Identification of calcium-regulated heat-stable protein of 24 kDa (CRHSP24) as a physiological substrate for PKB and RSK using KESTREL

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A substrate for PKBα (protein kinase Bα) was detected in liver extracts, and was purified and identified as CRHSP24 (calcium-regulated heat-stable protein of apparent molecular mass 24 kDa). PKBα, as well as SGK1 (serum- and glucocorticoid-induced protein kinase 1) and RSK (p90 ribosomal S6 kinase), phosphorylated CRHSP24 stoichiometrically at Ser^{52} *in vitro* and its brain-specific isoform PIPPin at the equivalent residue (Ser^{58}). CRHSP24 became phosphorylated at Ser^{52} when HEK-293 (human embryonic kidney) cells were stimulated with IGF-1 (insulin-like growth factor-1) and this was prevented by inhibitors of PI3K (phosphoinositide 3-kinase), but not by rapamycin [an inhibitor of mTOR (mammalian target of rapamycin)] or PD 184352, an inhibitor of the classical MAPK (mitogen-activated protein kinase) cascade and hence the activation of RSK. IGF-1 induced a similar phosphorylation of CRHSP24 in ES (embryonic stem) cells from wild-type mice or mice that express the PDK1 (3-phosphoinositide-dependent kinase 1) mutant (PDK1[L155E]) that activates PKBα normally, but cannot activate SGK. CRHSP24 also became phosphorylated at Ser^{52} in response to EGF (epidermal growth factor) and this was prevented by blocking activation of both the classical MAPK cascade and the activation of PKBα, but not if just one of these pathways was inhibited. DYRK2 (dual-specificity tyrosine-phosphorylated and -regulated protein kinase 2) phosphorylated CRHSP24 at Ser^{30}, Ser^{32} and Ser^{41} *in vitro*, and Ser^{41} was identified as a site phosphorylated in cells. These and other results demonstrate that CRHSP24 is phosphorylated at Ser^{52} by PKBα in response to IGF-1, at Ser^{52} by PKBα and RSK in response to EGF, and at Ser^{41} in the absence of IGF-1/EGF by a DYRK isoform or another proline-directed protein kinase(s).

Key words: calcium-regulated heat-stable protein of 24 kDa (CRHSP24), kinase substrate tracking and elucidation (KESTREL), protein kinase B (PKB), p90 ribosomal S6 kinase (RSK).

INTRODUCTION

Signals that activate PI3K (phosphoinositide 3-kinase) and elevate the intracellular concentration of PtdIns(3,4,5)P_3 trigger the activation of PKBα (protein kinase B), also called Akt, which plays a central role in mediating many of the metabolic actions of insulin, as well as the anti-apoptotic effects of survival factors (reviewed in [1]). PtdIns(3,4,5)P_3 binds to the PH (pleckstrin homology) domains of PKBα and PDK1 (3-phosphoinositide-dependent protein kinase-1), co-localizing them at the plasma membrane. This allows PDK1 to phosphorylate PKBα at Thr^{308} in the 'activation loop' triggering its activation (reviewed in [1,2]).

PKB isoforms are members of the AGC subfamily of protein kinases that also include isoforms of SGK (serum- and glucocorticoid-induced protein kinase) and RSK (p90 ribosomal S6 kinase). Like PKBα, SGK [3–5] and RSK [6] have to become phosphorylated by PDK1 in order to become activated. However, in contrast with PKBα, the PH domain of PDK1 is dispensable for activation and, instead, these substrates first become phosphorylated at a C-terminal hydrophobic motif, allowing them to dock with and then be activated by cytosolic PDK1 [1]. In cells and tissues expressing a PDK1 mutant (PDK1[L155E]) in which this docking site on PDK1 has been disabled, PDK1 can still activate PKBα normally, but not SGK or RSK [1,7]. The protein kinase that is responsible for phosphorylating the hydrophobic motif varies from substrate to substrate. In the case of RSK isoforms, the hydrophobic motif is phosphorylated by the C-terminal kinase domain of RSK, whose activation is dependent on phosphorylation by MAPKs (mitogen-activated protein kinases) [ERK (extracellular-signal-regulated kinase) 1 and ERK2] of the classical MAPK cascade. This allows PDK1 to dock with and activate RSK via phosphorylation of its N-terminal kinase domain (reviewed in [1]).

Based on studies with synthetic peptide substrates, PKBα was found to phosphorylate serine and threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs [8]. However, SGK [3,4] and RSK isoforms [8,9] display similar specificities *in vitro* towards synthetic peptide substrates, making it difficult to predict substrates for PKBα simply by searching protein sequence databases with this motif. In order to identify novel physiological substrates for these protein kinases, we therefore decided to adopt the KESTREL (kinase substrate tracking and elucidation) approach [10]. In the present study, we identify CRHSP24 (calcium-regulated heat-stable protein of 24 kDa) as a protein in liver extracts that is phosphorylated efficiently by PKBα and go on to show that it is indeed a new physiological substrate for PKBα, as well as RSK, but not for SGK or S6K (p70 ribosomal protein S6 kinase).

Abbreviations used: CRHSP24, calcium-regulated heat-stable protein of 24 kDa; CSD, cold-shock domain; DYRK, dual-specificity tyrosine-phosphorylated and -regulated protein kinase; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; EST, expressed sequence tag; FCS, foetal calf serum; GST, glutathione S-transferase; HEK-293, human embryonic kidney; IGF-1, insulin-like growth factor-1; KESTREL, kinase substrate tracking and elucidation; LDS, lithium dodecyl sulphate; MAPK, mitogen-activated protein kinase; MKK1, MAPK kinase 1; MNK, MAPK-integrating kinase; PDK1, 3-phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PP, protein phosphatase; RSK, p90 ribosomal S6 kinase; S6K, p70 ribosomal protein S6 kinase; SGK, serum- and glucocorticoid-induced kinase; STYX, serine/threonine/tyrosine-interacting protein.

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MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP, ECL® (enhanced chemiluminescence) and materials for protein purification were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All cell culture media, pre-cast Bis-Tris SDS/12 % polyacrylamide gels, running buffer, transfer buffer, pre-stained markers and colloidal Coomassie Blue stain were from Invitrogen (Paisley, Renfrewshire, U.K.), unlabelled ATP, dithiothreitol and Complete™ protease inhibitor cocktail tablets were from Roche Molecular Biochemicals (Lewes, East Sussex, U.K.), FCS (foetal calf serum) was from Cambrex (Wokingham, Berks., U.K.) and Protran nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). PD 184352 was made by custom synthesis. Other chemicals were purchased from Merck or Sigma–Aldrich (both Poole, Dorset, U.K.) or from the sources given previously [11].

Purification of a 24 kDa substrate for PKBα in rat liver cytosol

Rat livers were removed from three Sprague–Dawley rats and placed on ice, and all subsequent procedures were carried out at 4 °C. The livers were cut into small pieces, then homogenized in a Potter–Elvehjem homogenizer in 3 vol. of 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 0.1 % (v/v) 2-mercaptoethanol and Complete™ protease inhibitor cocktail. The homogenate was centrifuged for 15 min at 16000 ***g***, the pellet was discarded, and the supernatant was re-centrifuged for 90 min at 100000 ***g***. The supernatant (cytosol) was removed, snap-frozen in liquid nitrogen and stored at − 80 °C until required.

The cytosol (60 ml) was passed though a 0.2-μm-pore-size filter, and desalted by passage through a 200 ml column of Sephadex G-25 at ambient temperature, equilibrated in 30 mM Mops, pH 7.5, 5 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol and 0.03 % (w/v) Brij-35 (Buffer A). The protein-containing fractions were pooled, re-filtered and applied to a 25 ml column of heparin HP–Sepharose equilibrated in Buffer A. The column was developed with a 520 ml non-linear salt gradient from 0 to 1 M NaCl in Buffer A at a flow rate of 5 ml/min. Fractions of 12 ml were collected, and an aliquot of each was diluted 10-fold into 30 mM Tris/HCl, pH 7.5, 0.1 EGTA, 0.1 % (v/v) 2-mercaptoethanol, 10 mM μg/ml aprotinin and 10 μg/ml leupeptin. Aliquots of the diluted fractions (20 μl) were then incubated for 4 min at 30 °C with 20 m-units of PKBα and 20 nM Mg-[γ-^{32}P]ATP (2.5×10^6 c.p.m.) in a total volume of 30 μl. Reactions were denatured in LDS (lithium dodecyl sulphate) heated for 10 min at 70 °C, subjected to SDS/PAGE, transferred on to nitrocellulose membranes and autoradiographed.

Fractions containing the 24 kDa substrate of PKBα were pooled, dialysed into Buffer A and loaded on to a 1 ml Source 15S (HR 5/5) column equilibrated in Buffer A. The column was washed with 5 ml of Buffer A and developed with a 20 ml non-linear salt gradient from 0 to 1 M NaCl in Buffer A. The flow rate was 1 ml/min, and fractions of 0.5 ml were collected. Fractions containing the 24 kDa substrate were identified as described above, pooled and dialysed into 30 mM Mops, pH 7.0, 5 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol and 0.03 % (w/v) Brij-35 (Buffer B). The material was then loaded on to a 0.24 ml column of Mini S (PC 3.2/3) 240, which was developed with a 1.7 ml non-linear gradient to 0.3 M NaCl in Buffer B. The flow rate was 0.1 ml/min, and fractions of 0.1 ml were collected and analysed as described in the Results section.

DNA constructs, protein expression and purification

The DNA encoding human CRHSP24 (GenBank® accession number NM_014316) was amplified from IMAGE EST (expressed sequence tag) 5737444 using the GC-rich PCR System (Roche) with the oligonucleotides 5′-GCGAATTCGCCACC-ATGTACCCATACGATGTGCCAGATTACGCCTCATCTGAG-CCTCCCCCACCAC-3′ and 5′-GCGTCGACCTAGGAGCTG-ATGACATGTCCAGACC-3′. The PCR product was digested using EcoRI and SalI and ligated into the same sites of pGEX6P-1 to produce pGEX6P-1HA-CRHSP24. The related protein PIPPin (GenBank® accession number BAA84704) was amplified from IMAGE EST 5759182 as described above, with oligonucleotides 5′-GCGAATTCCTAGGAGCCCACGACCTGGC-3′ and 5′-GC-GGATCCGCCACCATGTACCCATACGATGTGCCAGATTAC-GCCACTTCAGAGTCGACGTCACCCCC-3′, then digested with BamHI and EcoRI and ligated into pGEX6P-1 to produce pGEX6P-1-Pippin-His_6.

N-terminally tagged GST (glutathione S-transferase)–CRHSP24 and PIPPin tagged with GST at the N-terminus and a His_6 tag at the C-terminus were expressed in *Escherichia coli* BL21 CodonPlus-RIL (Merck), purified by affinity chromatography on glutathione–Sepharose, dialysed against 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 50 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol, 0.2 mM PMSF and 1 mM benzamidine and stored at − 20 °C. Active preparations of PKBα, SGK1 and RSK1 were expressed and purified as described, and DYRK2 (dual-specificity tyrosine-phosphorylated and -regulated protein kinase 2) as described for DYRK1A [12,13].

Protein kinase assays

Assays were carried out at 30 °C as described previously [12,13]. One unit of PKBα, SGK1 and RSK1 was that amount which catalysed the phosphorylation of 1 nmol of the standard substrate peptide Crosstide (GRPRTSSFAEG) in 1 min. One unit of DYRK2 was that amount which catalysed the phosphorylation of 1 nmol of Woodtide (KKISGRLSPINTEQ) in 1 min. The assay of DYRK2 was as described for DYRK1A [13].

Antibodies

Polyclonal antisera that recognize CRHSP24 phosphorylated at Ser^{52} and PIPPin at Ser^{58} were raised in sheep at Diagnostics Scotland (Edinburgh, U.K.) against the phosphopeptides CRTRTFS*ATVRA and CRTRTYS*ATARA (where S* is phosphoserine) coupled to both BSA and keyhole-limpet haemocyanin. The sequences correspond to residues 47–57 and 53–63 of human, rat or mouse CRHSP24 and PIPPin respectively, plus an N-terminal cysteine residue for coupling to the carrier proteins. Polyclonal antisera that recognize all forms of CRHSP24 and PIPPin were raised in sheep against full-length bacterially expressed GST-fusion protein. All antisera were affinity-purified on CH-Sepharose to which the antigens had been coupled covalently. The antibodies raised against GST–CRHSP24 and GST–PIPPin were also passed through GST–Sepharose to remove anti-GST antibodies. The phosphospecific antibodies were used for immunoblotting in the presence of 10 μg/ml unphosphorylated peptide antigen to neutralize antibodies present in the preparation that recognize unphosphorylated CRHSP24 or PIPPin. Rabbit anti-sheep IgG and goat anti-rabbit IgG, both conjugated to peroxidase, were obtained from Perbio Science (Tattenhall, Cheshire, U.K.). Other antibodies were from Cell Signaling Technologies (Hitchin, Herts., U.K.).

Cell culture and cell lysis

HEK-293 (human embryonic kidney) cells were grown at 37 °C in DMEM (Dulbecco's modified Eagle's medium) plus 10 % (v/v) heat-inactivated FCS, 100 units/ml penicillin and 100 mg/ml streptomycin in a 95 % air/5 % CO_2 atmosphere. Cells were

serum-starved for 15 h, then incubated without or with 100 nM wortmannin for 15 min or without or with 2 μM PD 184352 for 1 h before stimulating for 5 or 30 min with 20 ng/ml IGF-1 (insulin-like growth factor-1) or 40 ng/ml EGF (epidermal growth factor). Cells were then washed twice in ice-cold PBS and lysed in 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 50 mM sodium pyrophosphate, 0.27 M sucrose, 1 % (v/v) Triton X-100, 0.1 % (v/v) 2-mercaptoethanol and Complete^{TM} protease inhibitor cocktail. Lysates were centrifuged at 13 000 ***g*** for 10 min at 4 °C. Protein concentrations were determined by the Bradford method using BSA as a standard. Cell lysate was denatured in LDS sample buffer containing reducing agent and heated at 70 °C for 10 min before SDS/PAGE.

Use of the 4000 Q-Trap mass spectrometer to identify sites of phosphorylation in cells

HEK-293 cell lysate (10 mg of protein) was incubated for 90 min at 4 °C with 10 μg of anti-CRHSP24 antibody. A 40 μl volume of washed Protein-G-coated paramagnetic beads (Dynal) [a 1:1 slurry in 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 140 mM NaCl, 10 mM KCl, pH 7.4 (Buffer 1)] was added and incubation continued for a further 30 min. The beads were pelleted on a magnet, then washed three times in 0.5 ml Buffer A, once in 0.5 ml of 25 mM Tris/HCl, pH 7.1, and 150 mM NaCl, and prepared for SDS/PAGE as described above. Following electrophoresis, the gel was stained with colloidal Coomassie Blue, and the band corresponding to CRHSP24 was excised and digested with trypsin. The digests were separated by chromatography on a PepMapC_{18} (0.075 mm × 150 mm) column connected to an LC-Packings Ultimate HPLC system (Dionex, Camberley, Surrey, U.K). The C_{18} column was equilibrated in 0.1 % (v/v) methanoic (formic) acid in 2 % (v/v) acetonitrile at a flow rate of 350 nl/min. Peptides were eluted with a discontinuous acetonitrile gradient over 48 min, and the column eluate flowed into an in-line Tee (Upchurch, Oak Harbor, WA, U.S.A.), where it mixed with 80 % propan-2-ol delivered at 100 nl/min from a second pump. The combined flow was analysed by electrospray MS using an Applied Biosystems 4000 Q-Trap mass spectrometer, with a FS360-50-15-N Picotip (New Objectives) fitted to the micro-ion-spray source.

Phosphopeptides were detected by precursor ion scanning in the negative-ion mode, and, once a precursor generating a PO_3^- ion was detected, the polarity was switched automatically to positive-ion mode, and an enhanced resolution spectrum was recorded to obtain the charge state of the ion, followed by an MS/MS product ion scan and finally back to negative-ion mode. The duty cycle for this routine was 6 s. All the product ion spectra were searched using the Mascot search algorithm (Matrix Science) against in-house and public domain databases. Sites of phosphorylation were assigned from manual inspection of the MS/MS spectra using Analyst 1.4 software (MDS Sciex, Concord, Ontario, Canada).

RESULTS

Identification of a 24 kDa protein in liver extracts phosphorylated by PKBα

Desalted rat liver extracts were chromatographed on heparin HP–Sepharose, and aliquots of each fraction collected were phosphorylated with PKBα as described in the Materials and methods section. A protein was identified of apparent molecular mass 24 kDa, eluted at 0.18–0.20 M NaCl, that was phosphorylated by PKBα (Figure 1A) and therefore merited further investigation.

The fractions containing the PKBα substrate were pooled and fractionated further by successive chromatographies on Source S and Mini S (see the Materials and methods section). The peak fractions from the final step were pooled, concentrated, maximally phosphorylated by incubation with PKBα (1 unit/ml), 10 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (1000 c.p.m./pmol) and analysed by SDS/PAGE. The ^{32}P-labelled substrate co-migrated with a protein-staining band (Figure 1B), which was excised from the gel, digested with trypsin and analysed by MALDI–TOF/TOF (matrix-assisted laser-desorption ionization–tandem time-of-flight) MS and electrospray LC (liquid chromatography)–MS. This revealed that three peptides from the major protein-staining band in the preparation matched to the sequence of CRHSP24 [14], giving 23 % coverage of the sequence (results not shown). This suggested that CRHSP24 might be the PKBα substrate.

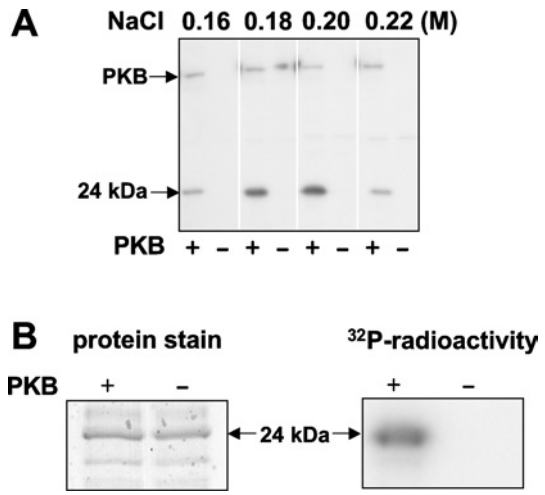

Figure 1 Identification of a hepatic protein of apparent molecular mass 24 kDa that is phosphorylated by PKBα *in vitro*

(**A**) Desalted rat liver extracts were chromatographed on heparin HP–Sepharose, and the eluted fractions were incubated with 2 mM $MgCl_2$ and 20 nM [γ-^{32}P]ATP in the presence (+) or absence (−) of PKBα as described in the Materials and methods section. The reactions were subjected to SDS/PAGE, transferred on to nitrocellulose membranes and autoradiographed. A substrate for PKBα of apparent molecular mass 24 kDa was detected that was eluted at 0.18–0.2 M NaCl. (**B**) After further purification by chromatography on Source S and Mini S as described in the Materials and methods section, fractions containing the PKBα substrate were maximally phosphorylated by incubation for 60 min with 2 units/ml PKBα, 2 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol), denatured in LDS, subjected to SDS/PAGE and stained with colloidal Coomassie Blue. The ^{32}P-labelled band co-migrated with a major protein-staining band in the preparation.

Identification of the residue in CRHSP24 phosphorylated by PKBα

To investigate whether the PKBα substrate and CRHSP24 were the same protein, the ^{32}P-labelled band from Figure 1(B) was digested with trypsin and chromatographed on a C_{18} column. One major phosphopeptide was detected (Figure 2A), whose molecular mass corresponded to that of the peptide comprising residues 50–56 of CRHSP24 plus one phosphate. Solid-phase Edman sequencing (Figure 2B) [15] identified the site of phosphorylation as Ser^{52}, establishing that the ^{32}P-labelled protein was indeed CRHSP24. The phosphorylated residue lies in the canonical consensus sequence for phosphorylation by PKBα (Arg-Xaa-Arg-Xaa-Xaa-Ser).

The amino acid sequence of CRHSP24 is given in Figure 2(C). The calculated molecular mass is 15.9 kDa, indicating that CRHSP24 migrates anomalously on SDS/polyacrylamide gels. The phosphorylation site lies N-terminal to the CSD (cold-shock domain) [16] that comprises over one-third of the molecule.

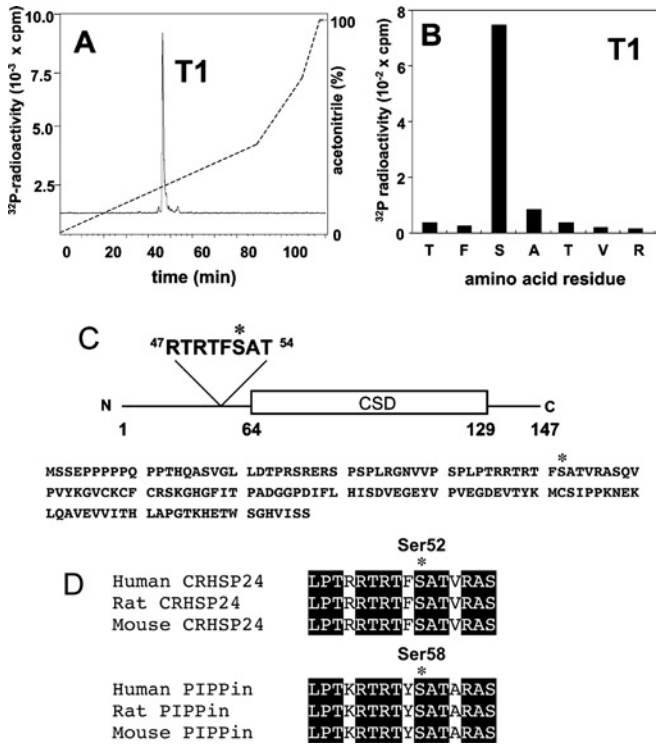

Figure 2 Identification of the residue in CRHSP24 phosphorylated by PKBα

(**A**) The ^{32}P-labelled PKBα substrate from Figure 1(B) was excised and digested with trypsin, and the resulting peptides were chromatographed on a Vydac C_{18} column equilibrated in 0.1 % (v/v) trifluoroacetic acid. The column was developed with an acetonitrile gradient (broken line) at 0.8 ml/min, and fractions of 0.4 ml were collected and ^{32}P-radioactivity was analysed by Cerenkov counting (continuous line). (**B**) The major phosphopeptide T1 was identified on a Q-TOF2 mass spectrometer, and the phosphorylation site was established by solid-phase sequencing [14], monitoring ^{32}P-radioactivity released at each cycle of Edman degradation. (**C**) Amino acid sequence of CRHSP24 and its domain structure, showing the phosphorylation site (asterisk) and CSD. (**D**) Comparison of sequences surrounding the phosphorylation site of CRHSP24 and PIPPin from several mammalian species. Identities between CRHSP24 and PIPPin are in white on a black background, and differences are in black on a white background.

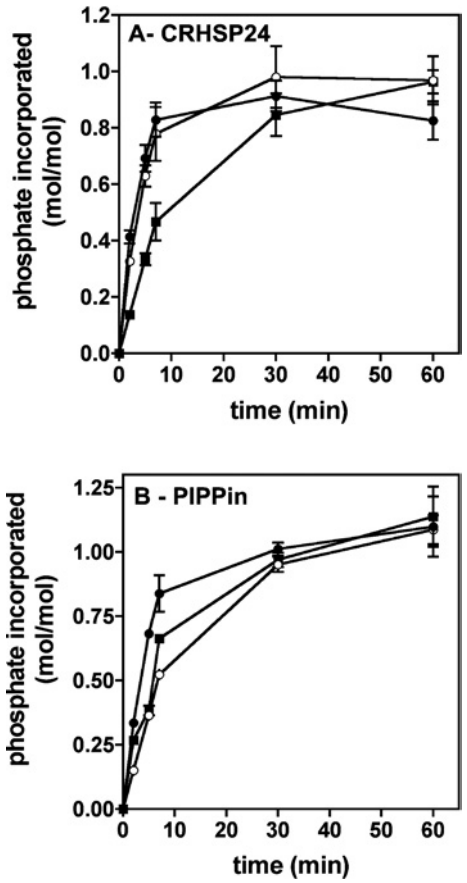

Figure 3 Phosphorylation of CRHSP24 and PIPPin by different protein kinases of the AGC subfamily

(**A**) Bacterially expressed GST–CRHSP24 (0.1 mg/ml) was phosphorylated at 30 °C for the times indicated with PKBα (●), SGK1 (○) and RSK1 (■), each at 1 unit/ml. The reactions were stopped by the addition of LDS and were subjected to SDS/PAGE. After staining with Coomassie Blue, the bands corresponding to GST–CRHSP24 were excised and counted. The stoichiometry of phosphorylation was calculated from the ^{32}P-radioactivity incorporated and the protein concentration of full-length GST–CRHSP24. The latter was estimated by the Bradford method, using BSA as a standard, and by calculating the proportion of the preparation that was full-length GST–CRHSP24. This was determined by densitometry of the Coomassie-Blue-stained gels. (**B**) Same as (**A**), except that GST–PIPPin replaced GST–CRHSP24.

CRHSP24 is most closely related to a brain-specific protein, termed PIPPin because of the presence of a Pro-Ile-Pro-Pro sequence just after the CSD [17]. The sequence surrounding Ser^{58} of PIPPin is very similar to that surrounding Ser^{52} of CRHSP24 (Figure 2D), and, after phosphorylation of bacterially expressed human PIPPin by PKBα, we identified Ser^{58} as the only site that was phosphorylated, using the same methodology used for CRHSP24 (results not shown).

Phosphorylation of CRHSP24 and PIPPin by other protein kinases of the AGC subfamily

PKBα, SGK1 and RSK1 were all capable of phosphorylating bacterially expressed CRHSP24 (Figure 3A) or PIPPin (Figure 3B) to a stoichiometry approaching 1 mol of phosphate incorporated per mol of protein.

Phosphospecific antibodies were raised against phosphopeptides corresponding to the sequences surrounding Ser^{52} and Ser^{58} of human, mouse and rat CRHSP24 and PIPPin respectively. Both phosphospecific antibodies required prior incubation with the unphosphorylated form of the peptide immunogen to prevent recognition of the unphosphorylated form of CRHSP24 (Figures 4A and 4B). The anti-pSer^{52} and -pSer^{58} antibodies recognized human CRHSP24 (Figure 4C) or PIPPin (Figure 4D) after phosphorylation by SGK and RSK, as well as PKBα, demonstrating that all three AGC family kinases phosphorylate the same sites *in vitro*. We also raised antibodies against full-length CRHSP24 and PIPPin that recognized the phosphorylated and unphosphorylated proteins equally well (lower panels of Figures 4C and 4D).

IGF-1 stimulates the phosphorylation of CRHSP24 at Ser^{52} in HEK-293 cells via the PI3K signalling pathway and PKBα

IGF-1 induced a rapid phosphorylation of CRHSP24 at Ser^{52}, which occurred in HEK-293 cells with a half time of < 2 min, was maximal after 10 min and sustained for at least 60 min. The phosphorylation of CRHSP24 paralleled the activation of PKBα, as assessed by the phosphorylation of Ser^{473} (Figure 5A). The same result was obtained if the extracts were immunoblotted directly (Figure 5A) or if CRHSP24 was first immunoprecipitated (results not shown). The IGF-1-induced phosphorylation of CRHSP24 observed after 5 or 30 min was prevented by pre-incubating the cells with 100 nM wortmannin, at which concentration it is a

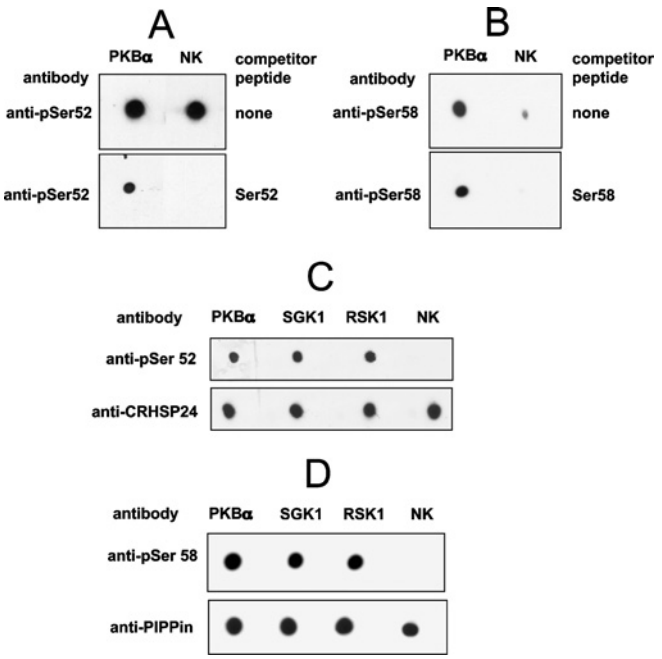

Figure 4 Characterization of antibodies that recognize CRHSP24 and PIPPin

(**A**) Characterization of a phosphospecific antibody that recognizes CRHSP24 phosphorylated at Ser^{52}. Bacterially expressed GST–CRHSP24 was maximally phosphorylated by incubation for 60 min at 30 °C with Mg-ATP in the presence of PKBα or absence of this protein kinase (NK, no kinase). Aliquots (100 ng) were spotted on to nitrocellulose membranes and immunoblotted using the phosphospecific antibody in the absence (none) or presence of 10 μg/ml of the unphosphorylated peptide immunogen (Ser^{52}). (**B**) Same as (**A**), except that GST–PIPPin replaced GST–CRHSP24 and the phosphospecific antibody that recognizes PIPPin at Ser^{58} was used. (**C**) GST–CRHSP24 was phosphorylated as in (**A**) using 1 unit/ml PKBα, SGK1 or RSK1, or in the absence of any protein kinase (NK, no kinase). Aliquots (100 ng) were spotted on to a nitrocellulose membrane and immunoblotted with the anti-Ser^{52} antibody in the presence of the unphosphorylated peptide immunogen, or with an antibody that recognizes the phosphorylated and unphosphorylated forms of CRHSP24 equally well (anti-CRHSP24). (**D**) Same as (**C**), except that GST–PIPPin replaced GST–CRHSP24, and the antibodies that recognize PIPPin were used instead of those that recognize CRHSP24.

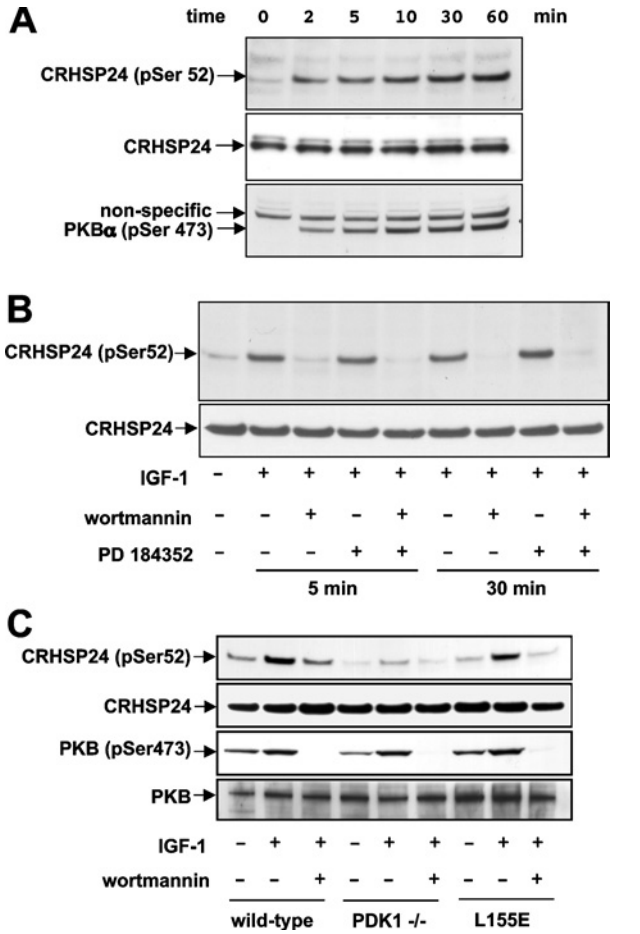

Figure 5 IGF-1 induces the phosphorylation of CRHSP24 at Ser^{52}

(**A**) HEK-293 cells were deprived of serum for 15 h, then stimulated for the times indicated with 20 ng/ml IGF-1. The cells were lysed, and the lysates (45 μg of protein) were subjected to SDS/PAGE, followed by transfer on to nitrocellulose membranes. The membranes were then probed with antibodies that recognize CRHSP24 phosphorylated at Ser^{52} (pSer^{52}), that recognize unphosphorylated and phosphorylated CRHSP24 equally well (anti-CRHSP24) or that recognize PKBα phosphorylated at Ser^{473} (pSer^{473}). (**B**) The experiment was performed similarly to (**A**), except that, after serum starvation, the cells were first incubated for 1 h without (−) or with (+) 2 μM PD 184352 or for 15 min without (−) or with (+) 100 nM wortmannin, before stimulation for 5 or 30 min with IGF-1. When PD 184352 and wortmannin were both present, the wortmannin was added to culture medium 45 min after the addition of PD 184352. (**C**) ES cells from wild-type mice, PDK1-deficient mice (PDK1−/−) and mice that express the PDK1[L155E] mutant (L155E) instead of wild-type PDK1 were deprived of serum for 4 h before incubation for 15 min with (+) or without (−) 100 nM wortmannin, and subsequent stimulation for a further 30 min with (+) or without (−) 20 ng/ml IGF-1. Cells were lysed and analysed by immunoblotting as before.

relatively specific inhibitor of PI3Ks. In contrast, the IGF-1-induced phosphorylation of CRHSP24 was unaffected by PD 184352 (Figure 5B), a potent and specific inhibitor of MKK1 (MAPK kinase 1) [12,18] at concentrations that prevent the activation of ERK1/ERK2 and hence the activation of RSK (results not shown). Phosphorylation was also unaffected by rapamycin (results not shown), a potent and specific inhibitor of mTOR (mammalian target of rapamycin), and hence the activation of S6K1, another AGC-family kinase with similar specificity requirements to PKBα [8,9].

In order to investigate whether a PKB or an SGK isoform was responsible for catalysing the IGF-1-stimulated phosphorylation of CRHSP24 at Ser^{52}, we studied the phosphorylation of this protein in ES (embryonic stem) cells from wild-type mice, mice that do not express PDK1 and mice that express the PDK1[L155E] mutant instead of the wild-type enzyme. The PDK1[L155E] mutant can activate PKBα normally, but cannot activate SGK or other substrates of wild-type PDK1. In contrast with serum-starved HEK-293 cells, there was a significant basal level of phosphorylation of CRHSP24 in serum-deprived ES cells from wild-type mice (Figure 5C), which was not reduced by prior incubation with wortmannin (results not shown). Phosphorylation was enhanced in IGF-1-stimulated cells and this was suppressed to basal levels if the cells were incubated with wortmannin before stimulation. The basal level of phosphorylation was lower in ES cells that lack PDK1 or express the PDK1[L155E] mutant. IGF-1-stimulated phosphorylation of Ser^{52} was greatly decreased (but not completely abolished) in PDK1-deficient ES cells (Figure 5C), suggesting that an IGF-1-stimulated protein kinase that is not activated by PDK1 may make a minor contribution to phosphorylation, if other PDK1-activated protein kinases are inactive. However, in ES cells expressing the PDK1[L155E] mutant that cannot activate SGK, IGF-1 induced a robust, wortmannin-sensitive, phosphorylation of Ser^{52}, similar to that observed in wild-type cells (Figure 5C). This demonstrated that a PKB isoform, rather than an SGK isoform, mediates the IGF-1-stimulated phosphorylation of CRHSP24 at Ser^{52}.

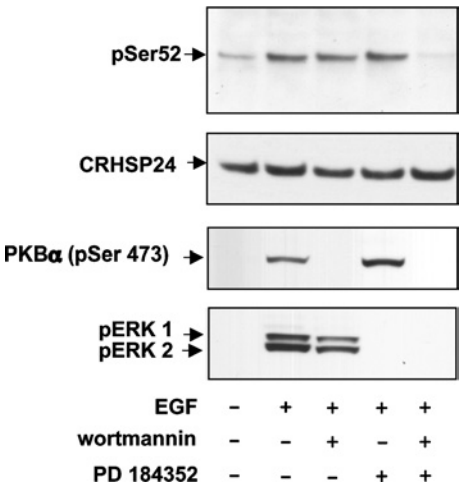

Figure 6 EGF-induced phosphorylation of CRHSP24 at Ser^{52} in HEK-293 cells is mediated by the classical MAPK cascade as well as by PKBα

The experiment was carried out as in Figure 5(B), except that the cells were stimulated for 5 min with 40 ng/ml EGF, and the nitrocellulose membranes were also immunoblotted with an antibody that recognizes ERK1 and ERK2 phosphorylated at their Thr-Glu-Tyr motifs.

The EGF-stimulated phosphorylation of CRHSP24 at Ser^{52} occurs via the classical MAPK cascade, as well as through the PI3K pathway

EGF stimulated a rapid phosphorylation of CRHSP24 at Ser^{52}, which occurred with a half-time of < 2 min, was maximal after 5 min and sustained for at least 60 min (results not shown). To investigate the signalling pathways involved in mediating the EGF-induced phosphorylation of CRHSP24, we examined the effects of PD 184352 and wortmannin. Interestingly, neither wortmannin nor PD 184352 alone reduced the EGF-induced phosphorylation of CRHSP24 significantly after 5 min, but phosphorylation was blocked completely in the presence of both inhibitors added together (Figure 6). These results are considered further in the Discussion.

Identification of Ser^{41} as a site in CRHSP24 phosphorylated in cells

CRHSP24 was originally described as a protein that undergoes dephosphorylation at serine residues in response to signals that elevate calcium ions [14,19]. However, incubation of serum-fed HEK-293 cells (in which CRHSP24 is highly phosphorylated at Ser^{52}) with calcium ionophores did not induce any dephosphorylation of Ser^{52}, suggesting that dephosphorylation may occur at another site(s) (G. C. Auld, unpublished work). Since many proteins are phosphorylated at Ser-Pro sequences under basal conditions, we investigated whether CRHSP24 could be phosphorylated *in vitro* by DYRK2, a proline-directed protein kinase known to be constitutively active in cells [20,21]. We found that CRHSP24 was phosphorylated efficiently *in vitro* by DYRK2 to greater than 2 mol of phosphate per mol of protein, indicating that at least three sites were being phosphorylated (Figure 7A). Tryptic digestion followed by chromatography on a C_{18} column revealed three phosphopeptides, termed T1, T2 and T3 (Figure 7B). MS and solid-phase sequencing [15] revealed that T2 comprised residues 36–46 phosphorylated at Ser^{41}, while T3 comprised residues 28–46 and was a triphosphorylated derivative phosphorylated at Ser^{30} and Ser^{32}, as well as at Ser^{41} (Figure 7C). Peptide T1 comprised residues 28–35 phosphorylated at Ser^{30} and Ser^{32} (results not shown).

In order to investigate whether these three sites were phosphorylated in cells, we used MS with precursor ion scanning (see the Materials and methods section) to identify sites in CRHSP24 that were phosphorylated in serum-fed cells. This analysis revealed that Ser^{41} was phosphorylated in addition to the expected phosphorylation site at Ser^{52} (Figure 8A). A representative MS/MS spectrum proving that Ser^{41} is phosphorylated is shown in Figure 8(B). Ser^{41} was also phosphorylated in serum-starved cells (results not shown). We did not detect any signal from a phosphopeptide corresponding to CRHSP24 phosphorylated at Ser^{30}/Ser^{32}, although the methodology used may have failed to detect these sites.

DISCUSSION

In the present paper, we have used the KESTREL method to identify CRHSP24 as a new physiological substrate for PKBα and RSK. PKBα, SGK1 or RSK1 phosphorylated CRHSP24 at Ser^{52} *in vitro*. Moreover, Ser^{52} became phosphorylated in HEK-293 cells in response to IGF-1. In IGF-1-stimulated cells, the phosphorylation of CRHSP24 paralleled the phosphorylation (activation) of PKBα, and phosphorylation of both proteins was prevented by the PI3K inhibitor wortmannin, but not by PD 184352 or rapamycin. The lack of effect of PD 184352 and rapamycin excludes the involvement of RSK and S6K isoforms in the IGF-1-induced phosphorylation of CRHSP24, and these observations indicate that phosphorylation of CRHSP24 is catalysed by PKBα or another protein kinase, such as SGK, that lies downstream of PI3K. The finding that IGF-1-induced the phosphorylation of CRHSP24 at Ser^{52} in PDK1[L155E]-expressing ES cells (which cannot activate SGK isoforms) to a level similar to that observed in wild-type ES cells strongly suggests that phosphorylation is catalysed by a PKB and not an SGK isoform(s).

EGF also induced the phosphorylation of CRHSP24 but, in contrast with IGF-1, wortmannin did not suppress the effect of this agonist and neither did PD 184352 or rapamycin. However, phosphorylation was blocked completely if the cells were first incubated with both wortmannin and PD 184352 before stimulation with EGF. These observations indicate that at least two protein kinases are involved in mediating the EGF-stimulated phosphorylation of CRHSP24, one protein kinase being activated downstream of PI3K (PKBα) and the other activated downstream of MKK1, with the activity of either kinase being sufficient for maximal phosphorylation of CRHSP24. Since ERK1 and ERK2, the MAPKs of the classical MAPK cascade, phosphorylate serine and threonine residues that are followed by proline, they cannot be the enzymes that phosphorylate CRHSP24 at Ser^{52}, which is not followed by proline. The phosphorylation of Ser^{52} must therefore be catalysed by another protein kinase(s) that is activated by ERK1/ERK2. In the present paper, we demonstrate that RSK1, a protein kinase activated by ERK1/ERK2, phosphorylates CRHSP24 stoichiometrically at Ser^{52} *in vitro*, suggesting that it may be the relevant kinase involved in the EGF-induced phosphorylation of CRHSP24. In support of this conclusion, we have shown that a potent and specific cell-permeant inhibitor of RSK isoforms, which we have identified in collaboration with a pharmaceutical company, prevents the EGF-induced phosphorylation of CRHSP24 at Ser^{52} in HEK-293 cells when combined with wortmannin (results not shown). This compound does not inhibit PKBα and therefore does not inhibit the IGF-1-induced phosphorylation of CRHSP24 (G. C. Auld and P. Cohen, unpublished work). Moreover, this compound does not inhibit MNK1 (MAPK-integrating kinase 1), MNK2 or MSK1 (mitogen- and stress-activated protein kinase 1) *in vitro*, the other

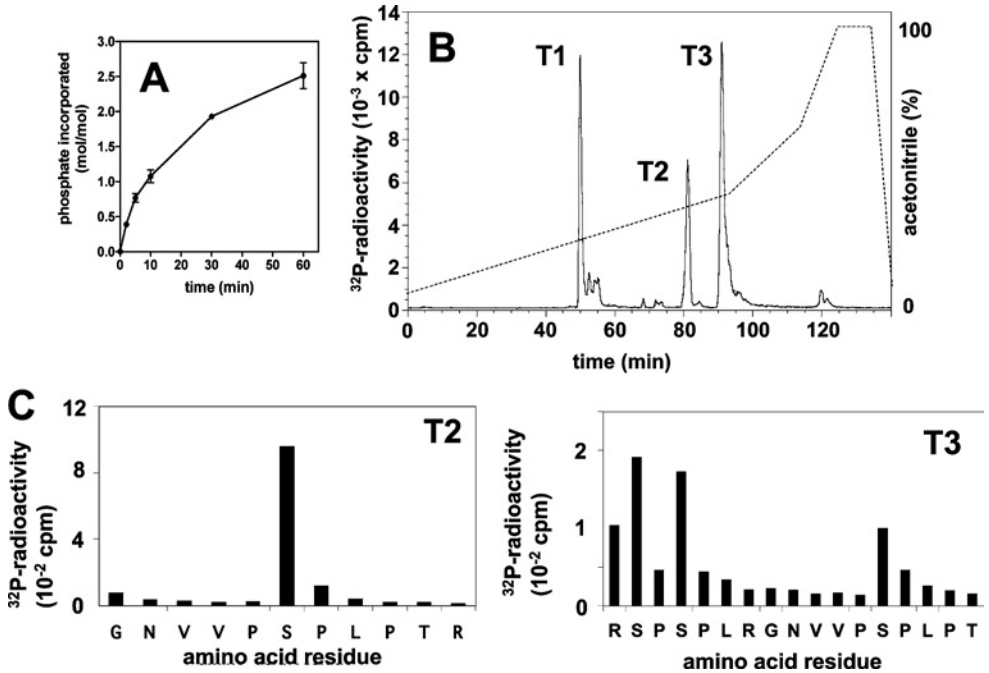

Figure 7 CRHSP24 is phosphorylated *in vitro* by DYRK2 at three serine residues

(**A**) Bacterially expressed GST–CRHSP24 (0.1 mg/ml) was phosphorylated at 30 °C for the times indicated with 1 unit/ml DYRK2 and Mg-[γ-^{32}P]ATP. The reactions were stopped by the addition of LDS and were subjected to SDS/PAGE. After staining with Coomassie Blue, the bands corresponding to GST–CRHSP24 were excised and phosphorylation stoichiometries were calculated as in Figure 3. (**B**) Phosphorylated GST–CRHSP24 (2 μg) from the 60 min time point in (**A**) was subjected to SDS/PAGE, and the protein was excised, digested with trypsin and chromatographed on a Vydac C_{18} column as in Figure 2(A). ^{32}P-radioactivity is shown by the continuous line and the acetonitrile gradient by the broken line. (**C**) Peptides T2 and T3 were identified by MS, and the phosphorylated residue was identified by solid-phase sequencing as in Figure 2(B). Before solid-phase sequencing, the N-terminal glutamate in peptide T3 was removed as described in [15] to prevent cyclization of the peptide. ^{32}P-radioactivity released after each cycle of Edman degradation is shown by the bars.

protein kinases that are known to be activated by ERK1/ERK2, neither does it inhibit 30 other protein kinases tested (G. Sapkota, J. Bain, P. Cohen and D. R. Alessi, unpublished work). Therefore, like glycogen synthase kinase 3 [22], the tuberosclerosis complex protein 2 [23] and METTL1 (methyltransferase-like protein 1) [24], CRHSP24, is a further example of a protein that is phosphorylated at the same site in cells by isoforms of PKB and/or RSK.

Our finding that CRHSP24 becomes phosphorylated at Ser^{52} in response to growth factors raises the question of the physiological role of this modification. CRHSP24 has been described previously as a protein that undergoes dephosphorylation at serine residues in response to elevated levels of calcium ions or cAMP, or in response to tumour-promoting phorbol esters in pancreatic acinar cells [14,19]. The calcium-induced dephosphorylation of CRHSP24 appears to be catalysed by the calcium-dependent PP2B (protein phosphatase 2B, also called calcineurin), because it is prevented by the immunosuppressant drug cyclosporin, a specific inhibitor of this enzyme [14]. In contrast, cAMP- or phorbol-ester-induced dephosphorylation is prevented by low concentrations of okadaic acid [19], suggesting that, under these conditions, dephosphorylation is catalysed by PP2A or a closely related enzyme, such as PP4 or PP5. However, in these studies, dephosphorylation was inferred by changes in the electrophoretic mobility of CRHSP24 and whether the site(s) that became dephosphorylated was Ser^{52} or another residue(s) is unknown. Our preliminary experiments suggest that Ser^{52} is unlikely to be one of the sites that become dephosphorylated, because calcium ionophores did not induce any dephosphorylation of this residue in HEK-293 cells, while PMA actually increased the phosphorylation of Ser^{52}, presumably by activating RSK isoforms (G. C. Auld, unpublished work). These findings suggest that agonist-induced dephosphorylation of CRHSP24 involves another residue(s), perhaps Ser^{41} which we have shown to be phosphorylated in serum-fed or serum-starved cells.

In testis extracts, CRHSP24 has been shown to co-immunoprecipitate with STYX (serine/threonine/tyrosine-interacting protein), a protein that is structurally related to protein tyrosine phosphatases, but is catalytically inactive [25]. Moreover, mice that do not express STYX have a 1000-fold reduction in the production of spermatozoa and display male sterility. We tried to investigate whether phosphorylation at Ser^{52} affected the interaction of CRHSP24 with STYX by co-expressing tagged versions of each protein and looking to see if interaction was affected by stimulation with IGF-1. However, under the conditions we tested, we were unable to detect a significant interaction between CRHSP24 and STYX in either wortmannin-treated or IGF-1-stimulated cells (results not shown).

CRHSP24 and its brain orthologue PIPPin both possess a CSD, which was reported to be flanked by two putative double-stranded RNA-binding motifs [26]. Indeed, PIPPin has been reported to bind to the 3′ untranslated region of the mRNAs encoding histone H1° and histone H3.3 [17]. Moreover, when added to *in vitro* translation systems, PIPPin inhibited the translation of the mRNAs encoding these histones [27]. However, we were unable to detect any significant binding of histone H1° RNA to our bacterially expressed CRHSP24 before or after phosphorylation by PKBα (results not shown).

It will clearly be necessary to identify the function of CRHSP24 before the role of Ser^{52} phosphorylation can be elucidated. However, the present study, in conjunction with our other previous reports [10,11,24,28,29] has again illustrated the power of the KESTREL method to find new physiological substrates of protein kinases.

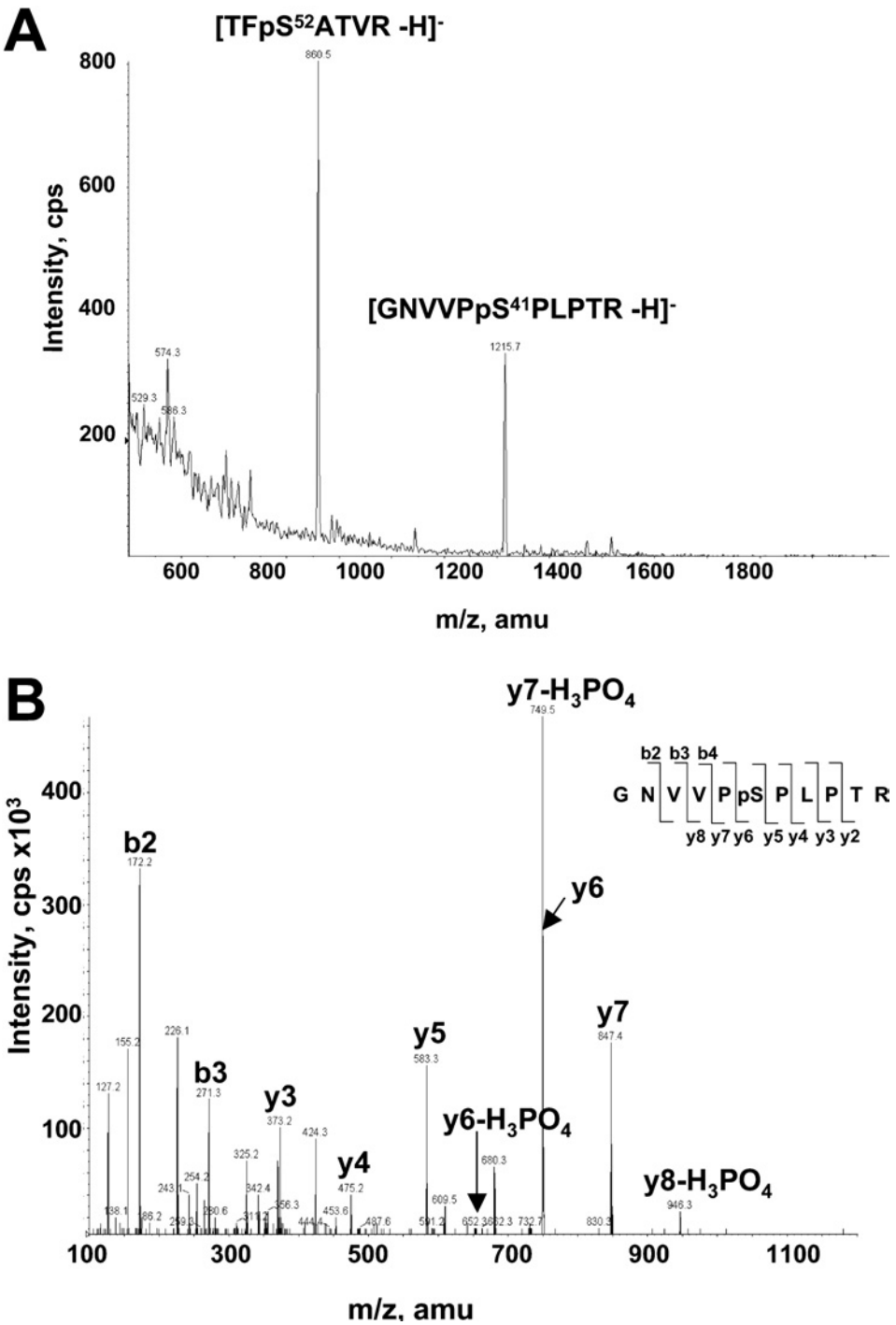

Figure 8 Identification of residues in CRHSP24 that are phosphorylated in cells

CRHSP24 was immunoprecipitated from serum-fed cells, and the tryptic phosphopeptides were separated and analysed on a 4000 Q-Trap mass spectrometer (see the Materials and methods section). (**A**) Using electrospray MS in precursor ion-scanning mode, the precursors of 79 scans obtained over the 45 min peptide separation were summed, and the two major phosphopeptide ions (detected as singly charged ions) annotated (pS, phosphoserine). (**B**) Positive-ion MS/MS spectrum of the phosphopeptide GNVVP(pS)LPTR by collision-induced dissociation of the doubly charged ion of $m/z = 608.76$. The mass difference between the y6-H_3PO_4 ion (652.3) and the y5 ion (583.3) corresponds to dehydroalanine, which is derived by β-elimination of phosphoric acid from phosphoserine and hence pinpoints the site of phosphorylation. amu, atomic mass units.

cDNA encoding DYRK2 was generously provided by Professor Walter Becker, Institute of Pharmacology and Toxicology, University of RWTH, Aachen, Germany, and the DNA encoding CRHSP24 and PIPPin was cloned by Dr Mark Peggie in our Unit. PDK1-deficient and PDK1[L155E]-expressing cells were developed by Dr Barry Collins and Professor Dario Alessi in our Unit and were a gift from these colleagues. We also thank our Protein Production and Antibody Purification Teams (co-ordinated by Dr Hilary McLauchlan and Dr James Hastie) for PKBα, SGK1 and RSK1, and the anti-CRHSP24 and anti-PIPPin antibodies. DNA sequencing was performed by the Sequencing Service (School of Life Sciences, University of Dundee, Scotland, U.K.; http://www.dnaseq.co.uk). This study was supported by the UK Medical Research Council, The Royal Society, AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck and Co., Merck KGaA and Pfizer.

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