKB. We have further identified serine318 on PIKfyve as the site phosphorylated in vitro by PKB, and shown that this site is phosphorylated in intact cells in the presence of constitutively active mutants of either PKB or PI3-kinase, in an insulin-stimulated and PI3-kinase-dependent manner. This indicates that PKB is responsible for phosphorylating serine318 in intact cells in response to insulin. Importantly, PKB-mediated phosphorylation of PIKfyve leads to a stimulation of its PtdIns(3)*P* 5-kinase activity. Taken together, the results provide a novel mechanism by which changes in the levels of plasma membrane PtdIns(3,4,5)P_3 may regulate the levels of another phosphoinositide lipid, PtdIns(3,5)P_2, on intracellular membranes. We further propose a role for PIKfyve phosphorylation in insulin-regulated GLUT4 trafficking.

Whether phosphorylation of serine318, which is near the N-terminal PtdIns(3)*P*-binding FYVE domain, enhances the affinity of PIKfyve for PtdIns(3)*P*-containing vesicles and so increases the formation of PtdIns(3,5)P_2 indirectly, or leads to direct catalytic activation of PIKfyve per se, has not be determined. However, the data suggests that insulin-dependent phosphorylation of PIKfyve via PKB may lead to a localised increase in PtdIns(3,5)P_2 generation on the cytosolic surface of the intracellular vesicles on which PIKfyve is found (Fig. 2B and Fig. 4), and which may include a sub-population of IRAP/GLUT4 positive vesicles (see Fig. 4 and below for further discussion). EGF stimulation of COS cells has been

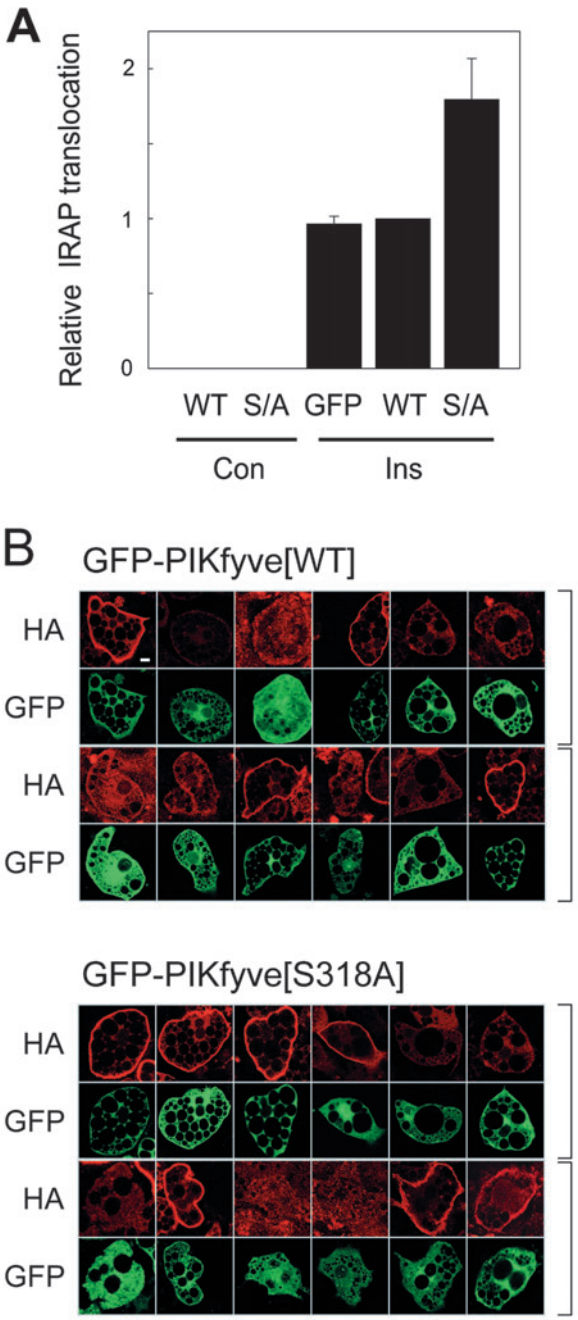

Fig. 5. The PIKfyve[S318A] mutant enhances insulin-stimulated IRAP translocation. (A) 3T3-L1 adipocytes stably expressing IRAP with an exofacial (luminal) HA tag, were microinjected with plasmids encoding GFP, wild-type GFP-PIKfyve (WT) or the PIKfyve[S318A] mutant (S318A). The cells were then treated in the absence (Con) or presence of insulin (Ins) as indicated. Cells were fixed, but not permeabilised, and stained with an anti-HA antibody that only detects surface-exposed IRAP. GFP-expressing cells exhibiting a clear surface-associated staining (i.e. a plasma membrane ring of fluorescence) were counted as having undergone IRAP translocation. In the absence of insulin, no cells were detected that exhibited surface staining. Quantitative data are provided for either two (control) or four (insulin) independent experiments. (B) Images of individual insulin-stimulated cells from one representative experiment are shown. HA-stained surface IRAP is shown in red, with the corresponding wild-type (WT, upper panel) or [S318A] mutant GFP-PIKfyve (lower panel) expression in the same cell in green. In this experiment 13 cells out of 33 wild-type GFP-PIKfyve expressing cells exhibited the translocation phenotype (39.3%). In cells expressing PIKfyve[S318A] this became 12 out of 19 (63%). Bar, 10 μm.

reported to lead to a small increase in PtdIns(3,5)P_2 formation (Tsujita et al., 2004) although it is unknown whether insulin has the same affect in adipocytes.

The data suggest that PIKfyve activity may be stimulated by insulin via a PKB-dependent phosphorylation of serine318. At first sight this would appear inconsistent with previous reports that PIKfyve was not stimulated by insulin (Sbrissa et al., 1999). However, in these experiments the PIKfyve was isolated in the absence of phosphatase inhibitors making it likely that the protein was dephosphorylated and inactivated during its isolation and assay. Thus the possibility that insulin itself regulates PIKfyve activity requires investigation.

Mammalian PIKfyve has been reported to play a role in the sorting of cargo between the late endosome and the lysosome (Ikonomov et al., 2001; Shisheva, 2001) and is a homologue of yeast Fab1p, a PtdIns(3)*P* 5-kinase important for sorting cargo from the late endosome to the multivesicular body (Cooke et al., 1998; Gary et al., 1998; Odorizzi et al., 1998). We now propose a novel phosphorylation-dependent regulatory role for mammalian PIKfyve in the trafficking of IRAP/GLUT4 in adipocytes.

PIKfyve and IRAP/GLUT4 exhibit only a very limited degree of colocalisation (Shisheva et al., 2001) (Fig. 4A). Importantly, however, close inspection by time-lapse confocal microscopy of cells coexpressing GFP-PIKfyve and IRAP-mRFP1 revealed the presence of numerous highly motile IRAP-mRFP1 vesicles that coexpressed GFP-PIKfyve (Fig. 4B and supplementary material). Many of these vesicles exhibited linear movements from the cell periphery towards the perinuclear region, a behaviour highly reminiscent of vesicle trafficking along microtubules. Indeed GLUT4, with which IRAP is known to colocalise, has been shown to traffic along microtubules and this has been demonstrated to be important for the ability of insulin to stimulate glucose uptake (Fletcher et al., 2000; Olson et al., 2001). These highly motile IRAP (and by inference GLUT4) vesicles are likely to be endosomes being delivered along microtubules for sorting in the perinuclear/trans-Golgi network (TGN) region of the cell. Consistent with this we find that some IRAP-mRFP1 and GFP-PIKfyve coexpressing vesicles enter the perinuclear region, where they appear to stall and undergo behaviour suggestive of fusion with neighbouring membrane compartments (e.g. see vesicle D in supplementary material). This suggests that PIKfyve plays a role in the dynamic sorting of IRAP and GLUT4 in adipocytes.

We found that overexpression of the PIKfyve[S318A] mutant in 3T3-L1 adipocytes enhanced insulin-stimulated, but not basal, IRAP translocation to the plasma membrane under conditions where the expression level of IRAP was not affected (Fig. 5). Thus the phosphorylation of this site on PIKfyve appears to play a role in insulin-regulated trafficking of IRAP/GLUT4. This data is, at first sight, inconsistent with observations of Shisheva and co-workers who have previously demonstrated that a kinase-inactive mutant of PIKfyve blocks

insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (Ikonomov et al., 2002). However, as this mutant promotes pronounced vacuolation of many cell types (Ikonomov et al., 2001) it is possible that the kinase-dead PIKfyve mutant redistributes GLUT4 into a non-insulin-responsive pool.

Although we have not firmly established the site at which PIKfyve acts, GLUT4 and IRAP are known to localise to numerous different tubulovesicular membrane compartments within adipocytes and muscle cells. Precisely how they traffic through these compartments, are sequestered away from the plasma membrane in the basal state and can undergo such a profound translocation to the plasma membrane upon insulin stimulation, is not fully understood despite intensive investigation. The fact that the PIKfyve[S318A] mutant enhances the ability of insulin to stimulate IRAP translocation to the plasma membrane suggests that this mutant is disrupting a key trafficking step and so increases the amount of IRAP available for translocation to the plasma membrane.

Insulin-stimulated exocytosis of GLUT4 to the plasma membrane occurs in a PKB-dependent manner and this step may be subject to control by PIKfyve. However, the fact that PIKfyve and IRAP colocalise on vesicles undergoing trafficking towards the perinuclear/TGN region, suggests that phosphorylation of serine318 by PKB plays a role in controlling an endosomal sorting step.

Trafficking of GLUT4 out of internalised endosomes and into the insulin-responsive sequestered compartment probably occurs via the TGN (Martin et al., 2000; Shewan et al., 2003; Slot et al., 1991a; Slot et al., 1991b). Sorting of GLUT4 out of endosomes via this pathway and into the sequestered pool is accelerated by insulin (Foster et al., 2001; Zeigerer et al., 2002) in a PKB- and PI3-kinase-dependent manner (Foster et al., 2001). PIKfyve phosphorylation in response to insulin may be responsible for stimulating the flux of IRAP and GLUT4 through this sorting step. Thus the PIKfyve[S318A] mutant may slow the flux of IRAP from endosomes into the TGN and thus divert the IRAP into recycling endosomes. IRAP would then traffic back to the plasma membrane but only in the presence of insulin, which is still necessary to mobilise this pool (Foran et al., 1999; Martin et al., 1996); this is consistent with our data (Fig. 5). The TGN holds a significant amount of cellular GLUT4 that does not appear to be insulin-responsive (Bao et al., 1995; Kono-Sugita et al., 1996; Slot et al., 1991b). As such, if the PIKfyve mutant slows the entry of IRAP into the TGN, then more IRAP might also be available for translocation to the plasma membrane in response to insulin. A full and complete understanding of the mode of action of the PIKfyve mutant will require a complex and detailed kinetic analysis of its effect on the flux of IRAP through these compartments.

To conclude, our data suggest that PIKfyve phosphorylation of serine318 by PKB plays an important role in IRAP/GLUT4 trafficking. Identification of PIKfyve as a substrate for PKB provides a novel signalling paradigm that may link plasma membrane-derived PtdIns(3,4,5)P_3 signals via a protein kinase signalling network to regulated PtdIns(3,5)P_2 production on intracellular vesicles. As such, this pathway may have wider implications for the regulation of vesicular sorting in response to other extracellular stimuli that control PKB activation. The degree to which this signalling cascade is involved in Ras-stimulated endocytosis (Barbieri et al., 1998) and integrin receptor recycling (Roberts et al., 2004) warrants further investigation, as both of these events have been reported to be regulated by PKB. The possible role of PIKfyve phosphorylation in GPCR internalisation and desensitisation (Luttrell and Lefkowitz, 2002), and EGF receptor uptake and degradation in the lysosome (Cozier et al., 2002; Gampel et al., 1999), conditions in which PKB is often activated, would also be of interest. Finally, the possibility that PtdIns(3,5)P_2 facilitates the budding and sorting of GLUT4 from vesicles by recruiting one of the recently identified PtdIns(3,5)P_2 binding proteins mVps24p, Ent3p or myotubularin (Friant et al., 2003; Tsujita et al., 2004; Whitley et al., 2003) is now an intriguing possibility.

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