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The Identification of ATP-citrate Lyase as a Protein Kinase B (Akt) Substrate in Primary Adipocytes*

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Protein kinase B (Akt) plays a central role in cellular regulation, although many of the physiologically relevant substrates for the kinase remain to be identified. In this study, we have isolated a protein from primary epididymal adipocytes with an apparent molecular weight of 125,000. This protein exhibited immunoreactivity, in an insulin-dependent manner, with a phosphospecific antibody raised against the protein kinase B substrate consensus sequence R*X*R*XX*(pS/pT) as well as a phosphospecific antibody that recognizes serine 21/9 of GSK-3*α*/*β*. MALDI-TOF mass spectrometry revealed the protein to be ATP-citrate lyase, suggesting that the two phosphospecific antibodies recognize phosphoserine 454, a previously reported insulin- and isoproterenol-stimulated ATP-citrate lyase phosphorylation site. Indeed, both insulin and isoproterenol stimulated the phosphorylation of this protein on the site recognized by the phosphospecific antibodies in a wortmannin-sensitive and -insensitive manner, respectively. In addition, transient expression of a constitutively active protein kinase B in primary adipocytes mimicked the effect of insulin on ATP-citrate lyase phosphorylation. Furthermore, ATP-citrate lyase was phosphorylated *in vitro* by recombinant protein kinase B on the same site. Taken together, these results demonstrate that serine 454 of ATP-citrate lyase is a novel and major *in vivo* substrate for protein kinase B.

The protein kinase B (PKB,[1] also known as Akt) family comprises three isoforms (PKB*α*, -*β*, and -*γ*) that are highly conserved serine/threonine kinases originally identified through their homology to the transforming retroviral oncogene v-Akt, cAMP-dependent protein kinase (PKA) and protein kinase C (1–3). PKB was subsequently found to be activated by a variety of stimuli such as insulin, growth factors, G-protein-coupled receptor agonists, and integrin binding and to function downstream of phosphoinositide 3-OH-kinase (PI3K) (reviewed in Refs. 4 and 5).

PKB is involved in numerous cellular responses to insulin and growth factors. This includes suppression of apoptosis, at least in part through its ability to phosphorylate the pro-apoptotic protein BAD (6, 7), and the regulation of gene expression at the levels of both transcription (via transcription factor phosphorylation (8–10)) and translation (via the regulation of eI4E-binding proteins (11)).

PKB is also involved in the stimulation of glucose uptake by insulin in muscle and adipose tissues. Transgenic mice that lack PKB*β*, the isoform of PKB that predominates in adipose tissue, have markedly impaired glucose tolerance and are insulin-resistant and diabetic (12). PKB has been implicated in mediating insulin-stimulated glucose uptake, at least in part through stimulating the translocation of the insulin-responsive glucose transporter, GLUT4, to the plasma membrane (13–15). PKB also plays an important role in the stimulation of glycogen synthase by insulin via the phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3*α* and GSK-3*β* (16)).

In many cases, for example GLUT4 translocation, the substrates of PKB that subsequently mediate the response have not been discovered. To identify potentially novel PKB targets in adipocytes, we have utilized a proteomic approach using a commercially available antibody raised against the minimal PKB consensus phosphorylation site found on almost all of its known substrates, R*X*R*XX*(pS/pT) (17, 18). In the process of our study, we identified ATP-citrate lyase (ACL) as a novel PKB substrate in primary rat adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Except where otherwise stated, all chemicals were from BDH (Lutterworth, Leicester, UK), and biochemicals were from Sigma Chemical Co. (Poole, Dorset, UK). Adenosine 5′-[^{32}P]trisphosphate was from ICN Radiochemicals (Basingstoke, UK). Plasmids containing wild-type PKB (HA-PKB), constitutively active myristoylated PKB (Myr-PKB), and constitutively active p110 subunit of PI3K (p110.CAAX) were kindly provided by Dr. D. Alessi (University of Dundee), Dr. B. Hemmings (Friedrich Miescher Institute, Basel), and Dr. J. Downward (Cancer Research UK, London), respectively. All the PKB sequences included an N-terminal HA tag and were subcloned into the vector pcDNA3 (Invitrogen) for expression in rat adipocytes. Anti-ACL antisera, raised in either rabbit or chicken, were provided by Prof. R. M. Denton (University of Bristol). Male Wistar rats (150–200 g) were fed *ad libitum* on a stock laboratory diet (B&K Universal Ltd., Hull, UK).

Preparation and Incubation of Epididymal Fat Cells—Adipocytes were isolated from the epididymal fat pads of Wistar rats as described previously (19). Cells were subsequently washed in Krebs-bicarbonate-HEPES buffer, pH 7.4 (130 mM NaCl, 4.7 mM KCl, 1.5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 15.5 mM $NaHCO_3$, 10 mM HEPES, and 11 mM glucose) without bovine serum albumin. The cells were pre-treated with 100 nM wortmannin or 10 *μ*M U0126 (Promega, Southampton, UK) for 30 min as indicated in the figure legends and subsequently incubated with 83 nM insulin or 10 nM isoproterenol for 10 min as required. The reaction was terminated by extracting the cells 1:1 (packed cell volume/volume) in ice-cold Nonidet P-40 extraction buffer (50 mM Tris, pH 7.5, containing 1 mM EDTA, 120 mM NaCl, 50 mM NaF, 40 mM *β*-glycerophosphate, 1 mM benzamidine, 1% Nonidet P-40, 1 *μ*M micro-

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[1] The abbreviations used are: PKB, protein kinase B (or Akt); PKA, cAMP-dependent protein kinase; PI3K, phosphoinositide 3-OH-kinase; ACL, ATP-citrate lyase; MALDI-TOF, matrix-assisted laser-desorption time of flight; GSK-3, glycogen synthase kinase-3; HA, hemagglutinin; MAPK, mitogen-activate protein kinase.

cystin, 7.2 mM mercaptoethanol, 5 mM orthovanadate, and 1 μg/ml each of pepstatin, leupeptin, and antipain). Alternatively, the cells were extracted (1:1, v/v) in ice-cold TES extraction buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 250 mM sucrose, 2 mM Na_3VO_4, 10 mM NaF, 1 mM $Na_4P_2O_7$, 100 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin, antipain, and leupeptin) for subsequent protein sub-fractionation studies. Cell extracts were centrifuged at 10,000 × *g* for 10 min at 4 °C, and the infranatant was taken for subsequent analysis.

Transfection of Primary Adipocytes—Cells were prepared as described previously (19) and washed in intracellular Krebs buffer (4 mM NaCl ,125 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 2.5 mM NaH_2PO_4, 15.5 mM $NaHCO_3$, 10 mM HEPES, and 11 mM glucose). A 0.4-cm electrode gap Gene Pulser cuvette (Bio-Rad, Richmond, CA) was used to electroporate 500 μl of cells (30% cytocrit) in the presence of 5 μg of plasmid DNA. Electroporation was performed by administering six shocks at 600 V and a capacitance of 25 microfarads using a Gene Pulser Transfection apparatus (Bio-Rad). After electroporation, cells were transferred to a 30-ml Universal tube (Bibby-Sterilin Ltd., Staffs, UK) and incubated for 30 min at 37 °C before replacing the medium with 4 ml of Dulbecco's modified Eagle's medium, pH 7.4 (containing 1% bovine serum albumin, 2 mM glutamine, 200 nM phenylisopropyladenosine, 100 μg/ml gentamicin, and 25 mM HEPES). The cells were incubated for another 5.5 h at 37 °C and 5% CO_2 and subsequently washed into Krebs-bicarbonate-HEPES buffer without bovine serum albumin prior to the experiment.

Protein Sub-fractionation—Cell extracts prepared as described above were clarified by centrifugation at 10,000 × *g* for 10 min and filtered using a 0.22-μm syringe-driven filtration unit to remove fat particles and loaded onto an Amersham Biosciences SMART system Mono-Q column PC 1.6/5 previously equilibrated in buffer A (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM dithiothreitol, and 1 μg/ml each of pepstatin, antipain, and leupeptin). A linear gradient of buffer A containing 1 M NaCl was developed over 2.5 ml at a flow rate of 50 μl/min, and 200-μl fractions were collected. Following Western blotting with the anti-$PKB_{substrate}$ antibody, Mono-Q fractions that contained the M_r 125,000 candidate PKB substrate band were pooled and applied to the SMART system Mono-S column PC 1.6/5, previously equilibrated in buffer A. Samples were fractionated against a linear gradient of buffer A containing 1 M NaCl developed over 2.5 ml at a flow rate of 100 μl/min, and 200-μl fractions were collected. Elution of the M_r 125,000 protein was determined by Western blotting, and its apparent purity was assessed by Coomassie Blue staining. The M_r 125,000 band was excised from the gel and digested using sequencing grade porcine-modified trypsin (Promega). The peptides produced were analyzed by MALDI-TOF using a PE Biosystems Voyager-DE STR MALDI-TOF mass spectrometer with a 337-nm nitrogen laser. The matrix used was made from 10 mg/ml α-cyano-4-hydroxycinnamic acid (recrystallized) in a 1:1 mixture of acetonitrile (Rathburn, Peebleshire, UK) and 0.1% trifluoroacetic acid. The spectrum was acquired over a range of 800–3500 Da, under linear conditions with an accelerating voltage of 2000 V and an extraction delay time of 125 ns. Samples were calibrated using trypsin autolysis products as internal standards. MALDI-TOF data was searched against the NCBInr data base using the ProFound website (20).

Immunoprecipitation of ACL—ACL was immunoprecipitated by incubating 2.5 μg (dry weight) of protein A-Sepharose beads and 5 μl of rabbit anti-ACL antiserum with 200 μl of cell lysate for 2 h at 4 °C. The beads were washed three times in extraction buffer and once in PKB assay buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 1 mM EDTA) and subsequently used for *in vitro* phosphorylation. Alternatively, cell lysates (400 μl) were pre-cleared by centrifugation at 70,000 × *g* for 30 min at 4 °C, and the supernatant was incubated with 10 μl of chicken anti-ACL antiserum for 2 h at 4 °C. The immunocomplexes were pelleted by centrifugation at 70,000 × *g* for 30 min at 4 °C and resuspended in Laemmli sample buffer.

Western Blotting—Protein samples were separated by SDS-PAGE on 7.5% acrylamide gels prior to transfer to a polyvinylidene difluoride membrane. Western blots using the anti-$PKB_{substrate}$ and phospho-GSK-3 antibodies (both from Cell Signaling Technology, Beverly, MA) were performed according to the manufacturer's recommendations. The chicken anti-ACL antiserum was used at a 1:1000 dilution. In all cases, immunoreactive bands were visualized with the use of a horseradish peroxidase-coupled anti-rabbit or anti-chicken secondary antibodies (Amersham Biosciences) as appropriate at a dilution of 1:10,000 followed by enhanced chemiluminescence detection (Amersham Biosciences).

In Vitro Phosphorylation—Rabbit anti-ACL immunoprecipitates were incubated for 30 min with 780 milliunits of recombinant PKB (a gift from Dr. Dario Alessi, University of Dundee, UK) in the presence of 50 μM ATP (40 kBq/mmol) and 5 mM $MgCl_2$, made up to 25 μl with PKB assay buffer. Reactions were stopped after 30 min by the addition of Laemmli sample buffer, prior to separation by 7.5% SDS-PAGE. Phosphorylation was visualized by autoradiography or Western blotting with the anti-$PKB_{substrate}$ antibody.

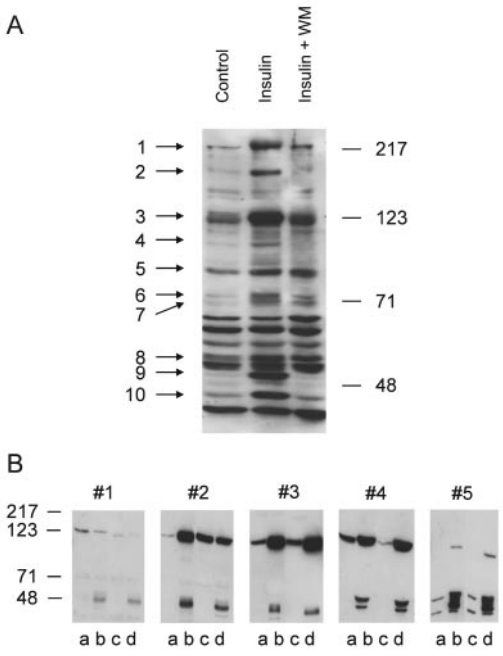

FIG. 1. **The use of an anti-$PKB_{substrate}$ antibody to identify novel substrates of protein kinase B.** *A*, rat adipocytes were preincubated in the absence or presence of 100 nM wortmannin (*WM*) for 30 min and subsequently stimulated with insulin (83 nM) for 10 min. Cells lysates were subjected to Western blotting with the $PKB_{substrate}$ antibody. Bands that display insulin-dependent phosphorylation are denoted with *arrows*. The migration of the molecular weight markers ($M_r \times 10^{-3}$) is indicated. *B*, rat adipocytes were preincubated in the absence (*a*, *b*) or presence of 100 nM wortmannin (*c*) or 100 μM U0126 (*d*) for 30 min. The cells were then further incubated in the absence (*a*) or presence (*b–d*) of 83 nM insulin for 10 min. Lysates were fractionated by Mono-Q chromatography as described under "Experimental Procedures." Fractions eluting were Western-blotted with the anti-$PKB_{substrate}$ antibody. Only fractions 1–5, corresponding to 0–200 mM NaCl, are shown. The migration of molecular weight markers ($M_r \times 10^{-3}$) is indicated. Results are typical of three experiments.

RESULTS

Insulin-stimulated Protein Phosphorylation in Primary Rat Epididymal Fat Cells—Freshly isolated primary adipocytes derived from rat epididymal fat pads were incubated in the absence or presence of insulin and lysates were subjected to SDS-PAGE followed by Western blotting with a phosphospecific antibody (anti-$PKB_{substrate}$) raised against the consensus sequence found in almost all known PKB substrates R*X*R*XX*(pS/pT). As shown in Fig. 1*A*, insulin stimulated the phosphorylation of many proteins, including those of apparent M_r 217,000, 181,000, 125,000, 107,000, 95,000, 78,000, 75,000, 52,000, 51,000, and 48,000 (indicated as *bands 1–10*, respectively, in Fig. 1*A*). Pre-treatment of the cells in the presence of the PI3K inhibitor wortmannin blocked the insulin-stimulated phosphorylation of many of these phosphoproteins (Fig. 1*A*), suggesting that they are regulated by one or more mechanisms downstream of PI3K.

Interestingly, the pattern of phosphorylation observed using the anti-$PKB_{substrate}$ antibody was very similar to the pattern of ^{32}P-labeled phosphoproteins observed after insulin stimulation of primary rat adipocytes metabolically labeled with [^{32}P]P_i (21, 22). This suggests that this antibody recognizes the majority of the most abundant insulin-stimulated phosphoproteins in rat fat cells.

We next incubated primary adipocytes in the presence of wortmannin or U0126 (which inhibits MAPK activation), followed by continued incubation in the absence or presence of insulin for 10 min. Subsequently, the cell lysates were subjected to Mono-Q anion exchange chromatography by SMART fast-protein liquid chromatography, and the resultant fractions were analyzed by Western blotting using the anti-$PKB_{substrate}$ antibody (Fig. 1*B*). Of particular interest was an abundant protein with M_r 125,000, which eluted from the Mono-Q column at low salt concentrations in fractions 2–4 (0–120 mM NaCl). The reactivity of the M_r 125,000 protein with the anti-$PKB_{substrate}$ antibody was low under basal conditions and increased significantly after insulin treatment in a wortmannin-sensitive but U0126-insensitive manner (Fig. 1*B*). A further three proteins of apparent M_r 48,000, 51,000, and 52,000, which were also wortmannin-sensitive and U0126-insensitive, eluted later in fractions 4 and 5 (120–200 mM NaCl, Fig. 1*B*). Taken together, these data indicate that the phosphorylation of the M_r 48,000, 51,000 52,000, and 125,000 proteins is regulated in a PI3K-dependent but MAPK-independent manner. Therefore, each of these four proteins represent potential insulin-stimulated PKB substrates.

Purification and Identification of the M_r 125,000 Phosphoprotein—The M_r 125,000 protein was subjected to further purification. To do this we took the peak fraction from the Mono-Q separation for further purification by cation exchange chromatography on a Mono-S column using SMART fast protein liquid chromatography. The resulting fractions were analyzed by Western blotting with the anti-$PKB_{substrate}$ antibody, and this revealed that the majority of the M_r 125,000 protein did not bind significantly to the column, being recovered in the column flow-through.[2] Coomassie Blue staining of the flow-though revealed the M_r 125,000 protein to be sufficiently pure to be excised from the gel, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. The experimentally determined tryptic peptide masses were then subjected to analysis using ProFound, and the identity of the protein band was confirmed as ATP-citrate lyase (ACL; probability = 1.00 and *Z* score = 2.37). Western blotting the Mono-S fraction with either of two distinct polyclonal antibodies to ACL verified the identity of the protein.[2]

Further Analysis of ACL Phosphorylation Using Phosphospecific Antibodies—Insulin has been previously reported to stimulate the phosphorylation of ACL in a wortmannin-sensitive manner (23), although the kinase involved has not been identified. Insulin has also been reported to induce the phosphorylation of a single serine on ACL in rat adipocytes, and this site has been identified as serine 454, which lies in the sequence ^{446}TPAPSRTA**S**F^{455} (24–26). Serine 454 of ACL bears considerable similarity to the consensus sequence for phosphorylation by PKB (R*X*R*XX*(pS/pT)Ψ, where Ψ is a bulky hydrophobic residue (17, 18)). Although the arginine in the −5 position is absent in the serine 454 sequence of ACL, it possesses a phenylalanine at +1, and relatively small amino acids at −1 and −2 (alanine and threonine, respectively). Consistent with the idea that PKB could phosphorylate serine 454, the threonine at the −2 position is reported to be particularly favored by PKB, and alanine is well tolerated (17, 18). Furthermore, the on-line motif alignment program Scansite (27) gives serine 454 a probability score of 0.2196, which is equivalent to the PKB sites in BAD and FKHRL1, which are known *in vivo* PKB substrates.

Concomitant to the induction of ACL serine 454 phosphorylation, insulin reduces the phosphorylation of serine 450 and threonine 446, which lie in the −4 and −8 positions relative to serine 454. These residues are known substrates for phosphorylation by glycogen synthase kinase-3 (GSK-3 (28–31)). GSK-3 is inhibited via phosphorylation by PKB on serine 21 (GSK-3α) and serine 9 (GSK-3β, reviewed in Ref. 32). The sequence surrounding serine 454 of ACL very closely resembles the PKB phosphorylation sites on GSK-3α and GSK-3β (^{449}PSRTA**S**F^{455} *versus* ^{16}RARTS**S**F^{22} and ^{4}RPRTS**S**F^{10}, respectively). Interestingly, therefore, we also found that a phosphospecific antibody raised against phosphoserine 21/9 of GSK-3α/β was reactive with ACL by Western blotting and that this reactivity was stimulated by insulin in a wortmannin-sensitive but U0126-insensitive manner (Fig. 2*A*).

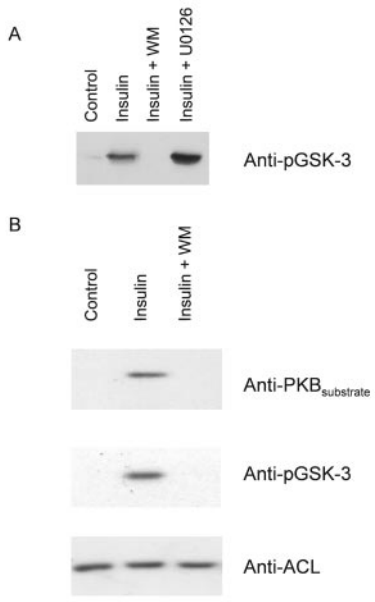

FIG. 2. **The anti-$PKB_{substrate}$ and anti-phospho-GSK-3α/β antibodies recognize ACL after insulin stimulation.** *A*, Mono-Q fraction 4 from untreated (*Control*), insulin-treated (*Insulin*), insulin and wortmannin-treated (*Insulin + WM*), and insulin and U0126-treated (*Insulin + U0126*) rat adipocytes were prepared as for Fig. 1*B* and Western-blotted with a phosphospecific antibody recognizing serine 21/9 of GSK-3α/β. *B*, ACL was immunoprecipitated with the chicken anti-ACL antibody from cell lysates prepared as described in the legend to Fig. 1. The immunoprecipitates were Western-blotted with the anti-$PKB_{substrate}$ antibody (*upper panel*), the phospho-GSK-3 antibody (*middle panel*), or the chicken anti-ACL antiserum (*lower panel*).

To confirm that the anti-$PKB_{substrate}$ and phospho-GSK-3 antibodies were reacting specifically with ACL, the enzyme was immunoprecipitated with anti-ACL antibodies from primary adipocyte lysates and then Western blotted with phosphospecific antibodies. As shown in Fig. 2*B*, ACL immunoisolated from primary adipocytes was reactive with both the anti-$PKB_{substrate}$ and phospho-GSK-3 antibodies, and this reactivity was increased by insulin in a wortmannin-sensitive manner.

The β-adrenergic agonist isoproterenol is well known to stimulate serine 454 phosphorylation on ACL to high stoichiometry via the activation of PKA (24, 25). As shown in Fig. 3, immunoreactivity of ACL toward the anti-$PKB_{substrate}$ and anti-phospho-GSK-3 antibodies was increased by isoproterenol to an extent equivalent to that seen with insulin but in a wortmannin-insensitive manner. Taken together the data demonstrate that insulin-stimulated serine 454 phosphorylation on ACL can be detected using either of the anti-$PKB_{substrate}$ and phospho-GSK-3 antibodies.

Insulin and isoproterenol also stimulated the phosphorylation of GSK-3α and GSK-3β (Fig. 3). As expected the effect of insulin on GSK-3 phosphorylation was almost completely blocked by wortmannin, whereas the effect of isoproterenol was only partially inhibited (Fig. 3). It should be noted, however,

[2] D. C. Berwick, K. J. Heesom, and J. M. Tavaré, unpublished data.

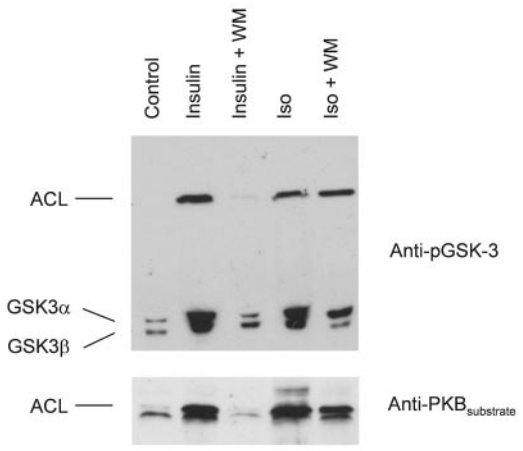

FIG. 3. **The anti-phospho-GSK-3 and anti-PKB$_{substrate}$ antibodies recognize ACL after insulin and β-adrenergic stimulation.** Rat adipocytes were preincubated in the absence or presence of wortmannin (*WM*) for 30 min and subsequently with insulin (83 nM) or isoproterenol (1 μM; *Iso*) for 10 min. Lysates were subjected to Western blotting with the phospho-GSK-3 antibody (*upper panel*) and PKB$_{substrate}$ antibody (*lower panel*). Bands representing ACL and GSK-3α and GSK-3β are indicated. Results are representative of three separate experiments.

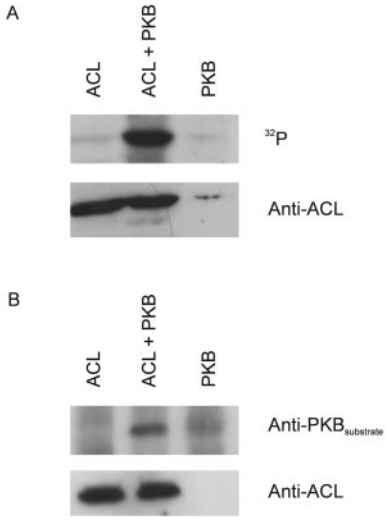

FIG. 4. **PKB phosphorylates ACL at serine 454 *in vitro*.** ACL was immunoprecipitated with the rabbit anti-ACL antibody and then incubated alone (*ACL*) or with recombinant PKB (*ACL + PKB*) in the presence of [γ-^{32}P]ATP. A control kinase reaction with PKB alone was also performed (*PKB*). Reactions were resolved by SDS-PAGE and transferred to membrane prior to visualization of phosphorylation by (*A*) autoradiography (^{32}*P*) or (*B*) Western blotting with the anti-PKB$_{substrate}$ antibody. The *lower panels* in each case show the loading of ACL in each lane. Results are representative of three separate experiments.

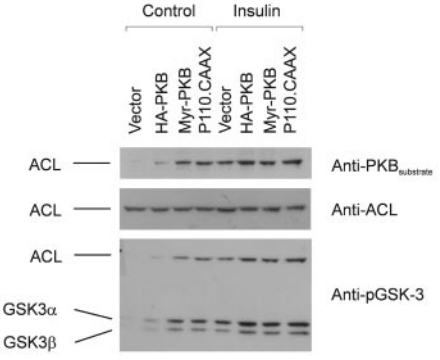

FIG. 5. **PKB activation is sufficient for phosphorylation of ACL on serine 454 *in vivo*.** Rat adipocytes were transfected with empty vector (*Vector*), wild-type HA-tagged PKB (*HA-PKB*), constitutively active myristoylated PKB (*Myr-PKB*), or a constitutively active mutant of the catalytic subunit of PI3K (*p110.CAAX*). After 6 h the cells were further incubated with or without insulin (83 nM) for 10 min as indicated. Cell lysates were subjected to Western blotting with the anti-PKB$_{substrate}$ antibody (*upper panel*), anti-ACL antiserum (*middle panel*), or the phospho-GSK-3 antibody (*lower panel*). In each panel ACL is indicated, and in the *lower panel* the bands corresponding to GSK-3α and GSK-3β are indicated.

that the proteins of apparent M_r 48,000, 51,000, and 52,000 found in cell lysates, and Mono-Q fractions 4 and 5, using the anti-PKB$_{substrate}$ antibody (Fig. 1*A*) were not GSK-3α or GSK-3β, because they were found to be unreactive with the phospho-GSK-3 antibodies.[2] As such these proteins may represent additional potentially novel PKB substrates, and their identities are currently under investigation in our laboratory.

ACL Is an in Vitro Substrate for PKB—Serine 454 of ACL has been demonstrated previously to be phosphorylated by an unknown insulin-activated wortmannin-sensitive kinase and by PKA *in vitro* (24–26). Our results suggest that PKB may be the insulin-activated wortmannin-sensitive cytosolic ACL serine 454 kinase identified by Czech and co-workers (26).

To establish whether PKB is indeed capable of directly phosphorylating ACL *in vitro*, recombinant active PKB was incubated with immunoprecipitated ACL in the presence of [γ-^{32}P]ATP. As shown in Fig. 4, ACL is indeed an *in vitro* PKB substrate, because ^{32}P incorporation into ACL was significantly increased when it was incubated in the presence of recombinant PKB. The PKB-dependent phosphorylation of ACL was recognized by the anti-PKB$_{substrate}$ antibody, suggesting that it was occurring on serine 454 (Fig. 4*B*). These results demonstrate that ACL is a direct substrate for phosphorylation by PKB *in vitro*.

Constitutively Active PKB and PI3K Promote Insulin-independent ACL Phosphorylation in Transiently Transfected Primary Rat Adipocytes—We next sought to determine whether active PKB could induce the phosphorylation of ACL in intact cells incubated in the absence of insulin. To do this we transiently transfected primary rat adipocytes by electroporation. This technique results in >80% of the cells becoming successfully transfected.[3] Primary rat adipocytes were transiently transfected with a constitutively active PKB targeted to the plasma membrane by artificial myristoylation (Myr-PKB) or a constitutively active PI3K catalytic subunit (p110.CAAX). Six hours post transfection, the cells were incubated in the absence or presence of insulin, and then ACL phosphorylation was assayed in cell lysates by Western blotting with the PKB$_{substrate}$ or phospho-GSK-3 antibodies.

[3] S. K. Moule, I. Hers, and R. M. Denton, unpublished data.

As shown in Fig. 5, both the constitutively active PKB and p110.CAAX stimulated ACL phosphorylation to the same extent as insulin as determined using both the PKB$_{substrate}$ and GSK-3 phosphospecific antibodies. The results mirrored the phosphorylation of GSK-3α and GSK-3β, which were increased by the transfected constitutively active Myr-PKB and p110.CAAX, as well as insulin (Fig. 5). Taken together, these results indicate that PKB activation is sufficient for the phosphorylation of ACL in intact adipocytes.

DISCUSSION

In this study we provide evidence to suggest that ATP-citrate lyase is an *in vivo* substrate for protein kinase B and that this kinase is responsible for the previously reported insulin-stimulated phosphorylation of serine 454. This not

only defines ACL as a novel *in vivo* PKB substrate, but also further characterizes the phosphorylation consensus recognition sequence that can be tolerated by PKB on an intact protein substrate.

Evidence to suggest that serine 454 on ACL is a direct substrate for PKB includes the facts that: (i) two distinct phosphospecific antibodies reactive with the PKB consensus sequence cross-react with ACL after insulin stimulation and in a wortmannin-sensitive manner (Figs. 1–3); (ii) extensive peptide mapping experiments have revealed that serine 454 is the only known insulin-stimulated phosphorylation site on ACL (29) and that it lies in a sequence that overlaps with the epitopes used to raise the $PKB_{substrate}$ and phospho-GSK-3 antibodies; (iii) isoproterenol-induced ACL phosphorylation, which occurs on serine 454 via the activation of PKA (24, 25), is also recognized by the $PKB_{substrate}$ and phospho-GSK-3 antibodies in a wortmannin-insensitive manner (Fig. 3); (iv) PKB directly phosphorylates ACL *in vitro* on a site that is recognized by the $PKB_{substrate}$ antibody (Fig. 4); and (v) a constitutively active PKB (Myr-PKB) or a constitutively active PI3K (p110.CAAX) that activates PKB in transfected primary adipocytes[3] stimulate ACL phosphorylation in the absence of insulin.

ACL is a homotetrameric cytosolic enzyme that catalyzes the formation of oxaloacetate and acetyl-CoA from citrate in an ATP-dependent manner and was first identified as a phosphoprotein in 1979 (33, 34). The cytosolic acetyl-CoA produced by this enzyme is utilized by acetyl-CoA carboxylase to generate malonyl-CoA, which is then used for the synthesis of fatty acyl CoA by fatty acid synthase.

Three phosphorylation sites have been identified on ACL, namely threonine 446, serine 450, and serine 454. Insulin and β-adrenergic receptor agonists induce an increase in serine 454 phosphorylation, the latter agonist acting directly via the activation of PKA. Insulin causes a concomitant decrease in the phosphorylation of the threonine 446 and serine 450 at least in part through the inactivation of GSK-3, although the activation of a phosphatase may also be involved (35, 36).

Despite many years of extensive work from several groups, neither isoproterenol or insulin treatment of cells nor *in vitro* phosphorylation by PKA have any apparent effect on ACL activity (37–39).[4] Recently, Potapova *et al.* (36) reported that recombinant ACL purified from *Escherichia coli* was activated *in vitro* when serine 454 was phosphorylated by PKA, whereas the phosphorylation of threonine 446 and serine 450 brought about by GSK-3 was without effect. They proposed that the inability to observe changes in the activity of ACL purified from rodent sources was due to extensive phosphorylation during its isolation and subsequent assay; *i.e.* the bacterially expressed ACL would lack such modifications. However, it is difficult to reconcile a serine 454 phosphorylation-induced activation of ACL by insulin and isoproterenol with their respective stimulatory and inhibitory effects on fatty acid synthesis. As a result, therefore, phosphorylation may also be important in regulating other properties of ACL such as its stability, subcellular localization, and/or interaction with other proteins.

Benjamin and co-workers (40) have reported that the phosphorylation of threonine 446 and serine 450 is reduced by insulin but not by isoproterenol in rat adipose tissue. Thus these two hormones may bring about differential effects on ACL function through differences in the relative phosphorylation status of threonine 446, serine 450, and serine 454. In the case of isoproterenol, PKA-induced serine 454 phosphorylation provides the priming phosphoserine required for phosphorylation of threonine 446 and serine 450 by GSK-3 (36, 41, 42). In the case of insulin, serine 454 phosphorylation would predominate over threonine 446 and serine 450 as a result of GSK-3 inhibition and the activation of a putative threonine 446 and serine 450 phosphatase (35, 36). PKB-induced serine 454 phosphorylation may prime for threonine 446 and serine 450 rephosphorylation when the insulin stimulus is removed, and GSK-3 is consequently reactivated. Clearly, considerable and more extensive studies are necessary to examine these possibilities.

Our data suggest that the effect of insulin on serine 454 phosphorylation, and perhaps ACL function, is mediated by PKB. Therefore, we propose that insulin receptor activation leads to the phosphorylation of insulin receptor substrates, recruitment of PI3K, production of plasma membrane phosphoinositide (3,4,5)-trisphosphate, activation of PKB, via PDK1- and "PDK2"-dependent phosphorylation, and finally ACL serine 454 phosphorylation. The other lipogenic steps regulated by insulin are pyruvate dehydrogenase (PDH) and acetyl-CoA carboxylase, which are stimulated via increases in PDH phosphatase and acetyl-CoA carboxylase kinase activity, respectively (43). However, the mechanism by which insulin regulates pyruvate dehydrogenase phosphatase is not known, nor has the insulin-stimulated kinase that phosphorylates acetyl-CoA carboxylase been identified.

The phosphospecific PKB substrate antibody that we used in this study, which was raised to the PKB consensus sequence R*X*R*XX*(pS/pT)Ψ, does not appear to strictly require the arginine in the −5 position for phosphoprotein recognition, because this residue is lacking in ACL. Indeed, the fact that PKB phosphorylates an ^{451}R*XX*S^{454} motif in ACL is significant. To date we are aware of only one other study that suggests that PKB can phosphorylate a protein substrate that lacks the arginine in the −5 position relative to the phosphorylation site; this being serine 133 of CREB (10). Interestingly, serine 133 of CREB lies in the sequence ^{128}LSRRP**S**Y^{134}, which, like serine 454 of ACL, is also a target for phosphorylation by PKA (10). All other known sequences in protein substrates for PKB appear to conform strictly to the R*X*R*XX*(S/T)Ψ motif (44, 45).

The consensus PKB phosphorylation site, R*X*R*XX*(S/T)Ψ, has been derived from a number of studies, including two that utilize synthetic peptide libraries (17, 18). The peptide sequence *ARK*RE<u>R</u>AYSF*GHHA* was reported to be the optimal short peptide substrate for phosphorylation by recombinant PKB (18). Substitution of the arginine at −3 relative to the phosphoserine (underlined) obliterated the ability of this peptide to serve as a substrate. In contrast, a peptide with an arginine at −5 substituted for an alanine was still phosphorylated but with a decrease of ~20-fold in V_{max}/K_m. Substitution of the alanine at position −2 for a threonine (as would be the case for serine 454 in ACL), which is a more optimal amino acid at this position, might be expected to increase the V_{max}/K_m, although this remains to be proven.

It is important to note that, because the PKB substrate consensus sequence has been derived from studies using short peptides, it may not represent the situation for an intact protein substrate *in vivo*. For this reason caution should be exercised when performing homology searches for PKB substrates based on the R*X*R*XX*(S/T)Ψ sequence. Our results raise the possibility that tertiary topology could also be important in making a "sub-optimal" site amenable to phosphorylation by PKB. Furthermore, Alessi and co-workers (44) have recently postulated the existence of a PKB docking site on some substrates in a manner analogous to the δ-domain on c-Jun that acts as a JNK docking site. This could make a sub-optimal site

[4] R. M. Denton, personal communication.

for PKB phosphorylation an excellent substrate *in vivo*. It remains to be established if ACL possesses such a PKB docking site, or whether such a site actually exists in any PKB substrate identified to date.

In conclusion, our data show that serine 454 on ACL is a novel *in vivo* substrate for PKB. Furthermore, we demonstrate that the use of a phosphospecific antibody to the generalized PKB substrate consensus sequence is a viable proteomic approach for discovering novel PKB substrates.

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The Identification of ATP-citrate Lyase as a Protein Kinase B (Akt) Substrate in Primary Adipocytes

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