Functional consequence of targeting protein kinase B/Akt to GLUT4 vesicles

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Summary

We have investigated the role of protein kinase B (Akt) in the insulin-stimulated translocation of vesicles containing the insulin-responsive isoform of glucose transporter (GLUT4) to the plasma membrane of adipocytes. Previous reports have suggested that protein kinase B can bind to intracellular GLUT4 vesicles in an insulin-dependent manner, but the functional consequence of this translocation is not known. In this study we have artificially targeted constitutively active and kinase-inactive mutants of protein kinase B to intracellular GLUT4 vesicles by fusing them with the N-terminus of GLUT4 itself. We examined the effect of these mutants on the insulin-dependent translocation of the insulin-responsive amino peptidase IRAP (a bona fide GLUT4-vesicle-resident protein). A kinase-inactive protein kinase B targeted to GLUT4 vesicles was an extremely effective dominant-negative inhibitor of insulin-stimulated IRAP translocation to the plasma membrane. By contrast, a kinase-inactive protein kinase B expressed in the cytoplasm did not have an effect. The results suggest that protein kinase B has an important functional role at, or in the vicinity of, GLUT4 vesicles in the insulin-dependent translocation of those vesicles to the plasma membrane of adipocytes.

Key words: Insulin, GLUT4, Signalling, Protein kinase B/Akt, Adipocytes

Introduction

A major physiological effect of insulin is the rapid stimulation of glucose uptake into its target tissues, namely muscle and fat. This occurs via the ability of insulin to promote the translocation of intracellular vesicles containing the insulin-responsive glucose transporter isoform 4 (GLUT4) to the plasma membrane (James and Piper, 1994; Kandror and Pilch, 1996; Holman and Kasuga, 1997). A detailed molecular description of how insulin promotes this translocation is presently lacking, although some of the components that are likely to be involved have been identified.

It is well established that phosphoinositide 3-kinase (PI 3-kinase) is pivotally involved in insulin-induced glucose uptake (Kanai et al., 1993; Okada et al., 1994; Hara et al., 1994), although a parallel PI 3-kinase-independent pathway, comprising CAP, Cbl, C3G and TC10, has also been proposed to play a role (Baumann et al., 2000; Chiang et al., 2001). The mechanism by which PI 3-kinase mediates the effect of insulin on GLUT4 translocation is controversial. Two protein kinases that lie downstream of PI 3-kinase have received considerable attention, namely protein kinase B (PKB) and the atypical protein kinase C-family members ζ and λ (PKCζ and PKCλ).

PKB is composed of an N-terminal pleckstrin homology domain (PH domain), a protein kinase domain and a C-terminal regulatory domain containing the phosphorylation site Ser473 (reviewed in Vanhaesebroeck and Alessi, 2000). Binding of phosphoinositide-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] to the PH domain allows recruitment of PKB from the cytoplasm to the plasma membrane, where it is phosphorylated and activated by PDK1 on Thr308 and, by an as yet ill-defined kinase, on Ser473. PKB can be rendered constitutively active by substitution of Thr308 and Ser473 for aspartic acid residues, which mimic phosphorylation [PKB[*DD*] (Alessi et al., 1996)], or by insertion of a myristoylation signal sequence from c-*src* at the N-terminus [Myr-PKB (Andjelkovic et al., 1997)]. The latter allows constitutive membrane localisation and thus phosphorylation on Thr308 and Ser473 (Andjelkovic et al., 1997).

Constitutively active mutants of PKB mimic the ability of insulin to promote glucose uptake and GLUT4 translocation in muscle and adipose cells (Kohn et al., 1996; Tanti et al., 1997), suggesting that this protein kinase may be a crucial mediator of insulin's effect on glucose transport. Studies using a kinase-inactive PKB mutant (Cong et al., 1997), in which the PDK1 and PDK2 phosphorylation sites were additionally mutated [PKB-AAA (Wang et al., 1999)], and inhibitory anti-PKB antibodies (Hill et al., 1999) were found to block insulin-stimulated glucose uptake. This is consistent with a role for PKB in mediating insulin-stimulated GLUT4 translocation. Furthermore, adipocytes isolated from diabetic rats or humans exhibited a parallel defect in insulin-stimulated glucose uptake and PKB activation and phosphorylation (Carvalho et al., 2000a; Carvalho et al., 2000b).

Equally, there is evidence against a role for PKB and this includes a report that a dominant-negative PKB failed to block insulin-stimulated glucose uptake in adipocytes under conditions where endogenous PKB activation was severely compromised (Kitamura et al., 1998). Also, we (L.M.F. and J.M.T., unpublished) (see Results) and others (Wang et al., 1999) have been unable to demonstrate inhibitory effects of a

cytosolic kinase-inactive PKB on insulin-stimulated GLUT4 translocation.

Constitutively active forms of PKCλ and PKCζ, in which the pseudo-substrate inhibitory region was deleted, promote GLUT4 translocation and glucose uptake in adipocytes in the absence of insulin (Bandyopadhyay et al., 1997; Standaert et al., 1997; Bandyopadhyay et al., 1999) (L.M.F. and J.M.T., unpublished). Furthermore, kinase-inactive mutants of PKCλ and PKCζ have been reported to block insulin-stimulated GLUT4 translocation. These data argue in favour of an additional role for the atypical PKCs in insulin action on glucose uptake.

Growth factors have been reported to induce a translocation of PKB from the cytosol to the plasma membrane and then to the nucleus (Meier et al., 1997; Goransson et al., 1998). Interestingly, insulin has also been reported to stimulate a recruitment of PKB to GLUT4-containing vesicles (Calera et al., 1998; Kupriyanova and Kandror, 1999). In the current study, therefore, we have examined the functional consequences of targeting kinase-inactive and constitutively active mutants of PKB to GLUT4 vesicles by fusion with GLUT4 itself. Our results suggest that PKB may indeed have an important functional role in GLUT4 vesicle translocation by acting at, or within the vicinity of, the GLUT4 vesicles themselves.

Materials and Methods

Cells and reagents

The murine 3T3-L1 fibroblast clone was obtained from ATCC (CL-173; Manassas, VA, USA). Tissue culture reagents were from GIBCO BRL (Paisley, Scotland) or the Sigma Chemical Co. (Dorset, UK). Mouse monoclonal anti-haemagglutinin (HA) antibodies were from Berkley Antibody Co. (Richmond, USA), mouse monoclonal anti-GFP antibodies were from Roche (East Sussex, UK) and transferrin-Alexa568 was from Molecular Probes (Oregon, USA). Unless otherwise stated, all other reagents were obtained from the Sigma Chemical Co. The GFP mutant used in this study was kindly provided by Jon Pines (Wellcome/CRC, Cambridge, UK) and has been previously described by us (Oatey et al., 1997).

Plasmids

The plasmid pIRAP-GFP comprises IRAP tagged at the C-terminus with GFP and was generated from a partial IRAP cDNA clone kindly provided by S. Keller (Dartmouth Medical School, Hanover, USA). An IRAP fragment (bases 66-533) was generated by PCR using the primers 5′-TTTTAAGCTTGCGAAGATGGAGACC-3′ and 5′-TTTTGGATCCAATCGGCTGAATGAG-3′. The resulting PCR product was cloned into the *Hin*dIII and *Bam*HI sites of the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, USA) to produce pcDNA3-IRAP. GFP was subcloned at the 3′ end of IRAP by first amplifying using PCR a GFP fragment using the primers 5′-TTTTGGATCCAGTAAAGGAGAAGAAGAA-3′ and 5′-TTTTCT-CGAGTTACCTCAGGTCCTCCTCCGAGATCAGCTTCTGCATTT-TGTATAGTTCATC-3′; this incorporates a Myc-epitope tag at the 3′end of the GFP moiety, and *Bam*HI and *Xho*I sites at the 5′ and 3′ ends of the PCR product, respectively. This PCR fragment was then subcloned into the *Bam*HI and *Xho*I sites of pcDNA3-IRAP. The GFP moiety in the resulting plasmid essentially replaces the lumenal amino peptidase domain of IRAP.

Plasmids (pCMV5) containing (i) wild-type PKB (PKB-WT), (ii) constitutively active PKB[Thr308Asp/Ser473Asp] (PKB[*DD*]), (iii) constitutively active myristoylated PKB (Myr-PKB), (iv) kinase-inactive PKB[Lys179Ala] (PKB[*KD*]) and (v) PKB lacking the PH-domain (PKB[*ΔPH*]; i.e. a deletion of amino acid residues Met1–Ala117) were kindly provided by D. Alessi (University of Dundee) and B. Hemmings (Friedrich Miescher Institute, Basel). All the PKB sequences included an N-terminal HA-tag. A kinase-inactive mutant of Myr-PKB (Myr-PKB[*KD*]) and a kinase-inactive PKB lacking the PH domain (ΔPH-PKB[*KD*]) were constructed by site-directed mutagenesis (thus introducing a K179A substitution) using the QuikChange™ site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA) and the plasmids Myr-PKB or PKB[*ΔPH*] described above as templates, respectively.

PKB mutants were fused to the N-terminus of GLUT4 as follows. PKB[*KD*]-GLUT4 and PKB[*DD*]-GLUT4 were constructed by PCR amplification of fragments of pPKB[*KD*] and pPKB[*DD*], respectively, between bases 550 and 1638 using the primers 5′-TTTTAAGCTTGACGAGATGTATCCTTACGACGTCCCCGACTA-CGCCAGTCTGATGGACTTCCGGTCG-3′ and 5′-TTTTGGATCC-GGCCGTGCTGCTGGC-3′; an N-terminal HA-tag is thus introduced into each construct. The resulting PCR product was cloned into the *Hin*dIII and *Bam*HI restriction sites of the plasmid pGFP-GLUT4 (Dobson et al., 1996), such that the PKB sequence replaces the GFP sequence at the 5′ end of full-length GLUT4 in the plasmid pcDNAI/Neo. Thus the PKB sequences within PKB[*KD*]-GLUT4 and PKB[*DD*]-GLUT4 lack the PH domain.

The human FKHR sequence (bases 386-2353) was amplified by PCR from HEK293 cDNA using the primers 5′-TTTT-CTCGAGATGGCCGAGGCGCCTCAGGTG-3′ and 5′-TTTTGT-CGACTCAGCCTGACACCCAGCTATG-3′ and was cloned into pGEM-TEasy (Promega). Att recombination sites were subsequently added by PCR at the 5′ and 3′ extremities of the FKHR cDNA and the product cloned downstream of EGFP (Clontech) in pCI-Neo using the Gateway™ Cloning System (Life Technologies).

Cell culture, adipocyte differentiation and microinjection

3T3-L1 fibroblasts were grown on coverslips, differentiated into adipocytes and microinjected with plasmid DNA as previously described (Oatey et al., 1997). Plasmids were injected at 30 to 150 μg/ml, and then the cells were 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 prior to any further manipulations. Where appropriate, 100 nM insulin was added for 30 minutes prior to fixation.

Immunofluorescence analysis

Cells were fixed and permeabilised using 4% paraformaldehyde and 1% Triton X-100 in phosphate-buffered saline (PBS), respectively. For IRAP detection, the cells were incubated for 45 minutes with a 1:500 dilution of anti-IRAP antiserum (a gift from S. Keller, University of Dartmouth, New Hampshire, USA) and then for 30 minutes with TRITC-conjugated anti-rabbit IgG. All antibody dilutions were in 3% bovine serum albumin in PBS. The presence of PKB expression was detected using 10 μg/ml of monoclonal anti-HA antibody (Berkley Antibody Co. Richmond, USA) followed by incubation with TRITC-conjugated anti-mouse IgG as described previously (Foran et al., 1999). For transferrin-Alexa568 uptake assays, the cells were serum starved and placed in medium containing 20 μg/ml transferrin-Alexa568 for 1 hour at 37°C and then fixed in 4% paraformaldehyde for subsequent immunofluorescence analysis as described above.

Confocal microscopy, image and statistical analysis

Laser-scanning confocal microscopy of fixed cells was performed with a Leica SP2 inverted Confocal Imaging Spectrophotometer controlled with Leica Confocal Software (Leica, Heidelberg,

Germany). Visualisation of GFP was achieved by excitation with a 488 nm laser and collection of fluorescence using a 500-530 nm emission window. Detection of TRITC-conjugated antibody staining was achieved by excitation with a 543 nm laser, and collection of emitted fluorescence was achieved by using a 570-620 nm window. Adipocytes were classified as exhibiting IRAP-GFP translocation to the plasma membrane by the visual presence of a well defined ring of GFP fluorescence around the plasma membrane as previously described (Foran et al., 1999; Thurmond and Pessin, 2000). The results were validated by double-blind analysis by two independent workers and are expressed as mean±s.d. of the percentage of insulin-responsive cells (i.e. the proportion of cells that displayed a translocated phenotype) in three independent experiments, with each data point comprising a minimum of 50 cells. In some experiments further quantification was achieved using Metamorph 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 IRAP-GFP in the plasma membrane was taken as $100(I_{outer}–I_{inner})/I_{outer}$ as previously described by us (Foran et al., 1999). For quantification of FKHR translocation, the fluorescence intensities were calculated within a region of interest drawn around the entire cell and another inside the nuclear envelope ($I_{nuclear}$ and $I_{cellular}$, respectively). The amount of FKHR in the nucleus, as a percentage of the total, was taken as $100(I_{nuclear}/I_{cellular})$.

Results

Subcellular localisation of targeted PKB mutants expressed in 3T3-L1 adipocytes

Wild-type PKB (PKB[*WT*]) and the kinase-inactive PKB mutant (PKB[*KD*]) transiently expressed in 3T3-L1 adipocytes by microinjection were expressed predominantly within the cytosol (Fig. 1A,B respectively). The constitutively active PKB[*DD*] mutant was expressed within the plasma membrane and is concentrated in a region that immediately underlies the plasma membrane (Fig. 1C). This region of 3T3-L1 adipocytes is rich in cortical actin (Oatey et al., 1997), and it is of interest that Hannigan and co-workers have recently found that PKB colocalises with F-actin at the leading edge of chemotactic neutrophils (Hannigan et al., 2002). The constitutively active PKB mutant containing an N-terminal myristoylation signal sequence (Myr-PKB) was expressed both within the plasma membrane of the adipocyte and also extensively in intracellular vesicles (Fig. 1D).

Placing a 27 kDa green fluorescent protein (GFP) tag at the N-terminus of GLUT4 appears not to perturb the distribution or trafficking of GLUT4 relative to that expected of the native protein (Oatey et al., 1997; Fletcher et al., 2000). By contrast, placing GFP at the C-terminus of GLUT4 causes mistargeting, at least in some cells (L.M.F. and J.M.T., unpublished). We reasoned, therefore, that in order to target PKB mutants to GLUT4 vesicles we should place the PKB moiety at the N-terminus of GLUT4. Furthermore, the PH domain was removed from the PKB moiety to prevent inadvertent targeting of the construct to the plasma membrane.

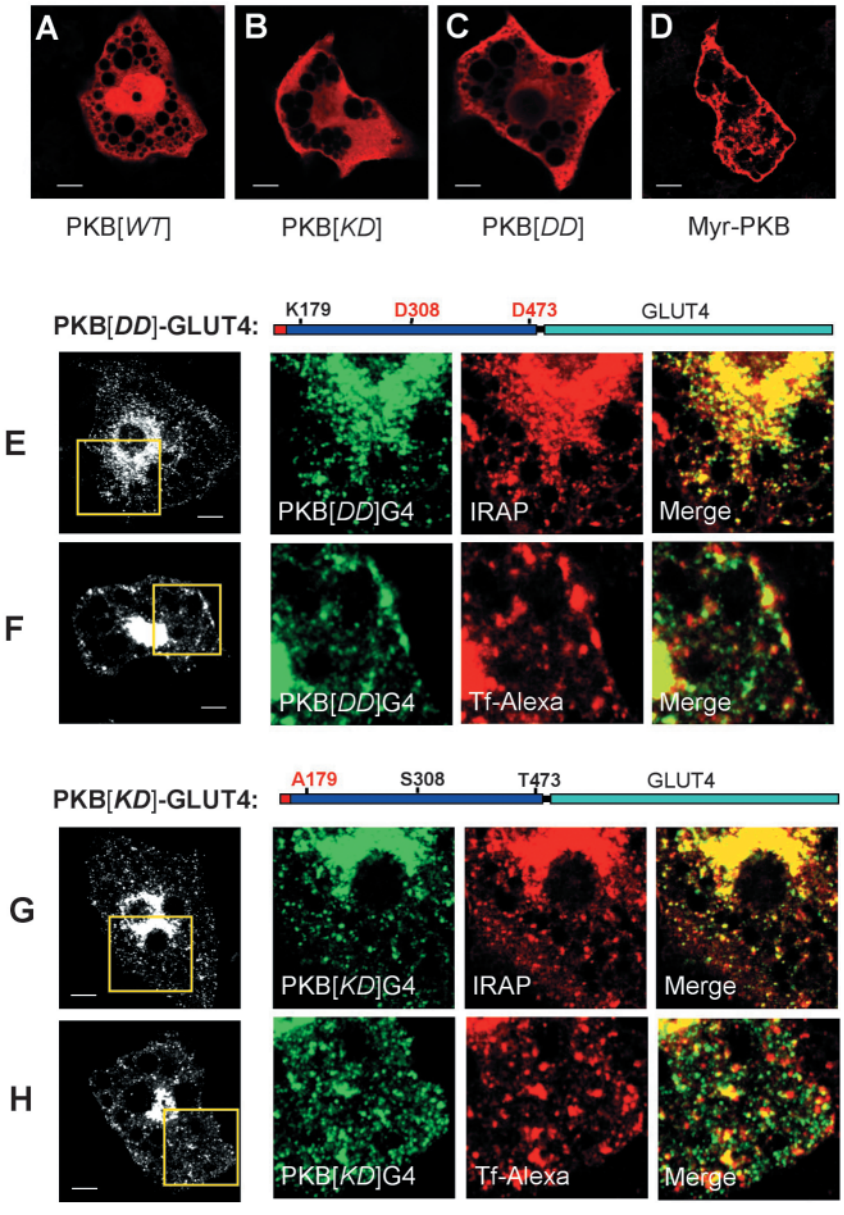

Fig. 1. Subcellular localisation of PKB mutants in 3T3-L1 adipocytes. 3T3-L1 adipocytes were microinjected with plasmids encoding wild-type PKB (A; PKB[*WT*]), kinase-inactive PKB (B; PKB[*KD*]), constitutively active PKB (C; PKB[*DD*]), constitutively active myristoylated PKB (D; Myr-PKB), constitutively active PKB fused to the N-terminus of GLUT4 (E,F; PKB[*DD*]G4) and kinase-inactive PKB fused to the N-terminus of GLUT4 (G,H; PKB[*KD*]G4). 16 hours later the cells were serum starved for 2 hours and then incubated in the absence (A-E,G) or presence (F,H) of transferrin-Alexa568 for 30 minutes (as indicated by 'Tf-Alexa') and then fixed and stained with anti-HA antibodies to visualise the PKB construct (as indicated in the panel) and anti-IRAP antibodies (as indicated by 'IRAP'), as required. A merged image is also provided of the regions indicated by the yellow box. Bars, 10 μm. The schematic of the PKB-GLUT4 fusion proteins illustrate the position of the N-terminal HA-tag (red), PKB (blue), GLUT4 (cyan) and the activatory (T308D/S473D) and inhibitory (K179A) mutations.

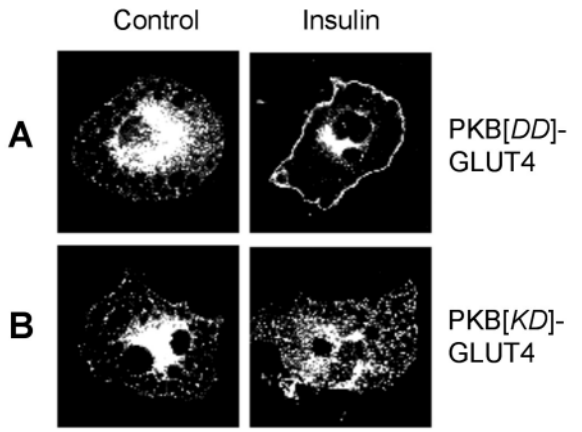

Fig. 2. Insulin induces the translocation of PKB[*DD*]-GLUT4 but not PKB[*KD*]-GLUT4 to the plasma membrane of 3T3-L1 adipocytes. 3T3-L1 adipocytes were microinjected with plasmids encoding constitutively active PKB fused to the N-terminus of GLUT4 (A; PKB[*DD*]-GLUT4) and kinase-inactive PKB fused to the N-terminus of GLUT4 (B; PKB[*KD*]-GLUT4). 16 hours later the cells were serum starved for 2 hours and then incubated, as required, in the presence or absence of insulin (30 minutes at 100 nM), as indicated. The cells were fixed and stained with anti-HA antibodies and imaged by confocal microscopy.

The PKB[*DD*]-GLUT4 (Fig. 1E,F) and PKB[*KD*]-GLUT4 (Fig. 1G,H) constructs were expressed in 3T3-L1 adipocytes by microinjection, and the cells were then either fixed and stained with antibodies to the GLUT4-vesicle-resident protein, IRAP (Fig. 1E,G), or the cells were incubated prior to fixation in the presence of transferrin-Alexa568 to fluorescently label recycling endosomes to equilibrium (Fig. 1F,H).

It is well established that GLUT4 and IRAP colocalise in adipocytes, and IRAP can be considered to be an excellent surrogate GLUT4 vesicle marker (Kandror and Pilch, 1994; Martin et al., 1997). Given that the PKB constructs were tagged to GLUT4 we could not examine their distribution relative to native GLUT4. For this reason, we examined the colocalisation of the targeted PKB constructs with endogenous IRAP. The results demonstrate that, in the basal state, PKB[*DD*]-GLUT4 and PKB[*KD*]-GLUT4 were targeted to intracellular vesicles that also contain endogenous IRAP, as would be expected of native GLUT4 (see Fig. 1E for PKB[*DD*]-GLUT4 and Fig. 1G for PKB[*KD*]-GLUT4). Indeed, the colocalisation of the PKB-GLUT4 constructs and endogenous IRAP was extremely high.

GLUT4 and IRAP co-exist within a highly specialised intracellular membrane pool in adipocytes called ‘GLUT4 storage vesicles’ [GSVs (Rea and James, 1997)]. Both proteins, however, are also found within recycling endosomes that contain transferrin receptors and GLUT1. To examine the degree to which the PKB-tagged GLUT4 constructs were excluded from the endosomal pool we labelled recycling endosomes to equilibrium with transferrin-Alexa568. Both PKB[*DD*]-GLUT4 and PKB[*KD*]-GLUT4 displayed a partial colocalisation with transferrin receptors with significant targeting to vesicles that exclude the fluorescent dye (Fig. 1F,H) as exhibited by native GLUT4. Taken together, these data demonstrate that PKB mutants can be efficiently targeted to bona fide GLUT4/IRAP vesicles in 3T3-L1 adipocytes by fusion at the N-terminus of GLUT4.

We next examined whether insulin was capable of inducing a translocation of the PKB[*DD*]-GLUT4 and PKB[*KD*]-GLUT4 fusion constructs to the plasma membrane as it does native GLUT4. The PKB[*DD*]-GLUT4 and PKB[*KD*]-GLUT4 constructs were expressed in 3T3-L1 adipocytes, which were then treated with or without insulin for 30 minutes. Insulin induced a translocation of PKB[*DD*]-GLUT4 to the plasma membrane as we have previously demonstrated for a GFP-tagged GLUT4 (Fig. 2A) (Oatey et al., 1997; Foran et al., 1999). By contrast, insulin appeared to be incapable of promoting a translocation of the PKB[*KD*]-GLUT4 fusion protein to the plasma membrane (Fig. 2B).

IRAP-GFP is a suitable surrogate marker for GLUT4 trafficking in 3T3-L1 adipocytes

We next wanted to examine the effect of the fusion proteins on insulin-dependent translocation of endogenous GLUT4. However, as we were already overexpressing GLUT4 in the cells and because we are unable to distinguish native GLUT4 from the transfected PKB[*DD*]-GLUT4 and PKB[*KD*]-GLUT4 constructs, we examined the translocation of IRAP instead.

To visualise IRAP translocation in transfected cells, we used an IRAP-GFP fusion protein in which the GFP moiety was placed at the C-terminus such that the GFP effectively replaced

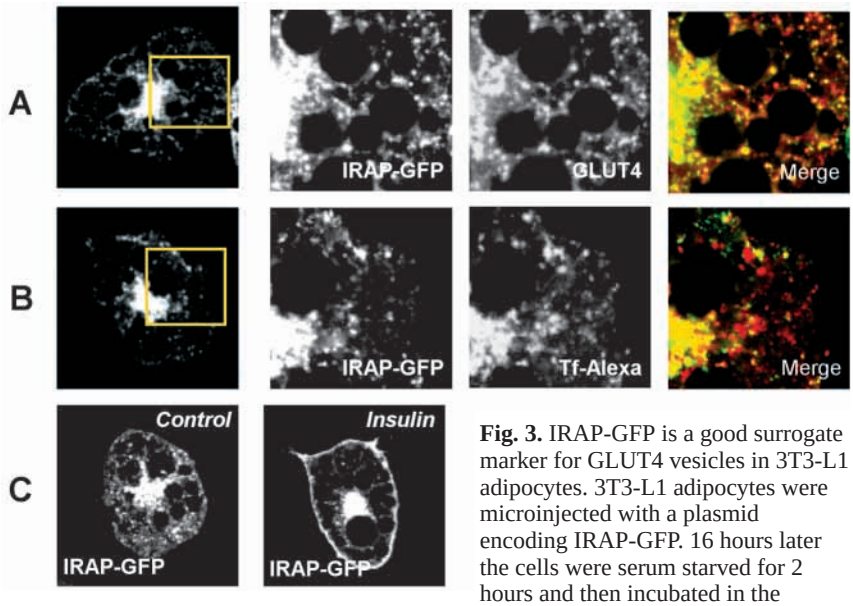

Fig. 3. IRAP-GFP is a good surrogate marker for GLUT4 vesicles in 3T3-L1 adipocytes. 3T3-L1 adipocytes were microinjected with a plasmid encoding IRAP-GFP. 16 hours later the cells were serum starved for 2 hours and then incubated in the absence (A,C) or presence (B) of transferrin-Alexa568 for 30 minutes. In C, the cells were incubated for 30 minutes in the absence or presence of insulin, as indicated. The cells were then fixed and stained with anti-GLUT4 antibodies (A, as indicated with GLUT4) as required. The images show the distribution of IRAP-GFP (visualised as GFP fluorescence), GLUT4 immunofluorescence or Tf-Alexa labelling, as indicated. A merged image is also provided.

the lumenal amino peptidase domain (see Materials and Methods). This IRAP-GFP fusion protein almost completely colocalised with endogenous GLUT4 (Fig. 3A) and showed only a partial overlap in expression with transferrin-Alexa568 labelled recycling endosomes (Fig. 3B). The construct translocates to the plasma membrane in response to insulin (Fig. 3C) and re-internalises efficiently after the withdrawal of the insulin signal (L.M.F. and J.M.T., unpublished). This makes IRAP-GFP an excellent surrogate marker for GLUT4 vesicle distribution in these studies. Indeed, it appears to be an improvement on our previously reported GFP-GLUT4 construct (Oatey et al., 1997), which we find does not efficiently re-internalise upon insulin withdrawal (Powell et al., 1999).

Effects of PKB-tagged GLUT4 fusion proteins on IRAP translocation to the plasma membrane

Cong et al. reported that a PKB[K179A] mutant has a dominant-negative effect on insulin-stimulated GLUT4 translocation in rat adipose cells (Cong et al., 1997). However, we have been consistently unable to demonstrate any significant dominant-negative effect of a PKB[*KD*] mutant on insulin-stimulated IRAP-GFP translocation (Fig. 4A, Fig. 5A,B) or on GFP-GLUT4 translocation (P. Oatey and J.M.T., unpublished) in 3T3-L1 adipocytes.

In contrast to the apparent lack of a dominant-negative effect of the PKB[*KD*], when this mutant was targeted to GLUT4 vesicles by fusion with GLUT4 (i.e. PKB[*KD*]-GLUT4), we found an unexpectedly profound inhibition of insulin-stimulated IRAP-GFP translocation to the plasma membrane (Fig. 4B) whether we quantified the response by determining the number of responding cells (Fig. 5A) or whether we determined it more quantitatively by establishing the amount of IRAP-GFP residing at the plasma membrane (Fig. 5B). This was consistent with the fact that the PKB[*KD*]-GLUT4 construct itself did not translocate to the plasma membrane in response to insulin (Fig. 2B). We also found that a mutant PKB possessing an N-terminal myristoylation signal sequence and a Lys179Ala mutation, which renders it kinase-deficient (Myr-PKB[*KD*]), also had a dominant-negative effect on insulin-stimulated IRAP-GFP translocation (Fig. 4C; Fig. 5A,B).

The PKB[*KD*]-GLUT4 construct differs from the PKB[*KD*] in two respects; (i) it is targeted to the GLUT4 vesicle, and (ii) it lacks the PH domain. To ensure that it was the targeting of PKB[*KD*] to GLUT4 that induced dominant-negativity, rather than the removal of the PH domain, we next examined the effect of a untargeted PKB[*KD*] mutant in which the PH domain was deleted. When this mutant was co-expressed with IRAP-GFP it exhibited no apparent dominant-negative effect (Fig. 5C), thus demonstrating that dominant-negativity of PKB[*KD*]-GLUT4 was indeed the result of targeting the PKB moiety to GLUT4 vesicles.

To determine whether the PKB[*KD*]-GLUT4 blocked any other effects of insulin we examined the ability of insulin to induce the translocation of the Forkhead transcription factor, FKHR, out of the nucleus, an effect which is PKB-dependent and has been proposed to underlie the ability of insulin to repress the expression of several metabolic genes in liver cells (Guo et al., 1999; Rena et al., 1999). We overexpressed a GFP-tagged FKHR in 3T3-L1 adipocytes and found that, as expected, insulin caused a pronounced exit of FKHR out of the nucleus (Fig. 6A,C). The ability of insulin to promote FKHR translocation out of the nucleus was unaffected by the presence of the PKB[*KD*]-GLUT4 construct (Fig. 6B,C).

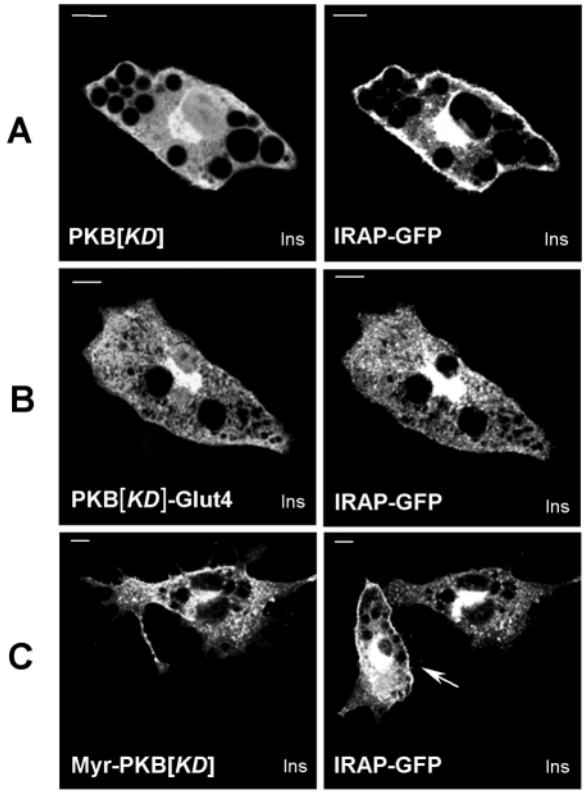

Fig. 4. PKB[*KD*]-GLUT4 and Myr-PKB[*KD*] act as dominant-negative inhibitors of insulin-stimulated IRAP-GFP translocation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were microinjected with plasmids encoding IRAP-GFP (A-C) and either kinase-inactive PKB (A; PKB[*KD*]), kinase-inactive PKB fused to the N-terminus of GLUT4 (B; PKB[*KD*]-Glut4) or kinase-inactive myristoylated PKB (C; Myr-PKB[*KD*]). 16 hours later the cells were serum starved for 2 hours and then incubated in the presence of insulin at 100 nM for 30 minutes. The cells were then fixed and stained with anti-HA antibodies to visualise the PKB constructs (as indicated). Bars, 10 μm. The arrow in C indicates a cell that expresses IRAP-GFP but little detectable Myr-PKB[*KD*] and which still responds to insulin, unlike its neighbour which co-expresses significant amounts of Myr-PKB[*KD*].

We next examined the effect of expressing constitutively active PKB mutants on IRAP-GFP translocation in 3T3-L1 adipocytes. Others have previously demonstrated that membrane-targeted constitutively active PKB mutants promote an increased GLUT4 translocation and glucose uptake in both adipose and muscle cells (Tanti et al., 1997; Hajduch et al., 1998). We have previously demonstrated that a PKB mutant, rendered constitutively active by virtue of replacement of both its regulatory Thr308 and Ser473 phosphorylation sites with aspartic acid residues (PKB[*DD*]), also causes constitutive translocation of GLUT4 in the absence of insulin (Foran et al., 1999). This was also confirmed to be the case for IRAP-GFP (Fig. 7A,D). Indeed, as we have previously noted for GFP-tagged GLUT4, the PKB[*DD*] mutant promoted a visible translocation of IRAP-GFP in approximately 60% of the 3T3-L1 adipocytes that co-expressed PKB[*DD*] and IRAP-GFP

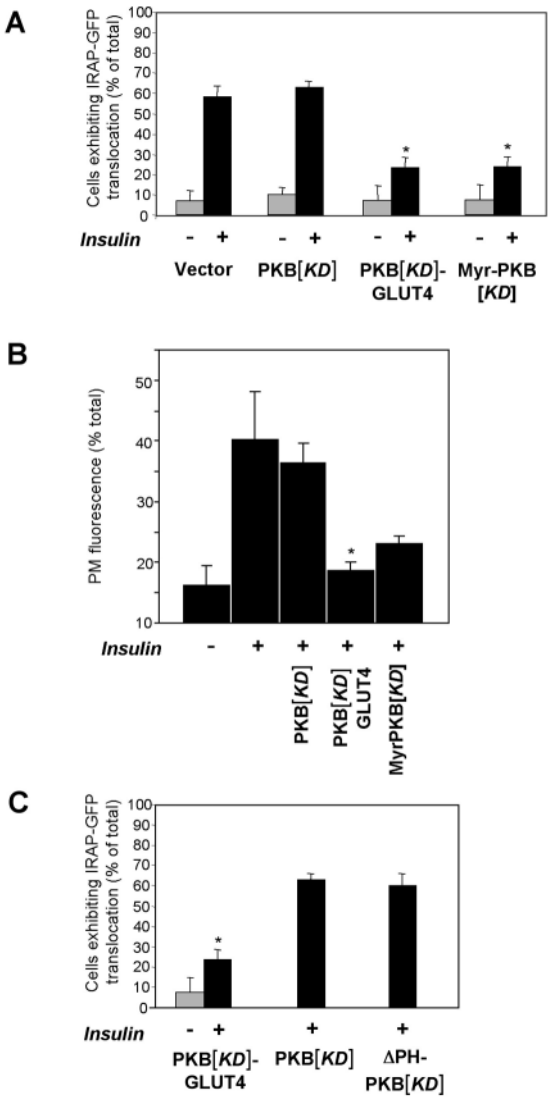

Fig. 5. Quantitative analysis of the dominant-negative effect of PKB[*KD*]-GLUT4 and Myr-PKB[*KD*]. In A and C, cells were scored for the visual presence of IRAP-GFP in the plasma membrane by double blind analysis by two independent workers as discussed in the Materials and Methods. The results shown represent means±s.d. for three independent experiments with each condition being represented by a minimum of 50 cells. **P*<0.01 versus the vector only injected cells incubated in the presence of insulin. In B the amount of IRAP-GFP residing in the plasma membrane was calculated as a percentage of the total expressed IRAP-GFP in that cell for each condition. The results are displayed as means±s.d. for a minimum of 10 cells for each condition, and they were collected from at least three separate experiments. *No significant difference from the control without insulin incubation. In C, adipocytes were microinjected with plasmids encoding IRAP-GFP and either kinase-inactive PKB (PKB[*KD*]), kinase-inactive PKB lacking the PH domain (ΔPH-PKB[*KD*]) or kinase-inactive PKB fused to the N-terminus of GLUT4 (PKB[*KD*]-GLUT4). 16 hours later the cells were serum starved for 2 hours and then incubated in the absence or presence of insulin for 30 minutes. The cells were then scored for the presence of IRAP-GFP in the plasma membrane as discussed in the Materials and Methods. The results shown represent means±s.d. for three independent experiments, with each condition being represented by a minimum of 50 cells. **P*<0.01 versus the PKB[*KD*]-injected cells incubated in the presence of insulin.

(Fig. 7D). This effect was indistinguishable from that of insulin alone (Fig. 7D). The remaining 3T3-L1 adipocytes (approximately 40%) appear to be considerably less sensitive to the actions of insulin and to the plasma-membrane-localised PKB[*DD*] mutant such that IRAP-GFP cannot be visibly detected at the cell surface.

In contrast to the effect of PKB[*DD*], when PKB[*DD*] was targeted to GLUT4 vesicles (PKB[*DD*]-GLUT4; Fig. 7B,D), it had a weak insulin-like effect on IRAP-GFP translocation (translocation occurring in approximately 25% of the cells (Fig. 7D), and this effect was additive with insulin. However, we consistently found that those cells that exhibited a visible translocation of IRAP-GFP also had high levels of expression of the PKB[*DD*]-GLUT4 mutant (data not shown).

The constitutively active Myr-PKB had a profound effect on IRAP-GFP translocation, such that approximately 80% of the cells responded, but the additional presence of insulin had no further effect (Fig. 7C,D).

Discussion

Several groups have reported that PKB translocates to the plasma membrane as well as to the nucleus in response to a variety of extracellular stimuli (Andjelkovic et al., 1997; Meier et al., 1997; Goransson et al., 1998). Additionally, PKB appears to be recruited to GLUT4 vesicles in response to insulin (Calera et al., 1998; Kupriyanova and Kandror, 1999). The functional consequence of this targeting has not been previously addressed. In the current study, we have examined the consequence of artificially targeting kinase-inactive and constitutively active mutants of PKB to GLUT4 vesicles by fusion at the N-terminus of GLUT4 itself. The PKB-GLUT4 fusions were efficiently targeted to GLUT4 vesicles as demonstrated by their almost complete colocalisation with endogenous IRAP and partial colocalisation with a recycling endosome marker (Fig. 1E-H). Furthermore, a PKB[*DD*]-GLUT4 fusion protein exhibited insulin-stimulated translocation to the plasma membrane (Fig. 2A). Thus GLUT4 appears to be able to tolerate the presence of PKB fused at the N-terminus, which is consistent with the ability of GLUT4 to tolerate a 27 kDa GFP moiety in this position (Oatey et al., 1997; Thurmond and Pessin, 2000; Lampson et al., 2000; Fletcher et al., 2000).

In the current study, we visualised GLUT4 vesicle translocation using IRAP, a well established bona fide GLUT4 vesicle-resident protein, tagged with GFP (Kandror and Pilch, 1994; Martin et al., 1997). The GFP moiety of IRAP-GFP is expressed either within the vesicle lumen or on the extracellular face of the plasma membrane after translocation although, despite the fact that GFP fluorescence is reported to be pH sensitive (Miesenbock et al., 1998), we do not find any detectable change in fluorescence intensity of the GFP moiety during exocytosis (data not shown). As we have previously shown for GFP-tagged GLUT4 (Oatey et al., 1997; Fletcher et al., 2000), IRAP-GFP expressed in 3T3-L1 adipocytes exhibited colocalisation with endogenous GLUT4, partial colocalisation with transferrin-Alexa568-labelled recycling endosomes and a translocation to the plasma membrane in

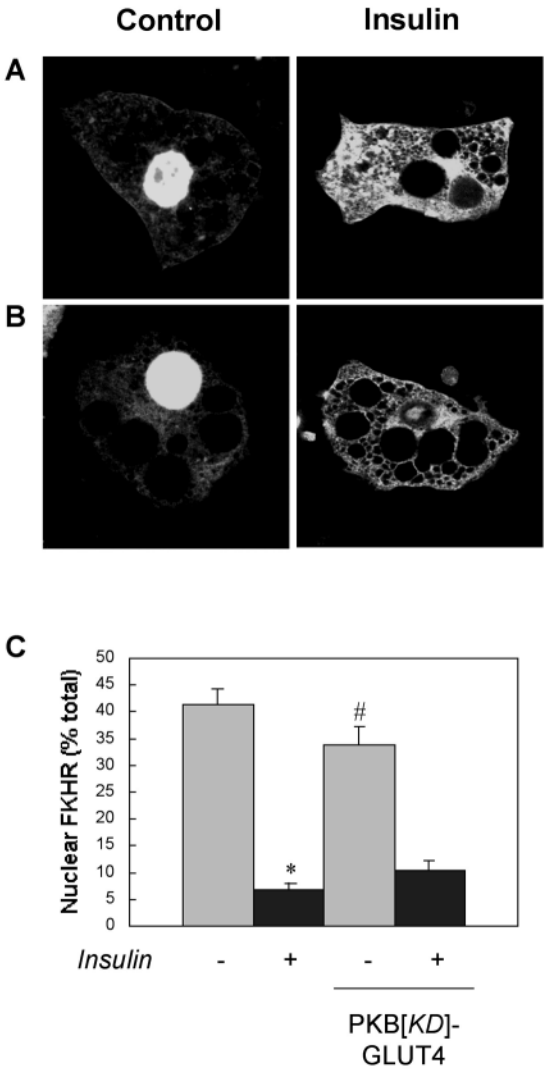

Fig. 6. Lack of a dominant-negative effect of PKB[*KD*]GLUT4 on insulin-stimulated Forkhead translocation out of the nucleus of 3T3-L1 adipocytes. 3T3-L1 adipocytes were microinjected with a plasmid encoding a GFP-tagged human FKHR in the absence (A) or presence (B) of the PKB[*KD*]-GLUT4 plasmid. After 24 hours the cells were serum starved and treated without (left-hand panels) or with (right-hand panels) insulin for 30 minutes as indicated. The cells were fixed and examined for the distribution of the FKHR-GFP by confocal microscopy. In C, the amount of nuclear FKHR was quantified as described in the Materials and Methods. The results shown represent means±s.d. for at least three independent experiments with each condition represented by a minimum of 11 cells. **P*<0.001 versus control incubated without insulin and no significant difference from the insulin-stimulated cells expressing PKB[*KD*]-GLUT4. #No significant difference from control in the absence of PKB[*KD*]-GLUT4.

response to insulin (Fig. 3); these are all characteristics expected of native IRAP and are also observed with the PKB[*DD*]-GLUT4 construct. IRAP-GFP was, therefore, used as a surrogate marker for the localisation for GLUT4/IRAP-containing vesicles.

It has been previously reported that a PKB[K179A] mutant has a dominant-negative effect on insulin-stimulated GLUT4 translocation in rat adipose cells, although the inhibition of the insulin effect was only approximately 20% (Cong et al., 1997). In 3T3-L1 adipocytes, we have consistently found no significant effect using a similar PKB mutant (PKB[*KD*]) construct that appears to be expressed predominantly within the cytosol (Fig. 1B, Fig. 4A), although small amounts were also found in the plasma membrane. The fact that insulin stimulated the translocation of PKB[*DD*]-GLUT4, but not PKB[*KD*]-GLUT4, to the plasma membrane gave us our first clue that PKB[*KD*]-GLUT4 may act as a dominant-negative protein capable of blocking GLUT4/IRAP vesicle translocation.

Our further investigations demonstrated that a PKB[*KD*] mutant targeted to the GLUT4 vesicle (PKB[*KD*]-GLUT4; Fig. 4B) had a potent dominant-negative effect on IRAP-GFP translocation (Fig. 5A,B). Furthermore, a kinase-inactive PKB containing a myristoylation signal sequence (Myr-PKB[*KD*]), which was expressed both at the plasma membrane and within intracellular vesicles containing endogenous IRAP (Fig. 4C), was also a potent dominant-negative inhibitor of IRAP-GFP translocation (Fig. 4C, Fig. 5A,B). As both PKB[*KD*]-GLUT4 and Myr-PKB[*KD*] are targeted to intracellular vesicles containing IRAP, we concluded that PKB[*KD*] must be targeted to intracellular membranes to exhibit a dominant-negative effect on insulin-stimulated GLUT4/IRAP vesicle translocation.

Interestingly, the dominant-negative effect of this GLUT4 vesicle-targeted PKB[*KD*] mutant was specific to blocking IRAP translocation, as the insulin-dependent translocation of FKHR out of the nucleus was not inhibited by the construct (Fig. 6). As the ability of insulin to promote FKHR translocation is dependent on the activation of PKB, this strongly suggests that PKB activation, and by inference PI 3-kinase and PDK1 activation, are largely intact in cells expressing the PKB[*KD*]-GLUT4 construct. Thus the ability of the PKB[*KD*]-GLUT4 construct to inhibit IRAP translocation is likely to be the result of a localised inhibition of phosphorylation of a specific PKB substrate that resides at or close to the GLUT4 vesicle.

GLUT4 continuously recycles between the cell interior and plasma membrane (Yang and Holman, 1993; Holman et al., 1994). Although we do not detect any of the PKB[*KD*]-GLUT4 fusion protein in the plasma membrane, it is possible that PKB[*KD*]-GLUT4 and Myr-PKB[*KD*] exhibit dominant-negative effects only when they reside in the plasma membrane. This is unlikely, however, because we find that the PKB[*KD*] mutant, which is clearly visible in the plasma membrane in some cells, is without any detectable dominant-negative effect on IRAP-GFP translocation in those cells.

Our data may provide an explanation for the inability of some groups to observe a dominant-negative effect of PKB mutants on GLUT4 translocation and glucose uptake. In studies where a dominant-negative effect has been observed (e.g. Cong et al., 1997; Wang et al., 1999), but not others (Kitamura et al., 1998), the PKB mutants may have been expressed at a level sufficient for the endogenous PKB associated with GLUT4 vesicles to be inhibited. In the study of Kitamura and colleagues (Kitamura et al., 1998), the dominant-negative PKB mutant (Akt-AA) potently (>80%) blocked the stimulation of endogenous PKB. However, this mutant may have lacked a dominant-negative effect on GLUT4 translocation because it did not block the activation of the

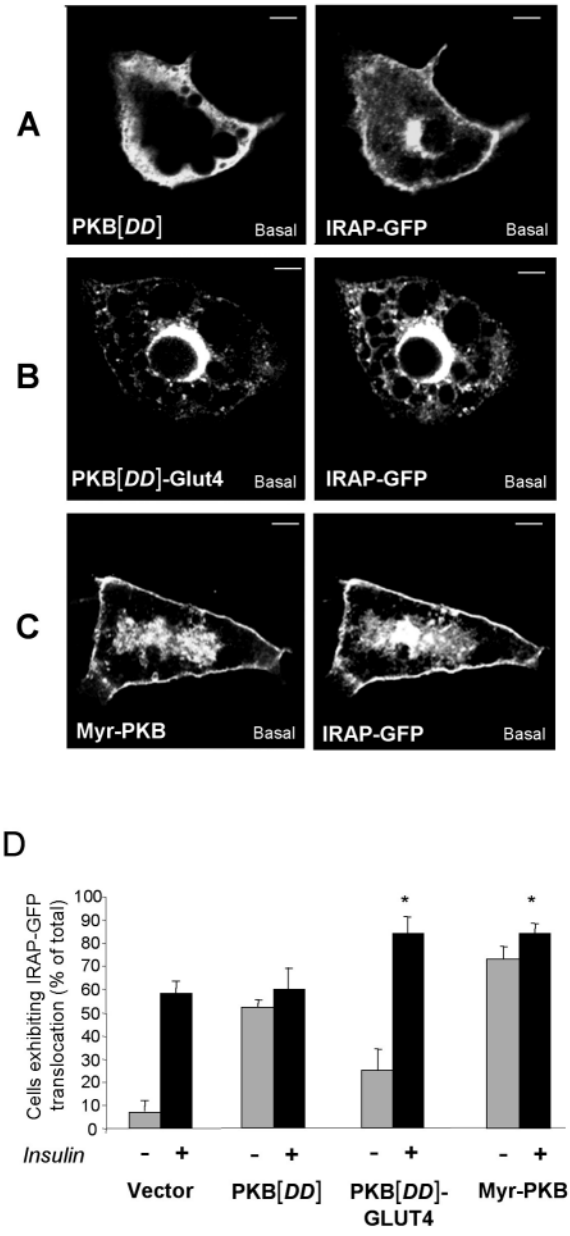

Fig. 7. The effect of constitutively active PKB constructs on IRAP-GFP translocation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were microinjected with plasmids encoding IRAP-GFP (A-C) and either constitutively active PKB (A; PKB[*DD*]), constitutively active PKB fused to the N-terminus of GLUT4 (B; PKB[*DD*]-Glut4) or a constitutively active myristoylated PKB (C; Myr-PKB). 16 hours later the cells were serum starved for 2 hours and then fixed and stained with anti-HA antibodies to visualise the PKB constructs (as indicated). Panels A-C show the distribution of the HA-tagged PKB constructs and GFP fluorescence resulting from the expression of the IRAP-GFP construct. Bars, 10 μm. In D, cells were scored for the presence of IRAP-GFP in the plasma membrane after incubation in the absence or presence of insulin for 30 minutes as discussed in the Materials and Methods. The results shown represent means±s.d. for three independent experiments with each condition being represented by a minimum of 50 cells. **P*<0.01 versus the vector only injected cells incubated in the presence of insulin.

GLUT4-resident pool of PKB, which may make up the residual (20%) activity that remained.

A logical conclusion from our data is that PKB plays a crucial role at, or in the vicinity of, GLUT4 vesicles. In our study we used PKBα (Akt1) in the fusion constructs, although others have reported that PKBβ (Akt2) localises to GLUT4 vesicles. Although Akt1$^{-/-}$ knockout mice exhibit normal whole body glucose tolerance and disposal (Cho et al., 2001a), a role for PKBα (Akt1) in adipose tissue glucose uptake cannot be excluded as (i) insulin-stimulated glucose uptake into adipocytes of the mice was not measured and (ii) the defect in insulin-stimulated glucose uptake into adipocytes (and muscle) from Akt2$^{-/-}$ mice was only partial (Cho et al., 2001b). Thus it remains possible that PKBα and PKBβ play redundant roles in insulin-stimulated GLUT4 translocation.

Lowering the temperature of 3T3-L1 adipocytes to 23°C causes an accumulation of GLUT4 vesicles underneath the plasma membrane in response to insulin as a result of a proposed block in the final fusion step (Elmendorf et al., 1999). Our data suggest that PKB does not act at this fusion step, since we do not observe IRAP-GFP vesicles accumulating under the plasma membrane in insulin-treated cells expressing PKB[*KD*]-GLUT4. As we observe no difference in IRAP-GFP distribution between non-injected and PKB[*KD*]-GLUT4-expressing cells, the dominant-negative PKB[*KD*]-GLUT4 must act on the initial mobilisation of GLUT4/IRAP vesicles by insulin.

Dominant-negative PKB[*KD*]-GLUT4 may act by preventing the phosphorylation of substrates by endogenous PKB in the vicinity of the GLUT4/IRAP vesicle. Indeed, partially purified PKBβ/Akt2 has been reported to phosphorylate several integral components of the GLUT4 vesicle (Kupriyanova and Kandror, 1999), although the identity and function of these substrates are currently not known. Alternatively, the dominant-negative PKB[*KD*]-GLUT4 could act by sequestering upstream activators such as PDK1, thus preventing the activation of endogenous PKBα and PKBβ. Thus, although we have used PKBα in our constructs, it may be able to block the activation of PKBβ by this mechanism. This is considered unlikely, however, as the PKB[*KD*]-GLUT4 mutant did not block the PKB-dependent translocation of FKHR out of the nucleus, suggesting that PKB activation is largely intact (Fig. 6). The dominant-negative PKB[*KD*]-GLUT4 may act by interfering with the stimulation of atypical PKCs ζ/λ by insulin in the vicinity of the GLUT4 vesicles, as these are also phosphorylated and activated by PDK1 and are reported to play a role in insulin-stimulated glucose uptake (Bandyopadhyay et al., 1997; Kotani et al., 1998). However, again we would consider this to be unlikely as the insulin-stimulated translocation of FKHR from the nucleus also requires PI 3-kinase and PDK1 activation (both of which are required for PKCζ activation), and our data suggest that these events must also be largely intact in cells expressing the PKB[*KD*]-GLUT4 construct.

A constitutively active PKB[*DD*] fully mimics the effect of insulin causing a visible translocation of IRAP-GFP in approximately 60% of the cells (Fig. 7A,D), although the effect of PKB[*DD*] and insulin were not additive. By contrast, PKB[*DD*] targeted to GLUT4 vesicles appears to only partially mimic the effect of insulin on IRAP-GFP translocation (Fig.

7B,D). Careful examination of many confocal images revealed that the cells that exhibit a high level of PKB[*DD*]-GLUT4 expression also contain significant amounts of IRAP-GFP in the plasma membrane; at lower levels of PKB[*DD*]-GLUT4 expression, both PKB[*DD*]-GLUT4 expression and IRAP-GFP are exclusively intracellular. This suggests that PKB[*DD*]-GLUT4 does not promote IRAP-GFP translocation unless, as a result of overexpression, sufficient PKB[*DD*]-GLUT4 leaks out to the plasma membrane from where it promotes GLUT4/IRAP vesicle translocation. This would be consistent with the fact that PKB[*DD*] causes insulin-independent GLUT4/IRAP vesicle translocation and is predominantly expressed within the plasma membrane (Fig. 7A,D). The effect of exclusive plasma membrane localisation of active PKB, using a K-Ras CAAX-targeting motif for example, is difficult to assess as this mutant has been reported to exhibit dominant-negative rather than constitutive activity (van Weeren et al., 1998). It should also be noted that we cannot exclude the possibility that the PKB[*DD*] moiety tethered to GLUT4 lacks dominant-negative activity because it has restricted access to the putative substrate located on the vesicle as a result of its orientation away from the vesicle surface.

In conclusion, we propose a model in which PKB activation at, or close to, the GLUT4 vesicle is necessary but not sufficient to induce GLUT4 vesicle translocation to the plasma membrane. PKB activation must also occur in the plasma membrane to promote efficient GLUT4 vesicle translocation, as suggested by the fact that a constitutively active plasma-membrane-localised PKB (i.e. the PKB[*DD*] mutant) can stimulate GLUT4/IRAP translocation in the absence of insulin. Identification of the intracellular protein(s) and/or substrates with which PKB[*KD*]-GLUT4 interacts to prevent GLUT4 vesicle translocation will provide important clues as to how insulin stimulates GLUT4 translocation in adipose cells.

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