Use of Akt Inhibitor and a Drug-resistant Mutant Validates a Critical Role for Protein Kinase B/Akt in the Insulin-dependent Regulation of Glucose and System A Amino Acid Uptake*

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Protein kinase B (PKB)/Akt has been strongly implicated in the insulin-dependent stimulation of GLUT4 translocation and glucose transport in skeletal muscle and fat cells. Recently an allosteric inhibitor of PKB (Akti) that selectively targets PKBε and -β was reported, but as yet its precise mechanism of action or ability to suppress key insulin-regulated events such as glucose and amino acid uptake and glycogen synthesis in muscle has not been reported. We show here that Akti ablates the insulin-dependent regulation of these processes in L6 myotubes at submicromolar concentrations and that inhibition correlates tightly with loss of PKB activation/phosphorylation. Similar findings were obtained using 3T3-L1 adipocytes. Akti did not inhibit IRS1 tyrosine phosphorylation, phosphatidylinositol 3-kinase signaling, or activation of Erks, ribosomal S6 kinase, or atypical protein kinases C but significantly impaired regulation of downstream PKB targets glycogen synthase kinase-3 and AS160. Akti-mediated inhibition of PKB requires an intact kinase pleckstrin homology domain but does not involve suppression of 3-phosphoinositide binding to this domain. Importantly, we have discovered that Akti inhibition is critically dependent upon a solvent-exposed tryptophan residue (Trp-80) that is present within the pleckstrin homology domain of all three PKB isotypes and whose mutation to an alanine (PKBW80A) yields an Akti-resistant kinase. Cellular expression of PKBW80A antagonized the Akti-mediated inhibition of glucose and amino acid uptake. Our findings support a critical role for PKB in the hormonal regulation of glucose and system A amino acid uptake and indicate that use of Akti and expression of the drug-resistant kinase will be valuable tools in delineating cellular PKB functions.

It is now widely accepted that the serine/threonine kinase PKB3 (also known as Akt) acts as a major node in the cellular signaling network downstream of phosphatidylinositol 3-kinase (PI3K) regulating diverse pathways that impact upon cellular metabolism, growth, proliferation, and apoptosis (1). Activation of PKB by insulin and growth factors relies upon its recruitment to the plasma membrane. Precisely how this occurs is unclear, but the process is facilitated by the binding of 3-phosphoinositides (such as phosphatidylinositol 3,4,5-triphosphate (PIP3)) to the N-terminal pleckstrin homology domain (PH) domain of the kinase (2). PIP3 binding induces conformational changes in PKB (3) that expose two regulatory sites, Thr308 and Ser473 (in PKBα), whose phosphorylation is required for full activation of the kinase. Phosphorylation of Thr308 is mediated by PDK1, whereas that of Ser473 relies upon the TORC2 complex (4, 5). Activated PKB is then able to target a number of important downstream molecules including, for example, members of the FOXO family of forkhead transcription factors (6), the pro-apoptotic protein BAD as well as TSC2 and PRAS40, which have been implicated in regulation of the mTOR pathway (7). PKB also targets a protein called AS160, a Rab-GAP that associates with membrane vesicles harboring the insulin-regulated glucose transporter, GLUT4 (8). PKB-mediated phosphorylation of AS160 has been proposed to suppress its Rab-GAP activity, thereby permitting Rab-dependent trafficking of GLUT4 to the plasma membrane where the transporter facilitates an increase in glucose uptake (9). Another important physiological PKB substrate is glycogen synthase kinase-3 (GSK3) whose phosphorylation is considered significant in alleviating the suppressive effect that GSK3 exerts upon glycogen synthesis, which in response to insulin, is thought to be simultaneously dephosphorylated and activated by protein phosphatase 1G (10). Consequently, through its ability to reg-

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1 The abbreviations used are: PKB, protein kinase B; PI3K, phosphoinositide 3-kinase; Akt, Akt inhibitor; PKC, protein kinase B and C; GSK3, glycogen synthase kinase 3; mTORC2, mammalian target of rapamycin complex 2; IRS, insulin receptor substrate; PDK1, 3-phosphoinositide-dependent kinase-1; GLUT4, glucose transporter 4; SNAT2, sodium-dependent neutral amino acid transporter 2; ERK, extracellular signal-regulated kinase; RSK, ribosomal S6 kinase; GRP1, general receptor of phosphoinositides 1; PIP3, phosphatidylinositol 3,4,5-triphosphate; PH, pleckstrin homology; Me-ALB, α-methylaminobutyrate; GST, glutathione S-transferase; wt, wild type; GFP, green fluorescent protein; DNP, 2,4-dinitrophenol; P(3,4)P2, d-phosphatidylinositol 3,4-diphosphate; GAP, GTPase-activating protein.

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ulate AS160 and GSK3, PKB-directed signaling serves to coordinate the insulin-dependent increase in glucose uptake and storage of glucose as glycogen in tissues such as skeletal muscle.

Our current appreciation of the diverse cellular functions regulated by PKB stems from a variety of technical approaches that include, for example, (i) suppression of PKB function (via expression of dominant-interfering PKB constructs or small interfering RNA-mediated knock-down of endogenous PKB), (ii) overexpression of specific PKB isoforms to mimic gain of function, and (iii) generation of genetic mouse models that either lack a single or combination of PKB isoforms (1, 11). Although such approaches have undoubtedly helped to define PKB substrates and functions, there is concern that experimental artifacts may arise from a loss or gain in PKB function in cells or the organism as a whole due to adaptation or compensation for changes in cellular PKB expression. Consequently, there is considerable interest in the development of small molecule inhibitors of PKB that will not only help to further characterize new substrates and kinase function but may be of therapeutic value in the treatment of conditions in which PKB is either overexpressed or chronically activated as, for example, in certain cancers (12). The generation of specific PKB inhibitors that act in an ATP-competitive manner has been hindered by the significant homology that exists within the ATP binding pocket of kinases that belong to the AGC (protein kinase A/protein kinase G/protein kinase C) kinase family. However, recent work detailing the structure activity relationships of PKB inhibitors that act at sites out with the highly conserved ATP binding domain represents a promising development with which to further delineate cellular roles of PKB (13). Lindsley et al. (13) described a tricyclic quinoxaline compound (termed 16h) that exhibited dual specificity for PKBo and PKBβ (Akt1 and Akt2, respectively), hereon referred to as Akti. This inhibitor was deemed to act allosterically, as kinase inhibition was non-competitive with respect to ATP and peptide substrate but dependent on the presence of the PH domain given that antibodies directed to this region of PKB antagonized inhibition by Akti (14). In the present study we have investigated the effects of Akti on the hormonal activation of PKB in cultured L6 myotubes and 3T3-L1 adipocytes and assessed how this impacts upon important cellular responses such as nutrient (glucose and amino acid) uptake and glycogen synthesis. We also present novel data showing that the inhibition of PKB by Akti is dependent upon a critical tryptophan residue (Trp-80) within the PH domain of the kinase and that mutation of this residue results in the generation of a drug-resistant form of the kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—α-Minimal essential medium, Dulbecco’s modified Eagle’s medium, and fetal bovine serum, and antibiotic/antimycotic solution were from Invitrogen. All other reagent grade chemicals including insulin and bovine serum albumin were obtained from Sigma-Aldrich. Wortmannin was purchased from Calbiochem-Novabiochem, C2-ceramide was obtained from Sigma-Aldrich. Wortmannin was purified from Tocris (Bristol, UK). Complete protein phosphatase chase from Calbiochem, C2-ceramide was purchased from Roche Diagnostics. d-[U-14C]Glucose was purchased from GE Healthcare, and [α-14C]methylaminoisobutyrate (Me-AIB) and 2-deoxy-1-[3H]glucose were from PerkinElmer Life Sciences. Phospho-PKB (Thr308), phospho-PKB (Ser[27/28]), phospho-p42/p44 MAPK (mitogen-activated protein kinase; Thr[202/Tyr[204]), phospho-GSK-3α/β (Ser[21/99]), phospho-AMP-activated protein kinase Thr[172], and anti-PAS antibodies were from Cell Signaling Technology (Beverly, MA). Anti-phosphatidylinositol 3-kinase p85, anti-IRS1, anti-AS160 antibodies were from Upstate Cell Technology (Beverly, MA). Anti-phosphatidylinositol 3-kinase p85, anti-IRS1, anti-AS160 antibodies were from Upstate Cell Signaling Solutions, phospho-p70S6K (Thr[389]) was from Santa Cruz, and anti-GST antibodies were a gift from Graham Hardie (University of Dundee). Synthetic peptides for generating isoform-specific antibodies against PKBγ, pan antibodies against PKB, PKCζ/λ and phospho-PKCζ/λ (Thr[410/413]), cross-tide, and PKI (peptide inhibitor of cAMP-dependent protein kinase) were synthesized by Dr Graham Bloomberg at the University of Bristol.

**PKB Inhibitor (Akti) Compound Synthesis**—The PKB α/β inhibitor was synthesized in-house based on a modified synthetic procedure reported previously by Lindsley et al. (13). Purity of the synthesized compound (termed Akti hereon or AKT inhibitor VIII in the Calbiochem catalogue) was established as >98% by 1H NMR and liquid chromatography-mass spectrometry and is a derivative of Akti-1/2a (a compound originally termed Akti-1/2 in Ref. 15) but possesses increased potency.

**Cell Culture and Cell Transfection**—L6 rat skeletal muscle cells were grown as described previously (16) in α-minimal essential medium containing 2% (v/v) fetal bovine serum and 1% (v/v) antimycotic-antibiotic solution. For some experiments, mononucleated L6 myoblasts were transfected using FuGENE with 1 μg of DNA encoding GST-tagged wild type PKBo or GST-tagged PKBoW80A and then maintained in culture until they differentiated into myotubes. 3T3-L1 cells were maintained in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) donor calf serum. Adipocyte differentiation was then induced by adding isobutylmethyloxanthine (100 μM), dexamethasone (0.25 μM), and insulin (1 μg/μl) for 2 days followed by 2 days culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and insulin (1 μg/μl) and then cultured only in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum for the duration of the differentiation period. Differentiated cells were usually harvested at 10 days after confluence.

**Cell Lysis**—L6 myotubes and 3T3-L1 adipocytes were incubated for the time and with the appropriate amount of effectors described in the figure legends. After treatment, cells were rinsed twice with ice cold phosphate-buffered saline and then lysed using lysis buffer (50 mM Tris/HCl, pH 7.4, 0.27 M sucrose, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, and protease inhibitors). Whole-cell lysates were centrifuged (15,000 × g at 4 °C for 10 min), and supernatants were removed and stored at −80 °C until required.

**Immunoblotting**—Cell lysates were subjected to SDS/PAGE and immunoblotted as previously reported (16). Nitrocellulose membranes were probed with various antibodies described in
the figure legends. Detection of primary antibodies were performed using appropriate peroxidase-conjugated IgGs, and protein signals were visualized using enhanced chemiluminescence by exposure to Kodak autodadiagnostic film. Quantification of immunoblots was done using Image J software.

Glucose and Amino Acid Uptake—L6 myotubes were incubated with insulin and/or Akti or wortmannin for times and at concentrations indicated in the figure legends. Cells were washed 2 times with Hepes-buffered saline (140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM MgSO₄, and 1 mM CaCl₂, pH 7.4). Glucose uptake was assayed by incubation with 10 μM 2-deoxy-D-[3H]glucose (1 μCi/ml) for 10 min as described previously (16). For amino acid transport, cells were incubated for 10 min with 10 μM [14C]Me-AIB (47.6 kBq/ml). Nonspecific binding was determined by quantifying cell-associated radioactivity in the presence of 10 μM cytochalasin B or a saturating dose of unlabeled Me-AIB. Medium was aspirated before washing adherent cells 2 times with 0.9% (w/v) ice-cold NaCl. Cells were subsequently lysed in 50 mM NaOH, and radioactivity was quantified using a Beckman LS 6000IC scintillation counter. Protein concentration in cell lysates was determined using the Bradford reagent (17).

Glycogen Synthesis—L6 myotubes were incubated with insulin and/or Akti or wortmannin for times and at concentrations indicated in the figure legends. Cells were washed twice with Hepes-buffered saline (140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM MgSO₄, and 1 mM CaCl₂, pH 7.4). Glycogen synthesis was assayed by incubation with 10 μM [14C]glucose (2 μCi/ml) for 1 h. The incubation was terminated by 2 washes with ice-cold 0.9% (w/v) NaCl before lysis in 60% (w/v) KOH. Cellular glycogen was precipitated from lysates using a method adapted from that described previously (18), and associated radioactivity was determined by liquid scintillation counting. Glycogen synthesis was expressed as a rate/mg of cell protein.

Immunofluorescence and GRP1 Translocation—The cell surface recruitment of the general receptor for phosphoinositides (GRP1) was used to assess stimulus-dependent intracellular synthesis of 3-phosphoinositides. DNA constructs (1 μg) encoding the PH domains of wild type (wt) GRP1 and GRP1 harboring a point mutation within this domain (K273A, which affects the 3-phosphoinositide binding properties of PKB, a protein Lubricant overlap assay was carried out using GST fusion proteins of wild type PKBα or PKBα harboring a W80A mutation within the PH domain as described previously (19, 20). Briefly, sn-2-stearoyl-3-arachidonyl (1,2)-dioleoylphophatidylinositol 3,4-diphosphate (PI(3,4)P₂) dissolved in chloroform/methanol/water (1:2:0.8) was spotted at concentrations indicated in the figures onto Hybond C-Extra membranes (Amersham Biosciences) and allowed to dry for 1 h at room temperature. Membranes were blocked with 5% bovine serum albumin (w/v) in Tris-buffered saline Tween (TBST; 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature. Membranes were then incubated in TBST containing 0.5 μg/ml concentration of either wt-PKB-GST or PKBW80A-GST with or without Akti at the concentrations indicated in the figure legends before being washed with TBST and incubation with an anti-GST antibody (1:1000). Membranes were then probed with anti-mouse horseradish peroxidase, and immunoreactivity was visualized by enhanced chemiluminescence.

Immunoprecipitation—For immunoprecipitation of IRS1, protein G-Sepharose beads (20 μl) were washed 3 times in phosphate-buffered saline (150 mM NaCl, 2.68 mM KCl, 12 mM NaH₂PO₄, 1.77 mM KH₂PO₄, pH 7.4) and incubated with anti-IRS1 for 4 h at 4 °C on an orbital platform shaker. Bead-antibody mix was washed 3 times in phosphate-buffered saline and incubated with 500 μg of L6 cell lysate protein overnight at 4 °C before washing, SDS-PAGE, and immunoblotting. For immunoprecipitation of RSK, PKBα, PKBγ, AS160, or GST, 500 μg of cell lysate protein was incubated at 4 °C for 1 h on a shaking platform with antibodies against the indicated proteins coupled to 10 μl of protein G-Sepharose. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 mM NaCl and once with 1 ml of buffer A (50 mM Tris, pH 7.5, 100 μM EGTA).

Analysis of Kinase Activities—The standard assay (50 μl) contained washed protein G-Sepharose immunoprecipitate, 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 2.5 μM PKI (TTYADFIAASGRTGRRNAIHD, peptide inhibitor of cyclic-AMP-dependent protein kinase), 10 mM magnesium chloride, 0.1 mM [32P]ATP (200 cpm/pmol), and 30 μM crosstide (GRPRRTSSFAEG) for assay of endogenous RSK and PKB activities (21). Assays were carried out for 30 min at 30 °C with continuous agitation to keep the immunoprecipitate in suspension and terminated by applying 40 μl of the reaction mixture onto P81 membranes. The P81 membranes were washed in phosphoric acid, and the incorporated radioactivity was measured by scintillation counting 1 million of kinase activity was that amount of enzyme that catalyzed the phosphorylation of 1 pmol of substrate in 1 min.

In some experiments GST-PKBα, GST-ΔPH-PKBα, and GST-PKBαW80A were expressed in 293 cells. Cells were stimulated with 20 ng/ml IGF1 for 15 min before lysis, and the activated GST-tagged kinases were immunoprecipitated and assayed as described above in the absence and presence of Akti at the concentrations indicated in the relevant figure.

In Vitro PKB Activation Using Recombinant PDK1 and mTORC2—In vitro activation of recombinant PKB using purified PDK1 is a two-step process and was performed as follows. During the first step 0.5 μg of GST–PKBα was added to a reaction mixture (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 2.5 μM PKI, 10 mM MgCl₂, 100 μM ATP, 100 μM phosphatidylserine, 100 μM phosphatidylincholine, 10 μM phosphatidyl inositol 3,4,5-trisphosphate) that either contained or lacked Akti at the concentrations indicated in fig-
PDK1 (10 ng) was added to the assay mixture for 30 min at 30 °C to activate PKB. This reaction was either terminated by the addition of 10 μl of 5× SDS buffer, and the readout for PKB activation by PDK1 was assessed on the basis of PKB Thr308 phosphorylation or, alternatively, followed by a second step in which phosphorylation of PKB peptide substrate was determined. This second step was initiated by the addition of 100 μM [γ-32P]ATP and 100 μM crosstide in buffer containing 50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1% mercaptoethanol, 2.5 μM PKI, 10 mM MgCl₂. The reaction was allowed to proceed for 10 min...
at 30 °C after which period it was terminated by spotting the reaction mixture onto p81 phosphocellulose paper and quantitating radioactivity as described above.

For in vitro activation of PKB using mTORC2, we used a slight modification of a procedure reported previously (22). Rictor was immunoprecipitated from 293 cells, and the protein G-Sepharose immunoprecipitate was washed and stored in kinase buffer containing 25 mM Hepes, pH 7.5, 50 mM KCl. 0.5 μg of GST–PKBα was added to the mixture in the absence or presence of 1 μM Akti for periods indicated in the figure legends. The reaction was initiated by the addition of buffer containing 0.1 mM ATP, 10 mM magnesium acetate and allowed to proceed for 30 min at 30 °C. Reactions were terminated by the addition of 10 μl of 5× SDS buffer, and samples were resolved by electrophoresis on 10% acrylamide gels and immunoblotted with PKB or phospho-PKBSer473 antibodies.

PI3K—Recombinant heterodimeric complexes of p110α/p85 or monomeric p110y (1 μg protein each) were used to assess the effects of Akti on in vitro PI3K activity using phosphatidylinositol as the substrate based on adaptation of a previous method (23). Assays were initiated with the addition of pre-mixed substrate vesicles and 5 mM ATP that included 5μCi of [γ-32P]ATP (Amersham Biosciences) and incubated at 37 °C for 30 min. The assay was terminated by the addition of 600 μl of 80:40:1 (by volume) CH3OH, CH2Cl2, 12 mM HCl. After the addition of a further 200 μl of CHCl3 and 320 μl of 0.1 M HCl, samples were mixed by vortexing and separated by centrifugation at 14,000 × g for 1 min. After removal of the upper phase, the lower phase was washed with a further 780 μl of synthetic upper phase, and the upper phase was again removed to waste. The final lower phase containing the product lipids was then neutralized with 15 μl of 1.0 M NH4OH in methanol and dried under vacuum. Lipids were finally dissolved in 30 μl of 2:1 CHCl3:CH3OH (by volume) and separated by thin-layer chromatography before final detection of [32P]phosphatidylinositol 3-phosphate using a Fuji phosphorimager.

Statistical Analyses—For multiple comparisons, statistical analysis was performed using one way analysis of variance. Data analysis was performed using GraphPad Prism software and considered statistically significant at p values < 0.05.

RESULTS

Akti Blocks the Insulin-dependent Activation of PKB in L6 Myotubes—To establish the efficacy of Akti in our experimental system, we assessed phosphorylation of PKB on its two regulatory sites (Ser473/474/472 and Thr308/309/305 in PKBα/β/γ, respectively) and activity in whole cell lysates. Fig. 1, A–C, shows that PKB phosphorylation and activation was reduced substantially in a dose-dependent manner being suppressed completely when muscle cells were preincubated with 0.5 μM Akti for 60 min. Based on in vitro inhibition studies (13, 14), Akti is commercially marketed as a selective inhibitor of PKBα/β (i.e., Akt1 and Akt2). Consequently, we were surprised that L6 myotubes, which primarily express PKBα and PKBγ (16, 25), did not display any residual PKB phosphorylation that could have been attributed to the gamma (Akt3) isoform after treatment with insulin and 1 μM Akti (Fig. 1A). To assess whether PKBγ was also sensitive to Akti, we immunoprecipitated the kinase using an isoform-specific antibody (25) and monitored its hormonal activation in cells that had been incubated in the absence or presence of Akti. Fig. 1D shows that insulin induced a 7-fold increase in PKBγ activity that was reduced in a dose-dependent manner (similar to PKBα) by Akti. It is important to stress that immunoprecipitating PKBγ did not deplete cellular PKBα activity, indicating that the PKBγ antibody does not cross-react with PKBα (data not shown). In separate experiments we also expressed and monitored the phosphorylation of GST-tagged PKBγ in L6 muscle cells, and consistent with the inhibition of native γ-kinase activity, the insulin-dependent (Thr305) phosphorylation of the expressed GST kinase was lost in cells treated with 1 μM Akti (Fig. 1E). In line with the observed inhibition of PKB, Akti caused an associated loss in GSK3 phosphorylation and that of AS160; both of which are established physiological downstream targets of PKB (Fig. 1, A and F), whereas tyrosine phosphorylation of IRS1 or phosphorylation/activation of Erk1/Erk2 and that of RSK were unaffected (Fig. 1, A and G). It has been suggested that atypical PKCζ/λ may play an important role in the hormonal activation of glucose transport in skeletal muscle (26). To exclude the possibility that Akti may also target atypical PKCs and thereby confound analysis of downstream PKB-regulated events, we assessed the effects of insulin and Akti on PKCζ/λ activation in L6 myotubes. Intriguingly, we could not observe any detectable activation of atypical PKCs in response to insulin despite observing a robust hormonal activation of PKB in the same population of muscle cells that remained fully sensitive to Akti (Fig. 1H). We have previously shown that atypical PKCs can be activated by ceramide, whereas the hormonal activation of PKB is antagonized by this sphingolipid (27). In line with our previous
Akti Does Not Inhibit PI3K Activity, IRS-1/p85 Association, or Insulin-dependent Glucose Uptake, Glycogen Synthesis, and System A Amino Acid Transport

Akti does not inhibit PI3K activity in vitro, IRS1/p85 association, or insulin-dependent glucose uptake, glycogen synthesis, and System A amino acid transport.

Akti Inhibits Insulin-dependent Glucose Uptake, Glycogen Synthesis, and System A Amino Acid Transport

The inhibition of PKB by Akti involves a loss in PI3K activation in vivo. We subsequently assessed the effects of the compound on the ability of insulin to induce association of the regulatory p85 subunit of PI3K with tyrosine-phosphorylated IRS1 proteins. We observed a 50–70% increase in p85 abundance in IRS1 immunoprecipitates from insulin-stimulated L6 myotubes, which did not decline upon coinubcation of myotubes with insulin and Akti but which was reduced in cells incubated with the PI3K inhibitor, wortmannin (Fig. 2B). These findings imply that it was highly unlikely that association of p85 with IRS1 serves as a target site for Akti action.

To further substantiate that Akti was unlikely to suppress PI3K, we transfected L6 myoblasts with the PH domain of GRP1 fused to GFP. The PH domain of wt-GRP1 acts as a sensitive and selective probe for detecting the generation of PIP(3,4)P_2 or PIP(3,5)_2, and its translocation to the plasma membrane in response to insulin correlates tightly with cellular production of these 3-phosphoinositides (28). In unstimulated L6 cells the GRP1-GFP fusion protein was predominantly perinuclear but also distributed diffusely throughout the cytosolic compartment (Fig. 3a). Cell stimulation with insulin induced a striking redistribution of the PH-GRP1-GFP to the cell periphery (Fig. 3b) that was suppressed markedly in cells that had been preincubated with wortmannin (Fig. 3c). Incubation of transfected myoblasts with wortmannin or 1 μMAkti alone had no effect upon the basal localization of PH-GRP1-GFP (Fig. 3, d and e). Although wortmannin curtailed the insulin-dependent recruitment of GRP1 to the plasma membrane, pre-treatment of muscle cells with 1 μM Akti did not restrain translocation of the GFP fusion protein to the cell periphery (Fig. 3f), suggesting that the inhibitor does not affect insulin-dependent 3-phosphoinositide synthesis or degradation. As an additional control we also transfected myoblasts with a PH-GRP1-GFP fusion construct harboring a point mutation of a critical lysine residue (Lys-273) within the PH domain (K273A) did not exhibit any detectable change in cellular distribution of the mutated GFP fusion protein upon treatment with insulin (Fig. 3g and h).
gen synthesis (~4-fold) but that activation of both processes is lost upon coincubating cells with Akti in a dose-dependent manner and that this tallies with the attendant loss in PKB activation (Fig. 4A immunoblots). Insulin also promotes activation of system A amino acid transport in skeletal muscle cells via a PI3K-dependent mechanism, but hitherto it has been uncertain as to whether PKB is involved in this activation. Fig. 4C shows that Akti also abolishes insulin-stimulated system A transport as assessed using Me-AIB, a non-metabolizable substrate for this amino acid transport system. Consistent with the involvement of PI3K in the hormonal regulation of glucose uptake, glycogen synthesis and system A amino acid transport, the ability of insulin to stimulate all three cellular processes was blocked by wortmannin (Fig. 4, A–C).

To establish that inhibition of insulin-stimulated glucose uptake in response to Akti is primarily due to a targeted loss in PKB activation, we also monitored the effects of Akti on glucose uptake in response to mitochondrial/hypoxic stress. We used 2,4-dinitrophenol (DNP), an uncoupler of mitochondrial oxidation, which has been shown to induce an increase in glucose uptake by recruiting glucose transporters to the plasma membrane of L6 myotubes independently of insulin (30). Fig. 5A shows that pretreatment of muscle cells with DNP led to a 2-fold stimulation in glucose uptake. However, unlike the stimulation induced by insulin, the increase in glucose uptake elicited by DNP was insensitive to Akti. This finding is consistent with our observation that DNP does not induce any detectable increase in PKB activation (Fig. 5B) and is also in line with previous work showing that DNP-stimulated glucose uptake in L6 cells is mediated via a PI3K-independent mechanism (30).

DNP-induced mitochondrial uncoupling would be expected to promote a reduction in cellular ATP and cause a concomitant activation of AMP-activated protein kinase. The lower panel of Fig. 5B shows that this was indeed the case, but unlike the insulin-independent activation of PKB, activation of AMP-activated protein kinase by DNP was unaffected by Akti.

To exclude the possibility that Akti-mediated inhibition of insulin-stimulated glucose uptake is restricted to our muscle cell line we also investigated effects of the inhibitor on PKB activation and glucose uptake in cultured 3T3-L1 adipocytes. Fig. 6A shows that insulin caused a robust activation of glucose uptake (~6.5-fold) and that of PKB and Erk signaling as judged by phospho-blot analysis. Consistent with our muscle cell data, Akti induced a striking dose-dependent reduction in glucose uptake that correlated closely with the loss in PKB phosphorylation (Fig. 6A and B). In contrast, the inhibitor had no detectable effect on insulin-stimulated Erk1/2 phosphorylation, which remained sensitive to the PI3K inhibitor, wortmannin (Fig. 6A).

A Critical Tryptophan Residue (Trp-80) within the PH Domain of PKB Confers Sensitivity to Akti—Barnett et al. (14) previously demonstrated that Akti inhibition requires the presence of an intact PKB PH domain. In an attempt to further understand the mechanism by which the drug inhibits PKB we reviewed previous work reporting the crystal structure of the PH domain of PKBα complexed with inositol head group of PIP_3 (3). An important finding to emerge from that work was the significant conformational change that occurs in the ligand binding pocket of the PH domain as well as in variable loops two and three (VL2 and VL3). Intriguingly, localized at the apex of
Effects of Akti on Insulin Action

FIGURE 5. Effects of Akti on DNP-stimulated glucose uptake in L6 myotubes. Muscle cells were treated as indicated with 0.5 mM dinitrophenol (1 h), 100 nM insulin (30 min), and/or 1 μM Akti (1 h) before assaying 2-deoxyglucose (2-DG) uptake or cell lysis (A) to assess PKB Thr308 and AMP-activated protein kinase Thr172 phosphorylation using 30 μg of cell lysate protein (B). The bar graph shows mean values ± S.E. from three separate uptake experiments each conducted in triplicate, whereas the blots are representative of two experiments.

VL3 is a tryptophan residue (Trp-80 in PKBα and β and Trp-79 in PKBγ, Fig. 7A) that, unusually for a hydrophobic residue, is completely solvent-exposed and which may, therefore, be important in facilitating interactions between PKB and other organic molecules. To test this proposition, we compared the effect of Akti on the in vitro activity of wild type PKBα, PKBα lacking the PH domain, or PKBα in which the tryptophan residue was replaced with alanine (PKBαW80A). Fig. 7B shows that Akti induces a dose-dependent inhibition of preactivated PKB with an IC50 of ∼0.1 μM, whereas PKBα lacking the PH domain or harboring a point (W80A) mutation were effectively rendered insensitive to Akti.

The requirement for an intact PH domain led us to test whether inhibition of PKB by Akti involves impaired 3-phosphoinositide binding to the kinase. Fig. 7C shows a protein-lipid overlay analysis in which the in vitro binding of PI(3,4)P2 and PI(3,4,5)P3 to PKB was assessed in the absence and presence of Akti at two different pharmacological concentrations. The spot intensity is indicative of protein/lipid association and reveals that whereas PKB binds both PI(3,4)P2 and PI(3,4,5)P3 with different efficiencies, the inhibitor has no effect on binding of either lipid when present at concentrations greatly in excess (i.e. 10–100-fold) of that which causes maximal inhibition of PKB activity within cells (Fig. 1) or in in vitro based assays (Fig. 7B). Consistent with our observation that activation of PKBW80A is not sensitive to Akti (Fig. 7B) the inhibitor also has no detectable effect on 3’-phosphoinositide binding to PKBW80A (Fig. 7C).

The dose response data shown in Fig. 7B indicate that Akti causes maximal inhibition of PKBα that has been preactivated at concentrations within the 1–10 μM range. However, our cell-based observations (Figs. 1 and 6) indicate that the hormonal activation of PKB is prevented at submicromolar concentrations of the inhibitor. These findings raise the possibility that Akti may be more efficacious against the inactive/unphosphorylated kinase and that the mode of inhibition may differ depending on whether the inhibitor is presented to PKB in its inactive or active state. To test this, we determined the effects of Akti on PKB activation by its upstream kinase PDK1 in vitro. Fig. 8A shows that in the absence of Akti, PDK1 induces a robust activation and phosphorylation of PKB. However, inclusion of the inhibitor in the in vitro assay significantly blunts PDK1-dependent activation of PKB with an IC50 of 0.35 μM. We show in Fig. 8B that if the inhibitor is present during the entire 30 min in vitro assay, it not only prevents the activation of PKB but also severely restricts phosphorylation of PKBThr308 and PKBSer473 by PDK1 and mTORC2, respectively. Intriguingly, however, if the inhibitor is absent during the first 15 min of the assay to allow activation of PKB by either of the two upstream kinases and then subsequently added to the assay for the last 15 min, it abolishes the catalytic PKB activity without any significant loss in phosphorylation of PKBThr308 or PKBSer473.

To further substantiate the importance of PKBThr308 in Akti action, we subsequently expressed wt PKBα-GST and PKBαW80A-GST in L6 muscle cells. Acute insulin treatment induced phosphorylation of both the endogenous and GST-tagged PKB proteins on Thr308 (Fig. 9A). Preincubation of muscle cells with Akti completely suppressed phosphorylation of the wild-type GST-activated protein kinase on Thr308, whereas that of the PKBαW80A-GST was retained (Fig. 9A). Because PKBαW80A-GST was expressed in higher amounts than the wild-type GST-tagged kinase in this set of experiments, we quantified the phospho-immunoblots by normalizing the band intensities with respect to GST expression, this analysis confirmed that whereas the wild type kinase was inhibited by Akti, the PKBαW80A mutant retains its drug resistance when expressed in cells (Fig. 9B).

Muscle Cells Expressing PKBαW80A-GST Exhibit Akti-resistant Insulin-stimulated Glucose and Amino Acid Transport—Given that PKBαW80A exhibits insensitivity to Akti, we postulated that expression of this mutant form of the kinase should confer resistance against the drug with respect to activation of key endpoints of insulin action such as glucose and system A amino acid transport. To test this proposition we transiently transfected wt-PKBα, PKBαW80A, and performed a mock (empty vector) transfection as control. Insulin induces a robust phosphorylation of the endogenous PKB, the GST-tagged wt-PKB,
and of the PKB\textsuperscript{W80A} proteins on Thr\textsuperscript{308}. In line with the data presented in Fig. 9A, phosphorylation of PKB-Thr\textsuperscript{308} was abolished in muscle cells transfected with the empty vector (mock) or wild type PKB after cell pretreatment with 1 \(\mu\)M Akti but retained in those expressing the mutated PKB\textsuperscript{W80A} (Fig 10A). Consistent with preserving the ability to activate PKB\textsuperscript{W80A} in the presence of Akti, we find, unlike in mock or wt-PKB-GST transfected cells, phosphorylation of GSK3, which lies downstream of PKB, is also retained in cells expressing the drug-resistant mutant (PKB\textsuperscript{W80A}) kinase. Because the hormonal activation of PKB is critically dependent upon 3-phosphoinositide synthesis, pretreatment of muscle cells with wortmannin substantially reduced the insulin-dependent phosphorylation of both wt-PKB and PKB\textsuperscript{W80A} and that of its downstream target, GSK3 (Fig. 9A). Having established that we had expressed Akti-sensitive and Akti-resistant forms of PKB in our

FIGURE 6. Effects of Akti on insulin-stimulated glucose uptake and PKB and Erk phosphorylation in 3T3 L1 adipocytes. 3T3 L1 adipocytes were treated as indicated with Akti for 1 h at the concentrations shown with 100 nM wortmannin (20 min) and 100 nM insulin (30 min) before assaying 2-deoxyglucose (2-DG) uptake or lysis and SDS-PAGE analysis/immunoblotting to determine PKB and Erk phosphorylation (\(p\�textsuperscript{-}\), \(A\), \(B\)). PKB phospho blots were quantified and expressed as a \(\pm\) fold signal change relative to cells that had not been treated. The bar values in \(A\) and \(B\) represent the mean \(\pm\) S.E. from three separate experiments. The asterisk indicates a significant change (\(p < 0.05\)) from the untreated control.

FIGURE 7. Effects of Akti on PKB activity and 3-phosphoinositide binding. A, sequence alignment for a 50-amino acid stretch of the PH domain of PKB\(\alpha, \beta,\) and \(\gamma\) highlighting the conserved tryptophan residue that is localized at the apex of variable loop 3. B, Akti was incubated at the concentrations indicated with either preactivated wt PKB\(\alpha\) (WT-PKB\(\alpha\)), PKB\(\alpha\) lacking its PH domain (PKB\(\alpha\)-\(\Delta\)PH), or PKB\(\alpha\) harboring a point mutation within its PH domain (PKB\(\alpha\)-W80A). Kinases were preactivated in 293 cells using IGF-1, immunoprecipitated, and assayed as described under “Experimental Procedures.” The in vitro phosphorylation of a synthetic peptide “crosstide” was used as a readout of kinase activity. C, nitrocellulose membranes were spotted with 1 \(\mu\)l of PIP\(_3\) or P(3,4),P\(_3\), containing the lipid amounts indicated. Membranes were then incubated overnight at 4 \(^\circ\)C in TBS buffer containing wild type GST-PKB\(\alpha\) (0.5 \(\mu\)g/ml) in the absence or presence of Akti at the concentrations indicated or with PKB\textsuperscript{W80A} alone. Membranes were subsequently washed, and lipid-bound PKB was detected by probing with an anti-GST antibody.

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muscle cells, we subsequently assayed glucose and amino acid uptake in the same population of cells using a dual (³H/¹⁴C) isotope labeling protocol (16). In these experiments insulin stimulated glucose and amino acid uptake by up to 2-fold. Consistent with our earlier data (Fig. 4), pretreatment of muscle cells expressing the empty vector or wt-PKB with 1/₁₉₂₆ M Akti or wortmannin abolished the hormonal stimulation of both processes. However, muscle cells expressing PKBW₈₀A still exhibited a significant insulin stimulation of glucose and amino acid uptake in the presence of Akti but which remains fully sensitive to wortmannin (Fig. 10B). It should be stressed that the residual sensitivity to Akti that is observed in our PKBW₈₀A-expressing cells is fully in keeping with the efficiency (~60%) with which L6 cells are transfected with PKBW₈₀A.

**DISCUSSION**

The original structure-activity report for Akti (13) indicates that this quinoxaline derivative inhibits PKB isoforms in vitro with IC₅₀ values within the nanomolar to low micromolar range (PKBα, 58 nM; PKBβ, 210 nM; PKBγ, 2119 nM). More recent analysis carried out at the University of Dundee against a panel of 70 different protein kinases supports selective Akti inhibition of PKBα, PKBβ, and the calcium/calmodulin-dependent protein kinase 1 (CamK1) when used at a concentration of 1 μM or less (15). The inhibitor is commercially touted as a selective PKBα/β (Akt1/2) inhibitor based on the relative IC₅₀ values for the three PKB isoforms. However, despite the reported differences in the in vitro PKB isoform inhibition profiles, our studies using L6 myotubes, which express a sizable PKBγ activity (16, 25), indicate that Akti is also effective at targeting the γ isoform (Akt3) when used in cell-based assays at submicromolar concentrations. These observations suggest that the mechanism by which the inhibitor targets the three PKB isoforms in vitro may differ compared with that in the intracellular environment (Fig. 11). These differences are likely to arise because the in vitro effects of Akti involve assessing the effects of the inhibitor upon the activated kinase, as shown for example in Fig. 7 of this study, whereas in cell-based assays the goal is to assess how it affects activation of the inactive kinase in response to a stimulus, such as insulin. Because Akti is able to attenuate PKBγ activation much more effectively within cells than seen in vitro, it is very likely that the inhibitor is considerably more efficacious in preventing the transition of the kinase from an inactive to active state than inhibiting the activity of a kinase that is already active. This proposition is supported by the data presented in Fig. 8A that indicate that Akti is much more potent in suppressing the in vitro activation of inactive/unphosphorylated PKBα (IC₅₀ 0.35 nM) compared with its ability to suppress the activity of the preactivated kinase (Fig. 7B, IC₅₀ ~ 100 nM). However, despite the increased efficacy of the inhibitor in cell-based assays, our findings (Fig. 1, c and 1d) indicate that the sensitivity of PKBγ to Akti is lower than that of PKBα as established previously using in vitro assays (13).
Inhibition of PKB by Akti is critically dependent upon the presence of an intact PKB-PH domain, but hitherto the precise role that this domain plays with respect to Akti action has been poorly understood (14). Close inspection of the PH domain amino acid sequence reveals that all three PKB isoforms have a conserved tryptophan residue (Trp-80 in PKBα, see sequence alignment, Fig. 7A) localized at the tip of variable loop 3, which unexpectedly for a hydrophobic residue is completely solvent-exposed. Analysis of the high resolution crystal structure of the PKB-PH domain complexed with the head group of PIP3 has unexpectedly for a hydrophobic residue is completely solvent-exposed. Analysis of the high resolution crystal structure of the PKB-PH domain complexed with the head group of PIP3 has been challenged with a maximally effective insulin concentration. The authors postulated that additional insulin-stimulated, but PKB-independent mechanisms may contribute to the modest increase in GLUT4 translocation/glucose uptake that persists in the presence of Akti (33). One potential mechanism suggested to account for the residual increase in glucose uptake involved the atypical PKCs (i.e. PKCζ/λ), which have been implicated by some investigators in the hormonal activation of glucose uptake in both skeletal muscle and adipose tissue (26, 34). However, our data do not support this possibility as insulin did not appear to cause any detectable activation of PKCζ, and as such, the kinase was insensitive to Akti even when activated in response to cell treatment with ceramide in L6 myotubes (Fig. 1F). Consequently, if atypical PKCs are involved in the regulation of insulin-stimulated glucose uptake, then they are likely to play a very marginal role in the cells used in our study. Consistent with this proposition, it is noteworthy that studies involving expression of constitutively active or dominant interfering forms of atypical PKCζ or RNAi-based gene silencing in 3T3-L1 adipocytes fail to support a significant role for PKCζ in the hormonal stimulation of glucose uptake (35, 36). One possible explanation for the inconsistency that exists between our findings and those reported by Gonzalez and McGraw (33) may pin on the relative efficacy of Akti used in the two studies. Gonzalez and McGraw (33) noted that although their commercial source of Akti completely blocks PKB activation and the associated increase in adipocyte glucose uptake in response to low insulin concentrations (1 nM), the inhibitor was only partially effective when cells were stimulated with a higher insulin concentration (170 nM). In contrast, our preparation of Akti, when used at the same concentration (1 μM) as that by Gonzalez and McGraw (33), remains effective in its ability to suppress PKB activation and glucose uptake in muscle or fat cells that have been challenged with a maximally effective insulin concentration (100 nM). Because PKB requires only modest activa-
to promote an increase in glucose uptake (16, 37), it is not inconceivable that if Akti fails to fully suppress PKB activation, the residual kinase activity may serve to partially induce GLUT4 translocation and glucose uptake as reported by Gonzalez and McGraw (33).

The data presented in this study strongly suggest that PKB mediates the principal signal feed for stimulating glucose uptake in response to insulin but excludes its involvement in regulating this process in response stress agents such as DNP. Akti had no suppressive effect on glucose uptake induced by DNP, an observation that is in accord with previous work showing that this mitochondrial uncoupling agent stimulates carrier translocation and glucose uptake by a mechanism that does not depend on PI3K signaling (30). It is possible that stress-induced signals may either feed in at a point downstream of PKB or act to regulate by alternative means distal components that regulate trafficking of glucose transporters in response to both insulin or cell stress. The differential effect that Akti has upon insulin and DNP-stimulated glucose uptake provides additional, albeit indirect evidence that this inhibitor selectively targets PKB and cellular responses that are directly under its physiological control. This tenet is further upheld when one considers regulation of glycogen synthesis. The activity of glycogen synthase is regulated by phosphorylation/dephosphorylation and allosterically by glucose 6-phosphate (10, 38, 39). The activation of glycogen synthase involves its dephosphorylation on multiple amino acid residues (including those that are GSK3 target sites) by the glycogen-associated form of protein phosphatase 1 (PP1G). However, for efficient enzyme (glycogen synthase) activation, phosphorylation and inactivation of GSK3 is also considered important as this alleviates its negative regulation of glycogen synthase in muscle (10, 38, 39). The insulin-dependent inactivation of GSK3β involves PKB-catalyzed phosphorylation of Ser21 or Ser9, respectively. Consequently, inhibition of PKB would result in constitutive activation of GSK3 and, by extension, sustained inhibition of glycogen synthase. Consistent with this scenario, Akti reduces insulin-dependent phosphorylation of GSK3 and the stimulation of glycogen synthesis in L6 myotubes. Because Akti also suppresses insulin-stimulated glucose uptake, an additional factor halting
glycogen synthase activation would be the reduced allosteric drive that would normally be provided by accumulation of glucose 6-phosphate. Collectively, our findings underscore the critical role that PKB activation plays with respect to not only stimulating glucose uptake but "channeling" the incoming glucose for storage as glycogen, whose synthesis is greatly facilitated by stimulating glycogen synthase as a result of an increase in glucose 6-phosphate and GSK3 inactivation.

The system A carrier belongs to the SLC38 family of solute transporters that currently comprise three proteins, SNAT1–3, that mediate the cellular uptake of short-chain neutral amino acids (41). System A is extensively regulated by changes in substrate availability with expression and stability of the SNAT2 protein being enhanced in cells subjected to amino acid withdrawal (42, 43). In addition to this adaptive increase in SNAT2 protein, System A activity has long been known to be stimulated acutely by insulin (32, 44, 45), growth factors (31), and cell stresses (31, 32). These stimuli induce a rapid increase in the $V_{\text{max}}$ of system A transport, which involves molecules participating in proximal insulin and growth factor signaling. We have previously reported that the stimulatory effects of insulin and IGF-1 on system A can be blocked by inhibitors of PI3K (45) and that expression of a constitutively active form of PKB enhances amino acid uptake in cultured muscle cells (16). Hitherto, it has been difficult to exclude the possibility that the ability of PKB to stimulate system A transport in these latter studies may have been a consequence of changes in transporter expression associated with stable overexpression of PKB (16). However, the observation that exposure of L6 muscle cells and 3T3-L1 adipocytes to 1 μM Akti inhibits the acute insulin activation of system A transport provides compelling evidence that PKB is a fundamental component of the signaling pathway promoting the hormonal activation of this transporter in these two cell types. It is highly unlikely that SNAT2 is a direct physiological target for PKB given that we have been unable to identify any putative PKB phosphorylation motifs within regions of the

![Diagram of PKB inhibition by Akti](image)

FIGURE 11. Proposed model of PKB inhibition by Akti **in vitro** and **in vivo**. A, in the absence of Akti, preactivated PKB is able to phosphorylate its peptide substrate **in vitro**. The addition of Akti to the **in vitro** assay results in its association with the PH domain of PKB, which may sterically hinder substrate access to the active site of the kinase and/or affect the catalytic activity of the kinase, thus, promoting a loss in substrate phosphorylation. This loss in activity is seen despite phosphorylation of PKB on its two regulatory sites. B, in **in vivo**, the stimulation of cells with insulin promotes IRS1/PI3K activation, PIP3 synthesis, and the recruitment and activation of PKB via phosphorylation of Thr308 and Ser473 by PDK1 and TORC2, respectively. PKB phosphorylation involves a conformational change in the kinase PH domain. Activated PKB then targets downstream molecules such as GSK3 and AS160, which promote GLUT4 (G4) translocation and stimulate glucose and amino acid uptake and glycogen synthase (GS). Incubation of cells with Akti does not affect IRS1/PI3K activation and PIP3 synthesis but disrupts PKB activation. PKB inhibition depends upon Akti binding to the PH domain (via Trp-80) and impairing the conformational change in the PH domain that is required to permit phosphorylation of Thr308 and Ser473 by PDK1 and TORC2, respectively.

2-DG, 2-deoxyglucose.
transporter that are predicted to be exposed to the cytoplasmic compartment. Because hormonal activation of system A involves recruitment of SNAT2 from an endosomal compartment (24), it is plausible that PKB may target molecules associated with the trafficking and/or fusion of SNAT2-containing vesicles with the surface membrane. One such molecule is AS160, a Rab-GAP implicated in the regulation of GLUT4 translocation. It is thought that in unstimulated cells, the GAP activity of AS160 helps restrain the movement of GLUT4 to the cell surface by maintaining Rab proteins participating in the GLUT4 trafficking process in their GDP-bound form. However, in response to insulin, PKB activation results in increase in AS160 phosphorylation, which is thought to inactivate its associated GAP activity and thereby relieve its inhibitory restraint upon GLUT4. Our findings indicate that Akti suppresses the insulin-dependent phosphorylation of AS160 on PKB target sites, which is likely to reduce transporter translocation and contribute to the functional loss in insulin-stimulated glucose transport. It remains unknown at present if AS160 also participates in control of SNAT2 trafficking, and although there is no experimental evidence to support intracellular colocalization of GLUT4 and SNAT2, it is plausible that trafficking of SNAT2 from the endosomal compartment may depend upon a protein that functions in a manner analogous to AS160. Establishing whether AS160 or some other molecular target for PKB regulates SNAT2 translocation and/or carrier activity, thus, remains an important investigative goal of future studies.

In summary, we have characterized biochemically the action of Akti both in vitro and in vivo and established that PKB plays a pivotal role in the insulin regulation of glucose uptake, glycogen synthesis, and system A-mediated amino acid transport in cultured skeletal muscle cells. In addition to PKBα and PKBβ, our studies also indicate that Akti is effective at targeting PKBγ in cell-based assays and that the molecular basis by which it selectively targets PKB isoforms involves a critical tryptophan residue within the kinase PH domain. Mutation of this important solvent-exposed hydrophobic residue yields a drug-resistant kinase whose expression along with use of the inhibitor ought to serve as a useful tool in delineating the role played by PKB in cellular metabolism, growth, and survival.

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