

# Activity, phosphorylation state and subcellular distribution of GLUT4-targeted Akt2 in rat adipose cells

Xiaoli Chen<sup>1</sup>, Hadi Al-Hasani<sup>2</sup>, Torbjorn Olausson<sup>3</sup>, Ann-Marie Wenthzel<sup>3</sup>, Ulf Smith<sup>3</sup> and Samuel W. Cushman<sup>1,\*</sup>

<sup>1</sup>EDMNS/DB, NIDDK, NIH, 8 Center Dr MSC 0842, Bethesda, MD 20892-0842, USA

<sup>2</sup>German Institute for Human Nutrition, Arthur-Scheuner-Allee 114-116, 14558 Bergholz-Rehbrücke, Germany

<sup>3</sup>Lundberg Laboratory for Diabetes Research, Department of Internal Medicine, The Sahlgrenska Academy at Gothenburg University, SE-413 45 Gothenburg, Sweden

\*Author for correspondence (e-mail: sam\_cushman@nih.gov)

Accepted 19 May 2003

Journal of Cell Science 116, 3511-3518 Published by The Company of Biologists Ltd  
doi:10.1242/jcs.00675

## Summary

In this study, fusion of the kinase domain of Akt2 to the cytosolic C terminus of exofacially-HA-tagged GLUT4 is used to investigate the activity, phosphorylation state and subcellular localization of Akt2 specifically targeted to the GLUT4-trafficking pathway in rat adipose cells. Fusion of wild-type (wt) Akt2, but not a kinase-dead (KD) mutant results in constitutive targeting of the HA-GLUT4 fusion protein to the cell surface to a level similar to that of HA-GLUT4 itself in the insulin-stimulated state. Insulin does not further enhance the cell-surface level of HA-GLUT4-Akt2-wt, but does stimulate the translocation of HA-GLUT4-Akt2-KD. Cell-surface HA-GLUT4-Akt2-wt is found to be phosphorylated on Ser<sup>474</sup> in both the absence and presence of insulin, and mutation of Ser<sup>474</sup> to Ala reduces the increased basal cell-surface localization of the fusion protein. While Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-KD is detected only in the insulin-stimulated state, trapping this fusion protein on the cell surface by coexpression of a dominant negative mutant dynamin does not induce Ser<sup>474</sup> phosphorylation. Phosphorylation on

Thr<sup>309</sup> is not detectable in either HA-GLUT4-Akt2-wt or HA-GLUT4-Akt2-KD, in either the basal or insulin-stimulated state, and mutation of Thr<sup>309</sup> to Ala does not influence the insulin-independent increases in cell-surface localization and Ser<sup>474</sup> phosphorylation. Expression of HA-GLUT4-Akt2-wt stimulates the translocation of co-transfected myc-GLUT4 to a level similar to that in the insulin-stimulated state; this increase is moderately reduced by mutation of Ser<sup>474</sup> to Ala and absent with the kinase-dead mutant. These results demonstrate that targeting Akt2 to the GLUT4-trafficking pathway induces Akt2 activation and GLUT4 translocation. Ser<sup>474</sup> phosphorylation is an autocatalytic reaction requiring an active kinase, and kinase activity is associated with a plasma membrane localization. Fusion of Akt2 to the C terminus of GLUT4 appears to substitute for Thr<sup>309</sup> phosphorylation in activating the autocatalytic process.

Key words: PKB/Akt, GLUT4, Translocation, Phosphorylation

## Introduction

Insulin stimulates glucose transport in adipose and muscle cells through the translocation of the GLUT4 glucose transporter isoform (Rea and James, 1997; Holman and Cushman, 1994). In the basal state, the majority of GLUT4 resides in a specialized intracellular tubulovesicular compartment, known as 'GLUT4-containing vesicles'. Insulin stimulation induces a rapid subcellular redistribution of GLUT4 from the intracellular pool to the plasma membrane. Numerous studies have demonstrated that phosphoinositide 3-kinase (PI3-kinase) plays a signaling role in insulin-triggered GLUT4 trafficking (Cheatham et al., 1994; Okada et al., 1994). Protein kinase B/Akt (a serine/threonine protein kinase) has also been identified as a major downstream component of the signaling pathway, which mediates insulin-stimulated GLUT4 translocation and glucose uptake (Kohn et al., 1996; Hill et al., 1999; Wang et al., 1999). PKB $\beta$ /Akt2 is the major isoform activated in adipose cells.

Until recently, a reasonably consistent picture has emerged

regarding the mechanism of activation of Akt. In the case of insulin action, PI3-kinase is activated, enhancing the concentration of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) in the plasma membrane and inducing the binding of Akt through its N-terminal PH domain (Alessi et al., 1997; Stokoe et al., 1997). This binding reaction exposes the Akt to constitutively active PDK-1 in the plasma membrane which phosphorylates Thr<sup>309</sup> in Akt2, Thr<sup>308</sup> in Akt1, leading through an unknown mechanism to phosphorylation of Ser<sup>474</sup> and activation of the Akt kinase activity. Signaling is then prorogated to GLUT4 translocation through a further series of unknown steps.

Multiple questions have recently been raised, however, regarding the early stages in Akt activation. One work using isolated human adipose cells suggests that Thr<sup>309</sup> phosphorylation of PDK-1 takes place in an intracellular compartment associated with the low-density microsomes and that subsequent translocation of the Thr<sup>309</sup>-phosphorylated Akt

to the plasma membrane is associated with subsequent Ser<sup>474</sup> phosphorylation and activation (Carvalho et al., 2000). Additional reports argue the case for the activity of a distinct Ser<sup>474</sup> kinase in the plasma membrane as against a Ser<sup>474</sup> autophosphorylation reaction by Akt itself, both requiring prior Thr<sup>309</sup> phosphorylation (Hill et al., 2001; Hill et al., 2002; Andjelkovic et al., 1997). Furthermore, two recent studies support the concept that Ser<sup>474</sup> phosphorylation does not require prior Thr<sup>309</sup> phosphorylation, one employing stem cells from a PDK-1 knockout mouse (Williams et al., 2000) and the other, a PDK-1 inhibitor (Hill et al., 2001). Finally, a study of isolated rat adipose cells suggests that Ser<sup>474</sup> is the primary phosphorylation site in response to insulin and that Thr<sup>309</sup> is only marginally phosphorylated (Goransson et al., 2002). A corollary question relates to the relationship between Akt activation and GLUT4 translocation; one report purports to demonstrate the recruitment of PI3-kinase (Heller-Harrison et al., 1996) and another, the recruitment of Akt2 itself to the GLUT4-containing vesicles in response to insulin (Calera et al., 1998; Kupriyanova and Kandror, 1999).

In collaboration with M. J. Quon and S. I. Taylor, we have developed a transfection technique which permits expression of exogenous GLUT4 tagged with an exofacial HA epitope detectable on the cell surface using an antibody binding assay. The subcellular trafficking of this HA-GLUT4 construct is indistinguishable from endogenous GLUT4 (Quon et al., 1994). We have further characterized HA-GLUT4 constructs in which GFP has been fused to either the N or C terminus; the subcellular trafficking of HA-GLUT4-GFP, the C-terminal construct, is also indistinguishable from endogenous GLUT4, whereas fusion of GFP to the N terminus of GLUT4 leads to a primarily plasma membrane localization (Dawson et al., 2001).

In the present study, we have fused the kinase domain of Akt2-wt (wild type) and various Akt2 mutants to the C terminus of HA-GLUT4 in order to investigate the activity, phosphorylation state and subcellular localization of Akt2 specifically targeted to the GLUT4-trafficking pathway. We observed that the HA-GLUT4-Akt2-wt, but not the HA-GLUT4-Akt2-KD (K179A), spontaneously associates with the plasma membrane in a manner similar to the response to insulin and becomes phosphorylated on Ser<sup>474</sup>, but not Thr<sup>309</sup>. HA-GLUT4-Akt2-KD translocates normally with insulin and becomes phosphorylated on Ser<sup>474</sup> in response to insulin, but is still not phosphorylated on Thr<sup>309</sup>. HA-GLUT4-Akt2-wt, but not HA-GLUT4-Akt2-KD, stimulates the translocation of co-transfected myc-GLUT4. These data suggest that targeting Akt2 to the GLUT4-trafficking pathway induces Akt2 activation and GLUT4 translocation. Ser<sup>474</sup> phosphorylation is an autocatalytic reaction requiring an active kinase, and kinase activity is associated with a plasma membrane localization. Fusion of Akt2 to the C terminus of GLUT4 appears to substitute for Thr<sup>309</sup> phosphorylation in activating the autocatalytic process.

While this work was in progress, the Tarvare laboratory published a report of a similar project using morphological rather than biochemical assays, and 3T3-L1 adipocytes rather than primary rat adipose cells (Ducluzeau et al., 2002). The results of that study are substantially different than our results for unknown reasons, however, our work further focuses on the relationship between phosphorylation state and subcellular

localization of GLUT4-targeted Akt2, a problem not addressed in the Tavare paper.

## Materials and Methods

### HA-GLUT4-Akt2 fusion constructs

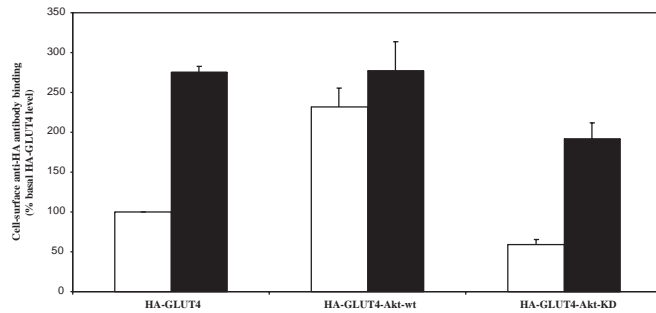
All constructs were generated in the pCIS2 mammalian expression vector (a generous gift from Dr C. Gorman). The wild type (wt), constitutively active (DE, i.e. S474D, T309E), kinase dead (KD: K179A) and triple mutant (A3: K179A, S474A, T309A) forms of human Akt2 cloned into the vector pLNCX (generous gifts from Dr Morris J. Birnbaum) were digested with *Bgl*III and *Bam*HI followed by Klenow fill-in reaction and then *Nco*I digestion, resulting in the inserts of ΔPH-Akt2 with 5'-*Nco*I and 3' blunt ends. The HA-GLUT4-*Nhe*I(CT) (GLUT4 containing *Nhe*I site in the C terminus) in pCIS2 was digested with *Nhe*I and *Hpa*I to generate the vector fragment for subcloning. To fuse the *Nco*I end of ΔPH-Akt2 inserts to the *Nhe*I end of HA-GLUT4 vector an oligonucleotide adapter (*Nhe*I-*Nco*I: 5' CTAGCGGGGCCGGGGCCGGGGC 3' forward and 5' CATG-GCCCCGGCCCCGGCCCCG 3' reverse) was used. The ΔPH-Akt2-wt, ΔPH-Akt2-DE, ΔPH-Akt2-KD and ΔPH-Akt2-A3 were then fused in-frame to HA-GLUT4 in pCIS2 using *Nhe*I and *Hpa*I (blunt) sites to generate HA-GLUT4-Akt2-wt, HA-GLUT4-Akt2-DE, HA-GLUT4-Akt2-KD and HA-GLUT4-Akt2-A3. HA-GLUT4-Akt2-S474A and HA-GLUT4-Akt2-T309A mutants were generated by site-directed mutagenesis using the QuickChange kit according to manufacturer's instructions (Stratagene, La Jolla, CA), and mutations were confirmed by BigDye sequencing. Construction of the HA-tagged GLUT4 and dominant-negative K44A dynamin1 in pCIS2 has been described previously (Quon et al., 1994; Al-Hasani et al., 1998). The myc-tagged GLUT4 (myc-GLUT4) construct was generated by restriction digestion of GLUT4 via a *Sau*I site contained within the extracellular loop between transmembrane domains one and two. For transfection experiments, the plasmids were purified in mg quantities using a maxiprep kit (Qiagen).

### Cell culture and transfection of rat adipose cells

Preparation of isolated rat epididymal adipose cells from male rats (CD strain, Charles River Breeding Laboratories, Inc.) was performed as described previously (Quon et al., 1994). Isolated cells were washed twice with Dulbecco's modified Eagle's medium containing 25 mM glucose, 25 mM Hepes, 4 mM L-glutamine, 200 nM N6-(2-phenylisopropyl)-adenosine, and 75 μg/ml gentamycin, and resuspended to a cytotric of 40% (5-6×10<sup>6</sup> cells/ml). 200 μl of the cell suspension were added to 200 μl of Dulbecco's modified Eagle's medium containing 100 μg of carrier DNA (sheared herring sperm DNA; Boehringer Mannheim) and expression plasmids as indicated. The total concentration of plasmid DNA in each cuvette was adjusted to 1 μg/cuvette for HA-GLUT4-Akt2 fusion proteins and 4.5 μg/cuvette for dominant-negative K44A dynamin1. In experiments where myc-GLUT4 and HA-GLUT4-Akt2 fusion proteins were co-transfected the total concentration of plasmid DNA in each cuvette was adjusted to 0.2 μg/cuvette and 0.5 μg/cuvette, respectively. Electroporation was carried out in 0.4-cm gap-width cuvettes (Bio-Rad) using a T810 square wave pulse generator (BTX). After applying three pulses (12 mseconds, 200 V), the cells were washed once in Dulbecco's modified Eagle's medium, pooled in groups of 4-10 cuvettes, and cultured at 37°C, 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium containing 3.5% bovine serum albumin.

### Cell-surface antibody binding assay

Rat adipose cells were harvested 20-24 hours post-transfection and washed in Krebs-Ringer bicarbonate Hepes buffer, pH 7.4, 200 nM adenosine (KRBH buffer) containing 5% bovine serum albumin. The cells from individual cuvettes were transferred into 1.5 ml



**Fig. 1.** Cell-surface expression of HA-GLUT4-Akt2 fusion constructs in rat adipose cells. Isolated cells were transfected with HA-GLUT4, HA-GLUT4-Akt2-wt, and HA-GLUT4-Akt2-K179A (KD), and cultured for 20 hours. After harvesting the cells, cell-surface levels of the constructs were determined in the transfected cells in the basal (white) and insulin-stimulated states (67 nM, black) using an HA antibody binding assay and the cell-surface-associated radioactivity was expressed relative to the basal HA-GLUT4 value in each experiment (1668, 1558, 2311 cpm). Relative cell-surface levels were then normalized to the total expression level of each construct determined by immunoblotting as described in Materials and Methods. Results are the means  $\pm$  s.e.m. of the means obtained from at least duplicate determinations in each of 3-5 independent experiments.

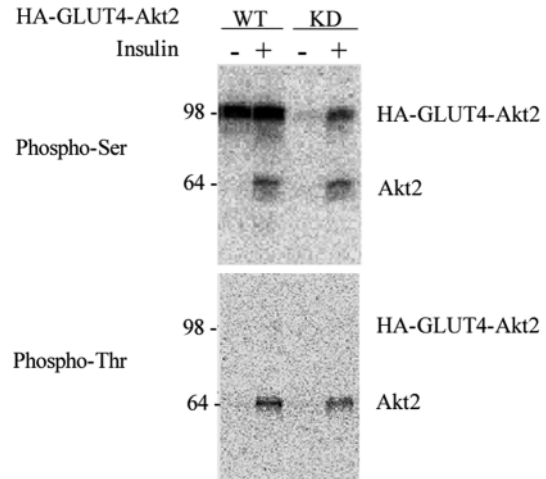
microcentrifuge tubes. After stimulation with 67 nM ( $1 \times 10^4$  microunits/ml) insulin for 30 min at 37°C, subcellular trafficking of GLUT4 was stopped by the addition of 2 mM KCN. All of the following steps were performed at room temperature. A monoclonal anti-HA antibody (HA.11, Berkeley Antibody Co.) or anti-myc antibody (Santa Cruz) was added at a dilution of 1:1000 or 1:10, respectively, and the cells were incubated for 1 hour. Excess antibody was removed by washing the cells three times with KRBH, 5% bovine serum albumin. Then 0.1  $\mu$ Ci of  $^{125}$ I sheep anti-mouse antibody (Amersham Pharmacia Biotech) was added to each reaction, and the cells were incubated for 1 hour. Finally, the cells were spun through dinonylphthalate oil to remove the unbound antibody, and the cell surface-associated radioactivity was counted in a  $\gamma$ -counter. The resulting counts were normalized to the lipid weight of the samples (Weber et al., 1998). Unless stated otherwise, the values obtained for pCIS-transfected cells were subtracted from all other values to correct for nonspecific antibody binding. Antibody binding assays were performed in duplicate or triplicate.

#### Membrane isolation and subcellular fractionation

Transfected cells were harvested after 20 hours of incubation. Crude total membrane fractions were prepared essentially as described previously (Chen et al., 1997). Homogenization and subcellular fractionation of adipose cells were carried out according to the method of Simpson et al. (Simpson et al., 1983). Briefly, cells were washed twice with TES buffer (25 mM Tris-HCl, 250 mM sucrose, 2 mM EDTA, pH 7.4) containing 0.12 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 10 g/ml aprotinin, and 10 g/ml leupeptin at 18°C, and homogenized with a Potter-Elvehjem teflon pestle. Subcellular membrane fractions, plasma membranes (PM), high-density microsomes (HDM), and low-density microsomes (LDM), were obtained by differential centrifugation.

Immunoblotting to detect expression of HA-GLUT4-Akt2 as well as Ser<sup>473</sup>- and Thr<sup>308</sup>-phosphorylated HA-GLUT4-Akt2 fusion proteins

Immunoblot analysis of HA-GLUT4-Akt2 fusion proteins was



**Fig. 2.** Phosphorylation of HA-GLUT4-Akt2 fusion proteins at Ser<sup>474</sup> and Thr<sup>309</sup> in rat adipose cells. Isolated cells were transfected with HA-GLUT4-Akt2-wt (WT) and HA-GLUT4-Akt2-K179A (KD), and cultured for 20 hours. After harvesting the cells, total cell membrane proteins were prepared from the transfected cells in the basal and insulin-stimulated states (67 nM) by differential centrifugation. 40  $\mu$ g of protein were subjected to immunoblotting with anti-Akt-phospho-Ser<sup>473</sup> or -phospho-Thr<sup>308</sup> antibody. These data are representative of 3 independent experiments.

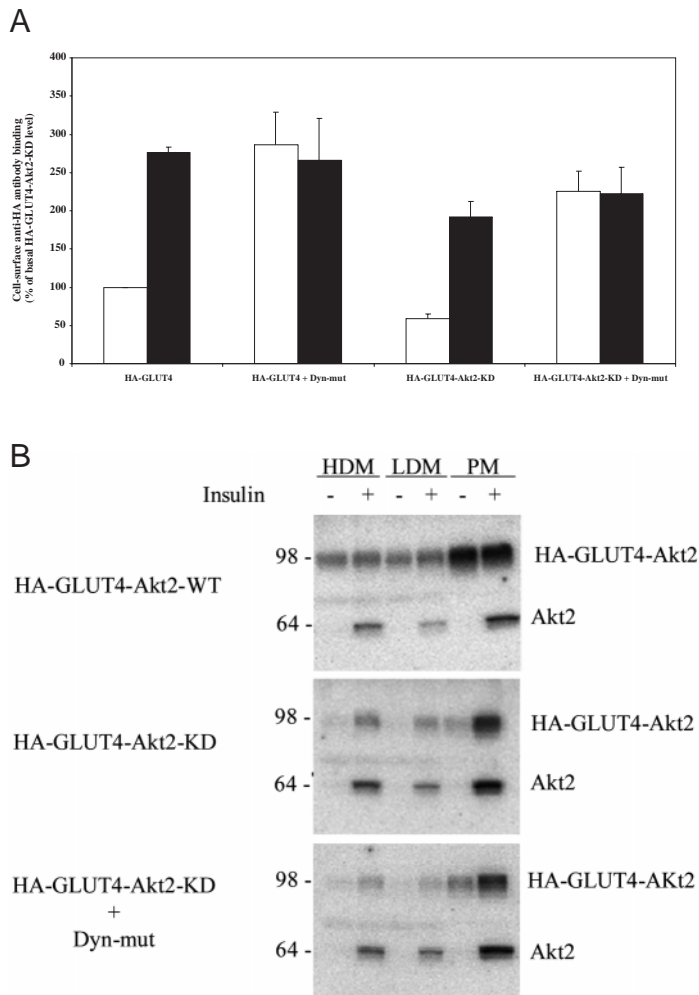
performed with a monoclonal anti-HA antibody (1:1000 dilution) and a polyclonal anti-serum against Akt-phospho-Ser<sup>473</sup> or Thr<sup>308</sup> (1:750 dilution). The crude total membrane or fractions were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters, and the filters were then incubated with 0.2  $\mu$ Ci/ml  $^{125}$ I-labeled protein A. HA-GLUT4-Akt2 and Ser<sup>474</sup>- or Thr<sup>309</sup>-phosphorylated HA-GLUT4-Akt2 fusion proteins were detected using anti-HA antibody and anti-Akt-phospho-Ser<sup>474</sup> or Thr<sup>308</sup>-specific antibodies, respectively, following the manufacturer's protocols. The signals of HA-GLUT4 and HA-GLUT4-Akt2 fusion proteins were quantified using Image Gauge V3.12, Science lab 98 (Fuji Photo Film Co., Ltd).

## Results

HA-GLUT4 with attachment of the kinase domain of wild-type, but not kinase-dead Akt2 constitutively targets to the cell surface

To examine the subcellular localization of Akt2 targeted to the GLUT4-trafficking pathway, HA-GLUT4-Akt2 fusion proteins were transfected into rat adipose cells and their cell-surface expression was detected by anti-HA antibody binding. Total expression was assessed by immunoblotting for the HA-epitope and used as a normalizing factor for cell-surface expression. As shown in Fig. 1, in the basal state (absence of insulin) cell-surface HA-GLUT4 is increased when Akt2-wt (or Akt2-DE, data not shown) is attached compared to that of HA-GLUT4 alone, and similar to that of HA-GLUT4 in the insulin-stimulated state. Insulin produces no further effect. The effect of fusing Akt2-wt to HA-GLUT4 is not blocked by wortmannin (data not shown). However, basal cell-surface HA-GLUT4 is slightly decreased with Akt2-KD (or Akt2-A3, data not shown) attached compared to HA-GLUT4 alone, although it still exhibits a normal insulin-stimulated translocation.





**Fig. 3.** Effects of dominant negative dynamin on cell-surface expression (A) and Ser<sup>474</sup> phosphorylation (B) of HA-GLUT4-Akt2-KD in rat adipose cells. Isolated cells were transfected with HA-GLUT4-Akt2-KD alone or co-transfected with HA-GLUT4-Akt2-KD and dominant negative dynamin, and cultured for 20 hours. After harvesting the cells, cell-surface levels of HA-GLUT4-Akt2-KD (A), and Ser<sup>474</sup> phosphorylation of Akt2 in membrane fractions (B) were detected in the transfected cells in the basal (white) and insulin-stimulated states (67 nM, black) using an anti-HA antibody binding assay and immunoblotting, respectively. Results in A are the means  $\pm$  s.e.m. of the means obtained from at least duplicate determinations in 3 independent experiments; results in B are representative of 2 independent experiments.

Subcellular fractionation reveals these same relationships (data not shown).

GLUT4-targeted Akt2-wt is phosphorylated on Ser<sup>474</sup>, but not Thr<sup>309</sup> in the basal state, while kinase-dead Akt2 is only Ser<sup>474</sup> phosphorylated in response to insulin

To address whether the Thr<sup>309</sup> and Ser<sup>474</sup> residues are phosphorylated when Akt2 is targeted to the GLUT4-trafficking pathway, total cell membrane proteins were isolated from adipose cells transfected with HA-GLUT4-Akt2 fusion proteins and immunoblotted with anti-Akt1-phospho-Ser<sup>473</sup>

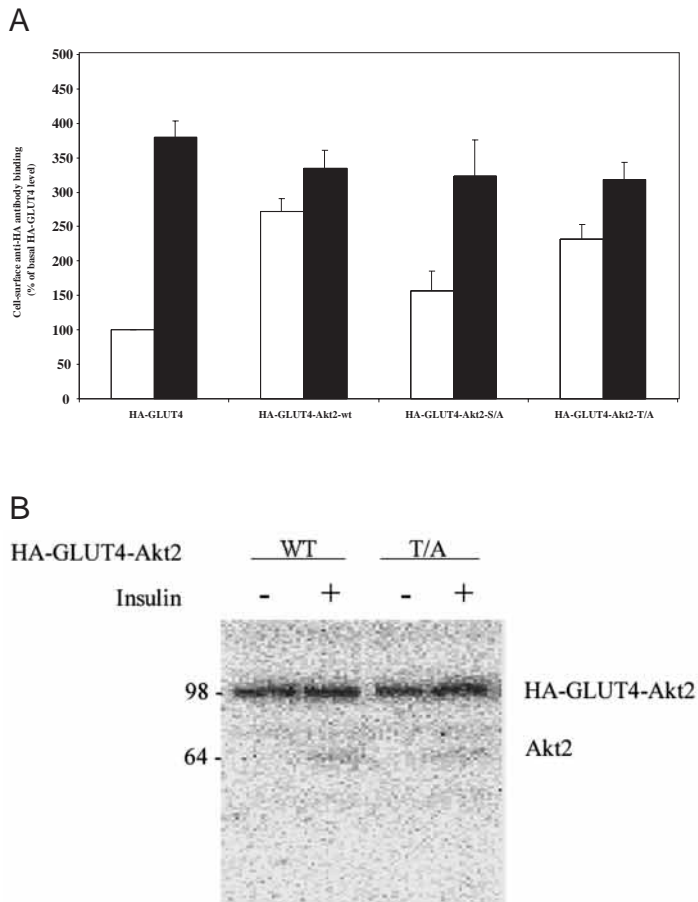
and -Thr<sup>308</sup>. As shown in Fig. 2, phospho-Ser<sup>474</sup> signals for both the fusion proteins (~95 kDa) and endogenous Akt2 (~62 kDa) are detected. HA-GLUT4-Akt2-wt is phosphorylated on Ser<sup>474</sup> in the basal state, with no further increase in response to insulin, and this Ser<sup>474</sup> phosphorylation is not inhibited by wortmannin (data not shown). In HA-GLUT4-Akt2-KD, little or no Ser<sup>474</sup> phosphorylation is detected in the basal state. Insulin treatment, however, leads to a significant increase in Ser<sup>474</sup> phosphorylation coincidentally with the marked increase in endogenous Akt2 phosphorylation and translocation of the KD fusion protein itself. Interestingly, phosphorylation at Thr<sup>309</sup> is not detectable in either HA-GLUT4-Akt2-wt or HA-GLUT4-Akt2-KD, in either the basal or insulin-stimulated state, although significant levels of Thr<sup>309</sup> phosphorylation of endogenous Akt2 are observed in the insulin-stimulated state (Fig. 2). Subcellular fractionation experiments further demonstrate that the proportions of Ser<sup>474</sup>-phosphorylated-HA-GLUT4-Akt2-wt and -HA-GLUT4-Akt2-KD relative to their respective expression levels are significantly higher in the plasma membranes than the other fractions (data not shown).

#### Coexpression of a dominant negative dynamin increases basal cell-surface expression, but not Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-KD

Whether Ser<sup>473</sup> phosphorylation of Akt occurs through an autophosphorylation mechanism remains to be determined. The data shown above suggest that Akt2 kinase activity might be required for Ser<sup>474</sup> phosphorylation. However, further evidence is needed to determine whether Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-KD in the insulin-stimulated state is mediated by endogenous activated Akt2 itself rather than another kinase located in the plasma membrane. Thus, HA-GLUT4-Akt2-KD was forced to the cell surface by co-expression of a dominant negative dynamin (Al-Hasani et al., 1998), and cell-surface expression and Ser<sup>474</sup> phosphorylation was examined. As illustrated in Fig. 3A, co-expression of mutant dynamin results in a significant increase in cell surface HA-GLUT4-Akt2-KD in the basal state, which is similar to the insulin-stimulated level. Insulin has no further effect. However, immunoblotting of both the plasma membrane fraction (Fig. 3B) and total cell membranes (data not shown) with anti-Akt1-phospho-Ser<sup>473</sup> demonstrates that forcing HA-GLUT4-Akt2-KD to the cell surface by a dominant negative mutant dynamin does not itself enhance Ser<sup>474</sup> phosphorylation to the level of GLUT4-Akt2-wt in the basal state; Ser<sup>474</sup> phosphorylation is still observed after insulin treatment where endogenous Akt2 is also activated.

#### T309A Mutation does not alter Ser<sup>474</sup> phosphorylation and cell-surface localization

In order to confirm that Ser<sup>474</sup> phosphorylation is important for targeting HA-GLUT4-Akt2 to the cell surface and that Thr<sup>309</sup> phosphorylation might not be necessary for Ser<sup>474</sup> phosphorylation when Akt2 is targeted to the GLUT4-trafficking pathway, cell-surface expression of the HA-GLUT4-Akt2-S474A and -T309A mutants was examined. The results, shown in Fig. 4, reveal that attachment of Akt2-S474A does not significantly increase the basal cell-surface level of



**Fig. 4.** Cell-surface expression (A) and Ser<sup>474</sup>-phosphorylation (B) of HA-GLUT4-Akt2-S474A and -T309A constructs in rat adipose cells. Isolated cells were transfected with HA-GLUT4, HA-GLUT4-Akt2-wt, HA-GLUT4-Akt2-S474A, and HA-GLUT4-Akt2-T309A, and cultured for 20 hours. After harvesting the cells, cell-surface levels of the constructs were determined in the transfected cells in the basal (white) and insulin-stimulated states (67 nM, black) using an HA antibody binding assay and the cell-surface-associated radioactivity was expressed relative to the basal HA-GLUT4 value in each experiment (1668, 1558, 2311 cpm). Relative cell-surface levels were then normalized to the total expression level of each construct determined by immunoblotting as described in Materials and Methods. Results are the means  $\pm$  s.e.m. of the means obtained from at least duplicate determinations in each of 3 independent experiments (A). Results in B are immunoblotting of total membrane proteins prepared from the transfected cells with anti-Akt-phospho-Ser<sup>473</sup> antibody. These data are representative of 3 independent experiments.

HA-GLUT4 compared to Akt2-wt (*t*-test,  $P > 0.05$ ), while attachment of Akt2-T309A has an effect not significantly different than that of Akt2-wt (*t*-test,  $P > 0.05$ ) (Fig. 4A). Moreover, significant levels of Ser<sup>474</sup> phosphorylation are still observed in HA-GLUT4-Akt2-T309A in the basal state (Fig. 4B).

HA-GLUT4-Akt2-wt, but not HA-GLUT4-Akt2-KD induces translocation of co-transfected myc-GLUT4 in the absence of insulin

To determine whether Akt2 targeted to the GLUT4- trafficking

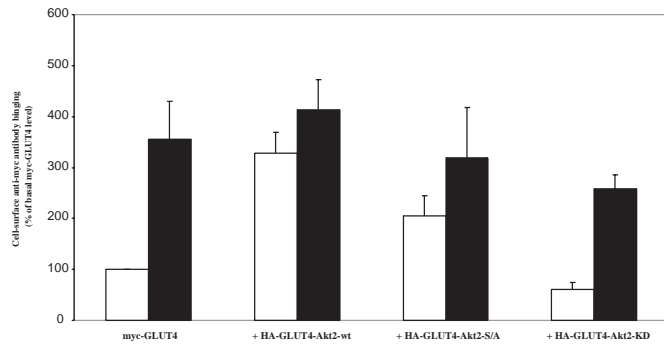
pathway is active in signaling, HA-GLUT4-Akt fusion proteins were co-transfected with myc-GLUT4 and the cell-surface expression of myc-GLUT4 was detected by an anti-myc antibody binding assay. In the absence of insulin, co-transfection of HA-GLUT4-Akt2-wt increases cell-surface myc-GLUT4 almost up to the level observed in the insulin-stimulated state, and insulin does not further enhance this effect. However, this effect is reduced by mutation of Ser<sup>474</sup> to Ala and not observed at all with HA-GLUT4-Akt2-KD (Fig. 5).

## Discussion

In this study, we used HA-GLUT4-Akt2 fusion proteins to directly study the function and subcellular distribution of Akt2 targeted to the GLUT4-trafficking pathway by assessing the phosphorylation state and cell-surface expression of the HA-GLUT4-Akt2 fusion proteins, and determining the effects of the fusion proteins on the translocation of co-transfected myc-GLUT4. Our data show that Akt2 targeted to the GLUT4-trafficking pathway is constitutively phosphorylated on Ser<sup>474</sup> but not Thr<sup>309</sup>, resides primarily on the cell surface, and activates myc-GLUT4 translocation even in the absence of insulin. Both phosphorylation state and subcellular localization are critically dependent on the presence of an active kinase domain, suggesting an autocatalytic mechanism; signaling activity is also critically dependent on the presence of an active kinase domain.

Compared to normal HA-GLUT4, the HA-GLUT4-Akt2-wt fusion protein is found predominantly on the cell-surface and in the plasma membrane fraction even in the basal state, and insulin has no further effect. In contrast, HA-GLUT4-Akt2-KD behaves like normal HA-GLUT4, including translocating to the cell surface in response to insulin. We expected that Akt2 fused to GLUT4 would be found in the GLUT4 storage compartment and were surprised that it localized primarily to the cell surface. Because expressed HA-GLUT4 targets to the insulin-responsive compartment and the HA-GLUT4-Akt2-KD construct does so as well, and because Akt2 without the PH domain contains no targeting information, it appears that the two moieties must function together. Furthermore, attaching an Akt2 moiety to HA-GLUT4 does not appear to disrupt normal processing since the HA-GLUT4-Akt2-KD exhibits all of the properties of HA-GLUT4 and GLUT4. These considerations suggest that the trafficking of the GLUT4 moiety provides the mechanism through which the fusion proteins physically associate with the cell surface and that the presence of a functional Akt2 moiety is required for this association.

In order to confirm this interpretation, we examined the phosphorylation state of the Akt2 moiety. Surprisingly, our results show that Thr<sup>309</sup> phosphorylation is not detected in HA-GLUT4-Akt2-wt regardless of its targeting to the cell surface in the absence or presence of insulin. In addition, ablation of Thr<sup>309</sup> phosphorylation by replacing Thr<sup>309</sup> with Ala does not reverse the cell-surface appearance of the HA-GLUT4-Akt2 fusion protein seen with HA-GLUT4-Akt2-wt in the basal state. Finally, translocation of GLUT4-targeted Akt2-wt is not blocked by the PI3-kinase inhibitor wortmannin (data not shown) which is known to inhibit PDK1-activated Thr<sup>309</sup> phosphorylation. In contrast, Ser<sup>474</sup> phosphorylation of HA-



**Fig. 5.** Effects of HA-GLUT4-Akt2 fusion proteins on cell-surface expression of myc-GLUT4 in rat adipose cells. Isolated cells were co-transfected with myc-GLUT4 and either nothing, HA-GLUT4-Akt2-wt, HA-GLUT4-Akt2-S474A, or HA-GLUT4-Akt2-KD, and cultured for 20 h. After harvesting the cells, cell-surface levels of myc-GLUT4 were determined in the transfected cells in the basal (white) and insulin-stimulated states (67 nM, black) using a myc antibody binding assay, and all of the values were expressed relative to the mean basal myc-GLUT4 value in each experiment as described in Materials and Methods. Results are the means  $\pm$  s.e.m. of the means obtained from at least duplicate determinations in each of 3-5 independent experiments.

GLUT4-Akt2-wt is observed in the basal state at a level similar to that observed in the insulin-stimulated state. This level of Ser<sup>474</sup> phosphorylation is not reduced by replacing Thr<sup>309</sup> with Ala nor inhibiting PI3-kinase with wortmannin (data not shown). However, mutation of Ser<sup>474</sup> to Ala in the Akt2 moiety of the fusion protein significantly reduces the association of HA-GLUT4-Akt2 with the cell surface in the basal state.

The subcellular distributions show that a significantly high proportion of expressed HA-GLUT4-Akt2-wt in both the basal and insulin-stimulated states, and HA-GLUT4-Akt2-KD in the insulin-stimulated state are phosphorylated on Ser<sup>474</sup> in the plasma membrane fraction as compared to the other fractions, suggesting that Ser<sup>474</sup> phosphorylation occurs at the plasma membrane (Carvalho et al., 2000; Scheid et al., 2002). Because the Akt2 moiety cannot be released from the HA-GLUT4, Ser<sup>474</sup> phosphorylation appears to stabilize the fusion protein complex on the cell surface.

The present study using rat adipose cells provides evidence for the concept that Ser<sup>474</sup> phosphorylation is catalyzed by Akt2 itself. For instance, in the basal state, HA-GLUT4-Akt2-wt shows Ser<sup>474</sup> phosphorylation and a cell-surface localization, while HA-GLUT4-Akt2-KD mainly remains in the intracellular sites and does not show Ser<sup>474</sup>-phosphorylation. In response to insulin, Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-KD is significantly increased, coincident with both translocation to the plasma membrane and activation of endogenous Akt2. To rule out the possibility that other kinases at the plasma membrane are responsible for Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-wt, we co-expressed dominant negative dynamin-K44A to increase basal cell-surface HA-GLUT4-Akt2-KD without activating endogenous Akt2 (Al-Hasani et al., 1998; Kao et al., 1998). This manipulation fails to enhance Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-KD in the basal state despite the marked increase in cell-surface HA-GLUT4-Akt2-KD. Significant levels of Ser<sup>474</sup> phosphorylation of HA-GLUT4-Akt2-KD are

observed only when endogenous Akt2 is activated in response to insulin, and because cell-surface GLUT4 do not recycle in the presence of the mutant dynammin, this phosphorylation appears to take place at the plasma membrane.

These data suggest that Akt2 activity itself is required for Ser<sup>474</sup> phosphorylation and that Ser<sup>474</sup> is phosphorylated through an autophosphorylation mechanism. Additional support for this concept comes from the findings that Ser<sup>474</sup> phosphorylation is correlated with Akt kinase activity. For instance, ML-9, an inhibitor of Akt activity, inhibits insulin stimulation of Akt kinase activity, GLUT4 translocation, and Akt phosphorylation on Ser<sup>474</sup>, but not on Thr<sup>309</sup> in brown adipocytes (Hernandez et al., 2001). A similar relationship is observed in adipocytes from type II diabetic subjects, i.e., both Ser<sup>474</sup> phosphorylation and full activation of Akt are impaired, while Thr<sup>309</sup> phosphorylation is much less affected (Carvalho et al., 2000).

A key finding in this study is that targeting Akt2 specifically to the GLUT4-trafficking pathway results in an insulin-like effect on Akt2 activation and GLUT4 translocation. The targeting of Akt to membranes has previously been achieved by replacing the PH domain with a membrane-targeting sequence, such as a src myristylation signal or a viral gag sequence (Cross et al., 1984; Ahmed et al., 1993). Expression of Akt constructs with a myristylation site produces a constitutive insulin-like effect similar to that observed here (Kohn et al., 1996; Kohn et al., 1996). In this case, however, the constructs are found to be bound indiscriminately to all membranes and thus do not provide information regarding a specific site of action. In the present study, targeting Akt2 specifically to the GLUT4-trafficking pathway is a direct way to examine a specific site of Akt2 action. Our data show that HA-GLUT4-Akt2-wt stimulates the cell-surface translocation of co-transfected myc-GLUT4 to a level similar to that of myc-GLUT4 itself in the insulin-stimulated state. This effect is not observed with HA-GLUT4-Akt2-KD and is decreased with the HA-GLUT4-Akt2-S/A mutant. The effects of the fusion proteins on myc-GLUT4 translocation are comparable to the behavior of the fusion proteins themselves. These data suggest that targeting Akt2 specifically to the GLUT4 trafficking pathway activates Akt2 and stimulates GLUT4 translocation in the absence of insulin. Moreover, these results together with the phosphorylation and subcellular distribution data above for the fusion proteins indicate that the plasma membrane localization is associated with Akt2 activation. With myr-Akt1, both Thr<sup>309</sup> and Ser<sup>474</sup> are phosphorylated in the absence of insulin; GLUT4-targeted Akt2-wt is only phosphorylated on Ser<sup>474</sup>, but still shows a full effect on GLUT4 translocation. This suggests that Thr<sup>309</sup> phosphorylation is necessary for targeting and Ser<sup>474</sup> phosphorylation, for Akt2 activity. However, because the Ser/Ala mutant is still partially active, it is possible that the remaining activity and cell-surface localization of HA-GLUT4-Akt2-S/A is attributable to Tyr<sup>475</sup> phosphorylation since it has recently been reported that Tyr<sup>475</sup> phosphorylation is associated with Akt2 activation (Conus et al., 2002).

While this work was in progress, the Tarvare laboratory published a report of a similar project using morphological rather than biochemical assays, and 3T3-L1 adipocytes rather than primary rat adipose cells (Ducluzeau et al., 2002). In this study, the similar fusion proteins of HA-GLUT4-Akt1-wt and



-KD both showed a predominantly cytosolic localization, and the distribution of HA-GLUT4-Akt1-KD was not changed in response to insulin (Ducluzeau et al., 2002). These discrepancies are most likely related to a combination of the different approaches used for the construction of the fusion proteins, the different cell types used, and the quantitative differences in the assay techniques. In the Tavare study, Akt1 was fused to the N terminus of GLUT4 while we fused Akt2 to the C terminus of GLUT4. As we observed previously, fusion of GFP to the N terminus, but not the C terminus, of GLUT4 interferes with the targeting of GLUT4 to the cell surface (Dawson et al., 2001). This observation suggests that the discrepancy possibly lies in the different constructs used in the two studies although the behavior of Akt fusion proteins might not be exactly the same as that of GFP fusion proteins. In addition, our observations demonstrate that Akt2 kinase activity is required for Ser<sup>474</sup> phosphorylation and association of Akt2 with the plasma membrane. For example, fusion of Akt2-KD to HA-GLUT4 does not increase basal cell-surface levels of HA-GLUT4 as the fusion of Akt2-wt does, nor is HA-GLUT4-Akt2-KD phosphorylated on Ser<sup>474</sup> in the absence of insulin. Finally, we find that GLUT4-targeted Akt2 stimulates GLUT4 translocation without the addition of insulin.

In summary, we have fused the kinase domain of Akt2-wt and various Akt2 mutants to the C terminus of HA-GLUT4 in order to investigate the activity, phosphorylation state and subcellular localization of Akt2 targeted to the GLUT4-trafficking pathway. We observe that HA-GLUT4-Akt2-wt, but not HA-GLUT4-Akt2-KD spontaneously associates with the plasma membrane in a manner similar to the response to insulin and becomes phosphorylated on Ser<sup>474</sup>, but not Thr<sup>309</sup>. HA-GLUT4-Akt2-KD translocates normally with insulin and becomes phosphorylated on Ser<sup>474</sup> in response to insulin, but is still not phosphorylated on Thr<sup>309</sup>. HA-GLUT4-Akt2-wt, but not HA-GLUT4-Akt2-KD stimulates the translocation of co-transfected myc-GLUT4. These data demonstrate that targeting Akt2 to the GLUT4-trafficking pathway induces Akt2 activation and GLUT4 translocation. Ser<sup>474</sup> phosphorylation is an autocatalytic reaction requiring an active kinase, and kinase activity is associated with a plasma membrane localization. Fusion of Akt2 to the C terminus of GLUT4 appears to substitute for Thr<sup>309</sup> phosphorylation in activating the autocatalytic process.

We thank Dena R. Yver, Mary Jane Zarnowski, and Steven R. Richards for expert technical assistance.

## References

- Ahmed, N. N., Franke, T. F., Bellacosa, A., Datta, K., Gonzales-Portal, M. E., Taguchi, T., Testa, J. R. and Tschlis, P. N. (1993). The proteins encoded by c-akt and v-akt differ in post-translational modification, subcellular localization and oncogenic potential. *Oncogene* **8**, 1957-1963.
- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B $\alpha$ . *Curr. Biol.* **7**, 261-269.
- Al-Hasani, H., Yver, D. R. and Cushman, S. W. (1999). Overexpression of the glucose transporter GLUT4 in adipose cells interferes with insulin-stimulated translocation. *FEBS Lett.* **460**, 338-342.
- Al-Hasani, H., Hinck, C. S. and Cushman, S. W. (1998). Endocytosis of the glucose transporter GLUT4 is mediated by the GTPase dynamin. *J. Biol. Chem.* **273**, 17504-17510.
- Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M. and Hemmings, B. A. (1997). Role of translocation in the activation and function of protein kinase B. *J. Biol. Chem.* **272**, 31515-31524.
- Calera, M. R., Martinez, C., Liu, H., Jack, A. K., Birnbaum, M. J. and Pilch, P. F. (1998). Insulin increases the association of Akt-2 with Glut4-containing vesicles. *J. Biol. Chem.* **273**, 7201-7204.
- Carvalho, E., Eliasson, B., Wesslau, C. and Smith, U. (2000). Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic subjects. *Diabetologia* **43**, 1107-1115.
- Cheatham, B., Vlahos, C., Cheatham, L., Wang, L., Blenis, J. and Kahn, C. R. (1994). Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol. Cell. Biol.* **14**, 4902-4911.
- Chen, H., Wertheimer, S. J., Lin, C. H., Katz, S. L., Amrein, K. E., Burn, P. and Quon, M. J. (1997). Protein-tyrosine phosphatases PTP1B and syp are modulators of insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *J. Biol. Chem.* **272**, 8026-8031.
- Conus, N. M., Hannan, K. M., Cristiano, B. E., Hemmings, B. A. and Pearson, R. B. (2002). Direct identification of tyrosine 474 as a regulatory phosphorylation site for the akt protein kinase. *J. Biol. Chem.* **277**, 38021-38028.
- Cross, F. R., Garber, E. A., Pellman, D. and Hanafusa, H. (1984). A short sequence in the p60src N terminus is required for p60src myristylation and membrane association and for cell transformation. *Mol. Cell. Biol.* **4**, 1834-1842.
- Dawson, K., Aviles-Hernandez, A., Cushman, S. W. and Malide, D. (2001). Insulin-regulated trafficking of dual-labeled glucose transporter 4 in primary rat adipose cells. *Biochem. Biophys. Res. Commun.* **287**, 445-454.
- Ducluzeau, P. H., Fletcher, L. M., Weish, G. I. and Tavaré, J. J. (2002). Functional consequence of targeting protein kinase B/Akt to GLUT4 vesicles. *J. Cell. Sci.* **115**, 2857-2866.
- Goransson, O., Reslo, S., Ronnstrand, L., Manganiello, V. and Degerman, E. (2002). Ser-474 is the major target of insulin-mediated phosphorylation of protein kinase B  $\beta$  in primary rat adipocytes. *Cell. Signal.* **14**, 175-182.
- Heller-Harrison, R. A., Morin, M., Guilherme, A. and Czech, M. P. (1996). Insulin-mediated targeting of phosphatidylinositol 3-kinase to GLUT4-containing vesicles. *J. Biol. Chem.* **271**, 10200-10204.
- Hernandez, R., Teruel, T. and Lorenzo, M. (2001). Akt mediates insulin induction of glucose uptake and up-regulation of GLUT4 gene expression in brown adipocytes. *FEBS Lett.* **494**, 225-231.
- Hill, M. M., Andjelkovic, M., Brazil, D. P., Ferrari, S., Fabbro, D. and Hemmings, B. A. (2001). Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. *J. Biol. Chem.* **276**, 25643-25646.
- Hill, M. M., Clark, S. F., Tucker, D. F., Birnbaum, M. J., James, D. E. and Macaulay, S. L. (1999). A role for protein kinase B $\beta$ /Akt2 in insulin-stimulated GLUT4 translocation in adipocytes. *Mol. Cell. Biol.* **19**, 7771-7781.
- Hill, M. M., Feng, J. and Hemmings, B. A. (2002). Identification of a plasma membrane Raft-associated PKB Ser473 kinase activity that is distinct from ILK and PDK1. *Curr. Biol.* **12**, 1251-1255.
- Holman, G. D. and Cushman, S. W. (1994). Subcellular localization and trafficking of the GLUT4 glucose transporter isoform in insulin-responsive cells. *BioEssays* **16**, 753-759.
- Kao, A. W., Ceresa, B. P., Santeler, S. R. and Pessin, J. E. (1998). Expression of a dominant interfering dynamin mutant in 3T3L1 adipocytes inhibits GLUT4 endocytosis without affecting insulin signaling. *J. Biol. Chem.* **273**, 25450-25457.
- Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.* **271**, 31372-31378.
- Kohn, A. D., Takeuchi, F. and Roth, R. A. (1996). Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J. Biol. Chem.* **271**, 21920-21926.
- Kupriyanova, T. A. and Kandror, K. V. (1999). Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin. *J. Biol. Chem.* **274**, 1458-1464.
- Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994). Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J. Biol. Chem.* **269**, 3568-3573.

- Quon, M. J., Guerre-Millo, M., Zarnowski, M. J., Butte, A. J., Em, M., Cushman, S. W. and Taylor, S. I.** (1994). Tyrosine kinase-deficient mutant human insulin receptors (Met1153→Ile) overexpressed in transfected rat adipose cells fail to mediate translocation of epitope-tagged GLUT4. *Proc. Natl. Acad. Sci. USA* **91**, 5587-5591.
- Rea, S. and James, D. E.** (1997). Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes* **46**, 1667-1677.
- Scheid, M. P., Marignani, P. A. and Woodgett, J. R.** (2002). Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol. Cell. Biol.* **22**, 6247-6260.
- Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B. and Cushman, S. W.** (1983). Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochem. Biophys. Acta.* **763**, 393-407.
- Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T.** (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**, 567-570.
- Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R. and Klip, A.** (1999). Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* **19**, 4008-4018.
- Weber, T. M., Joost, H. G., Simpson, I. A. and Cushman, S. W.** (1998). Methods for assessment of glucose transport activity and the number of glucose transporters in isolated rat adipose cells and membrane fractions. In *Receptor Biochemistry and Methodology*, Vol. 12B (ed. C. R. Kahn and L. C. Harrison), pp. 171-187. New York: Allan R. Liss.
- Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D. R.** (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* **10**, 439-448.