WNK1, the kinase mutated in an inherited high-blood-pressure syndrome, is a novel PKB (protein kinase B)/Akt substrate

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Recent evidence indicates that mutations in the gene encoding the WNK1 [with no K (lysine) protein kinase-1] results in an inherited hypertension syndrome called pseudohypoaldosteronism type II. The mechanisms by which WNK1 is regulated or the substrates it phosphorylates are currently unknown. We noticed that Thr-60 of WNK1, which lies N-terminal to the catalytic domain, is located within a PKB (protein kinase B) phosphorylation consensus sequence. We found that PKB phosphorylated WNK1 efficiently compared with known substrates, and both peptide map and mutational analysis revealed that the major PKB site of phosphorylation was Thr-60. Employing a phosphospecific Thr-60 WNK1 antibody, we demonstrated that IGF1 (insulin-like growth factor) stimulation of HEK-293 cells induced phosphorylation of endogenously expressed WNK1 at Thr-60. Consistent with PKB mediating this phosphorylation, inhibitors of PI 3-kinase (phosphoinositide 3-kinase; wortmannin and LY294002) but not inhibitors of mammalian target of rapamycin (rapamycin) or MEK1 (mitogen-activated protein kinase kinase-1) activation (PD184352), inhibited IGF1-induced phosphorylation of endogenous WNK1 at Thr-60. Moreover, IGF1-induced phosphorylation of endogenous WNK1 did not occur in PDK1$^{-/-}$ ES (embryonic stem) cells, in which PKB is not activated. In contrast, IGF1 still induced normal phosphorylation of WNK1 in PDK1$^{L155E/L155E}$ knock-in ES cells in which PKB, but not S6K (p70 ribosomal S6 kinase) or SGK1 (serum- and glucocorticoid-induced protein kinase 1), is activated. Our study provides strong pharmacological and genetic evidence that PKB mediates the phosphorylation of WNK1 at Thr-60 *in vivo*. We also performed experiments which suggest that the phosphorylation of WNK1 by PKB is not regulating its kinase activity or cellular localization directly. These results provide the first connection between the PI 3-kinase/PKB pathway and WNK1, suggesting a mechanism by which this pathway may influence blood pressure.

Key words: Gordon's syndrome, hypertension, mass spectrometry (MS), 3-phosphoinositide-dependent protein kinase-1 (PDK1), phosphoinositide 3-kinase (PI 3-kinase), pseudohypoaldosteronism type II (PHAII).

INTRODUCTION

Growth factors and insulin trigger many diverse physiological processes through the activation of PI 3-kinase (phosphoinositide 3-kinase), which phosphorylates PtdIns(4,5)P_2 to generate the PtdIns(3,4,5)P_3 second messenger [1]. PtdIns(3,4,5)P_3 induces the activation of a group of serine/threonine protein kinases belonging to the AGC kinase [similar to PKA (cAMP-dependent protein kinase), PKG (protein kinase G) and PKC (protein kinase C) protein kinase] subfamily that include isoforms of PKB (protein kinase B) [2], S6K (p70 ribosomal S6 kinase) [3] and SGK (serum- and glucocorticoid-induced protein kinase) [4]. Following the activation of PI 3-kinase, these AGC subfamily kinases are activated by phosphorylation of their activation loop by the PDK1 (3-phosphoinositide-dependent protein kinase-1) [5,6]. Other AGC kinase members are also activated by different mechanisms. For example, RSK (p90 ribosomal S6 kinase) isoforms are not only activated by PDK1, but also by ERK1/ERK2 (extracellular-signal-regulated protein kinase 1/2) mitogen-activated protein kinase family members in response to growth factors and phorbol esters [7,8]. The MSK (mitogen- and stress-activated protein kinase-1) isoforms are activated *in vivo* by ERK1/ERK2 or the stress- and cytokine-activated p38 mitogen-activated protein kinase [9] but not PDK1 [10]. Similarly, the PKA is not activated by PDK1 but by agonists that stimulate adenylate cyclase [11].

AGC kinases regulate numerous processes, including cell growth, proliferation and survival, as well as many metabolic events involving the transport and storage of nutrients in the cell [12–14]. To date only a moderate number of *bona fide* substrates for these enzymes have been identified. One approach that can be employed to search for novel potential substrates is to scan databases for proteins possessing potential AGC kinase consensus motifs (Arg/Lys-Xaa-Arg-Xaa-Xaa-Ser/Thr-Hyd, where Hyd is a hydrophobic residue) [15–17]. Performing this analysis, we noticed that a recently identified protein kinase termed WNK1 [with no K (lysine) protein kinase-1] [18], possesses such a motif at its N-terminus (Thr-60). WNK1 is large protein kinase of 2382 residues, possessing a kinase catalytic domain at its N-terminus (residues 221–479) and three coiled-coil domains (Figure 1A) [19]. In humans, mutations in an intronic region of the WNK1 gene, which apparently increase WNK1 mRNA expression, have

Abbreviations used: PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; AGC kinase, similar to PKA, protein kinase G and PKC protein kinase; ERK, extracellular-signal-regulated protein kinase; ES, embryonic stem cells; GSK3, glycogen synthase kinase-3; GST, glutathione S-transferase; IGF1, insulin-like growth factor; KD, kinase dead; MBP, myelin basic protein; MSK1, mitogen- and stress-activated protein kinase-1; PDK1, 3-phosphoinositide-dependent protein kinase-1; PHAII, pseudohypoaldosteronism type II; PI 3-kinase, phosphoinositide 3-kinase; RSK, p90 ribosomal S6 kinase; S6K, p70 ribosomal S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase; WNK1, with no K (lysine) protein kinase-1; CREB, cAMP-response-element-binding protein; BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; GFP, green fluorescent protein.

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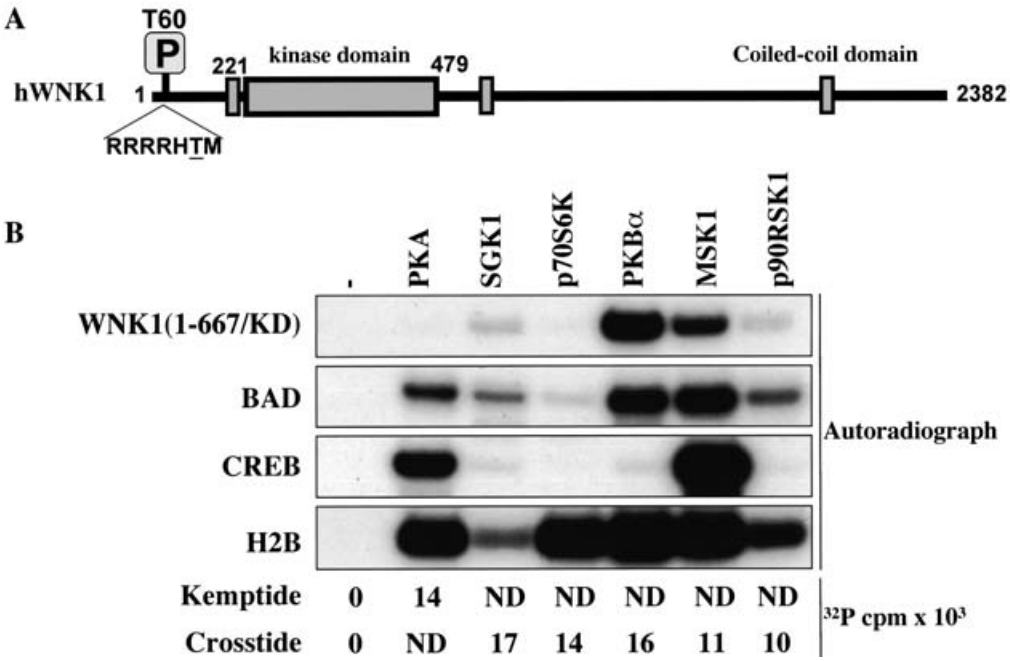

Figure 1 Phosphorylation of WNK1 by AGC kinases

(**A**) Schematic representation of the human (h) WNK1 coding sequence showing the location and surrounding residues of the T60 that lies in a conserved RXRXXT-Hyd (where Hyd is a hydrophobic residue) AGC kinase phosphorylation consensus sequence. (**B**) GST–WNK1(1–667/KD), GST–BAD, GST–CREB or histone 2B (H2B) and the peptide Crosstide or Kemptide were incubated with a final concentration of 0.1 units/ml of the indicated AGC kinase members in the presence of magnesium and [γ-^{32}P]ATP as described in the Experimental section. Phosphorylation of protein substrates was determined following electrophoresis on a 4–12 % gradient polyacrylamide gel by autoradiography of the Coomassie Blue-stained bands corresponding to each substrate. Phosphorylation of Crosstide and Kemptide was determined following adsorption of these peptides to phosphocellulose P81 paper (ND, not determined). Similar results were obtained in three separate experiments.

been linked to causing the inherited hypertension syndrome termed PHAII (pseudohypoaldosteronism type II) [20]. This disease is an autosomal dominant disorder in which patients present hypertension and hyperkalaemia (high serum potassium level). This hypertension, also known as Gordon's syndrome, is caused by increased renal salt re-absorption and the hyperkalaemia results from reduced renal potassium excretion [21]. WNK1 is widely expressed, including in the kidney, where it has been suggested it could play a role in regulating epithelial chloride flux, through the Na–Cl co-transporter [20,22,23]. Little is known regarding the mechanism by which WNK1 is regulated *in vivo*, nor have any physiological substrates for this enzyme been identified. In this study we provide biochemical, pharmacological and genetic evidence that PKB can phosphorylate WNK1 at this residue. This represents the first evidence implicating the PI 3-kinase/PKB pathways in targeting WNK1.

EXPERIMENTAL

Materials

Asp-N endoprotease, protease-inhibitor cocktail tablets Complete, FuGENE-6^{TM} transfection reagent and histone 2B were from Roche, IGF1 (insulin-like growth factor) was from Biosource, dialysed fetal bovine serum and other tissue culture reagents were from Life Technologies. G-418 was from Melford Laboratories (Chelsworth, Ipswich, Suffolk, U.K.). LY294002, H89, rapamycin, *N*-octylglucoside and wortmannin were from Calbiochem. [γ-^{32}P]ATP, Protein G–Sepharose and glutathione–Sepharose were from Amersham Biosciences. DMSO, forskolin, PMA, dimethyl pimelidate and PKA were from Sigma. PD184352 was custom-synthesized. The precast 4–12 % Bis-Tris gradient SDS/polyacrylamide gels and 3–8 % Tris/acetate were from Invitrogen. CREB (cAMP-response-element-binding protein) [9] and BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) [24] were expressed as GST (glutathione S-transferase) fusion proteins in *Escherichia coli* as described previously.

Antibodies

The Total-WNK1 antibody was raised in sheep against WNK1 protein encompassing residues 61–667, expressed in *E. coli* as a GST fusion protein and the GST moiety removed prior to immunization, as described below. The antibody was affinity purified on CH-Sepharose covalently coupled to the WNK1(61–667) protein. The phosphospecific antibody recognizing WNK1 phosphorylated at Thr-60 (termed T60-P antibody) was raised in sheep against the peptide EYRRRRHTMDKDSRGA (corresponding to residues 53–68 of human WNK1) in which the underlined residue is phosphothreonine. The antibody was affinity-purified on CH-Sepharose covalently coupled to the phosphorylated peptide, then passed through a column of CH-Sepharose coupled to the non-phosphorylated peptide. Antibody that did not bind to the latter column was selected. The following antibodies were raised in sheep and affinity-purified on the appropriate antigen: NT-WNK1 (residues 4–27 of human WNK1, GAAEKQSSTPGSLFLSPPAPAPKN), CT-WNK1 (residues 2360–2382 of human WNK1, QNFNISNLQKSISNPPGSNLRTT), Total human GSK3α (glycogen synthase kinase-3α; residues 471–483 of human GSK3α, CQSTDATPTLTNSS), Total mouse GSK3α (residues 314–327 of mouse GSK3α, CQPSDATTATLASSS), Total GSK3β (full-length human protein), Total S6K1 (residues 25–44 of rat S6K1, AGVFDIDLDQPEDAGSEDEL), Total RSK antibody (residues 712–734 of human p90 RSK2, RNQSPVLEPVGRSTLAQRRGIKK), Total MSK1 (residues 716–734 of

human MSK1, KATFHAFNKYKREGFCLQN), Total PKBα (residues 466–480 of rat PKBα, RPHFPQFSYSASGTA) and PKBα phospho-S473 (residues 467–477 of human PKBα, KHFPQFSpYSAS).

The following antibodies were purchased from Cell Signalling Technology and the catalogue numbers are indicated: PKB phospho-T308-P (no. 9275), GSK3α/GSK3β phospho-S21-P/S9-P (no. 9336), S6K1 phospho-T389-P (no. 9205), Total S6 Protein (no. 2212), phospho-Akt substrate antibody, which recognizes phosphorylated S6 protein [25] (no. 9611), Total ERK1/2 (no. 9102), ERK1/2 phospho-T202/Y204-P (no. 9101) and Total CREB (no. 9192). Antibodies recognizing CREB phospho-S133-P (no. 06–519) were from Upstate Biotechnology. Mouse monoclonal antibodies anti-FLAG M2 and mouse monoclonal antibodies recognizing GST were purchased from Sigma. Antibodies (sc-8334) recognizing GFP (green fluorescent protein) were from Santa Cruz Biotechnology. Secondary antibodies coupled to horseradish peroxidase were from Pierce.

General methods and buffers

Phosphoamino acid analysis of ^{32}P-labelled peptides, PCR, restriction enzyme digests, DNA ligations, site-directed mutagenesis and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing. This was performed by The Sequencing Service, School of Life Sciences, University of Dundee, U.K., using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers.

Lysis buffer was 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 % (by mass) Triton-X 100, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1 % (by vol.) 2-mercaptoethanol and Complete protease inhibitor cocktail (one tablet per 50 ml). Buffer A was 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 % (by vol.) 2-mercaptoethanol. Sample buffer was 2 % (by mass) lauryl dodecyl sulphate, 10 % (by vol.) glycerol, 200 mM Tris, pH 8.5, 25 mM dithiothreitol and 0.5 mM EDTA.

Cloning of full-length hWNK1 cDNA

In order to clone full-length WNK1 cDNA (NCBI accession nos. CAC15059 and NM_018979) four EST clones were ordered from the IMAGE consortium (NCBI accession nos. BG480518, BI825970, BG470798 and AL589547). These clones encode different regions of WNK1 and were used as templates for a PCR strategy to create a full-length WNK1 cDNA possessing an N-terminal FLAG tag which subcloned into the pEBG-2T vector. This construct codes for the expression of full-length GST–WNK1 in mammalian cells. In order to generate expression constructs encoding N-terminal FLAG-tagged WNK1 residues 61–667, PCR primers A and B were used; to create FLAG-tagged WNK1 residues 1–667, PCR primers C and B were employed: A, 5′-ATCGATGCCACCATGGACTACAAGGACGACGATGACAAGGACAAGGACAGCCGTGGGGCGGCCGCGACCACTACCACCACTGAGCA-3′; B, 5′-ATATCGATATCAAGATTCTGTGAAGACAGAGGATCCCTG-3′; C, 5′-ATTATCGATATTGCCACCATGGACTACAAGGACGACGATGACAAGTCTGGCGGCGCCGCAGAGAAGCAGAGCA-3′. The resulting PCR products were ligated into pCR 2.1 TOPO vector (Invitrogen), subcloned as a *Cla*I–*Cla*I fragment into the pEBG-2T vector and as *Bam*HI–*Bam*HI fragments into *E. coli* expression vector pGEX-6P-1 (Amersham Biosciences). The FLAG-tagged full-length WNK1 was subcloned into the pEGFP-C2 (Clontech) as an *Eco*RI–*Eco*RI fragment.

Expression of WNK1 in *E. coli*

The pGEX-6P-1 construct encoding GST–WNK1(61–667) was transformed into BL21 *E. coli* cells and a 0.5 litre culture was grown at 37 °C in Luria broth containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol until the absorbance at 600 nm was 0.6, and 0.3 mM isopropyl β-D-thiogalactoside was then added. The cells cultured for a further 16 h at 26 °C, resuspended in 24 ml of ice-cold lysis buffer and frozen in liquid nitrogen. After thawing, the suspension was sonicated and the lysates centrifuged at 4 °C for 30 min at 26000 ***g*** and incubated with 0.5 ml of glutathione–Sepharose for 1 h. The resin was washed in lysis buffer containing 0.5 M NaCl followed by buffer A. The resin was resuspended in 1 ml of buffer A containing 0.27 M sucrose and 5 μg of GST–PreScission Protease was added to cleave the GST tag from WNK1. Following overnight incubation, cleaved WNK1(61–667) was eluted and protein stored in aliquots at − 80 °C.

Expression of GST–WNK1 in HEK-293 cells

HEK-293 cells cultured in 10-cm-diameter dishes were transfected with 10 μg of the pEBG-2T construct encoding wild-type or indicated forms of WNK1, using a modified calcium phosphate method [26]. At 36 h post-transfection, the cells were lysed in 0.5 ml of ice-cold lysis buffer, the lysates pooled, centrifuged at 4 °C for 30 min at 26000 ***g*** and the GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose and stored in buffer A containing 0.27 M sucrose, as described previously for GST–PDK1 [27].

Cell culture, stimulation and cell lysis

HEK-293 cells were grown in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum, antibiotics (100 units of penicillin G and 0.1 mg/ml streptomycin) and 2 mM L-glutamine. PDK1$^{+/+}$, PDK1$^{-/-}$ [10], PDK1$^{L155E/L155E}$ [28] ES (embryonic stem) cells were grown on gelatinized tissue culture plastic in KnockOut Dulbecco's modified Eagle's medium containing 10 % KnockOut SR supplemented with 0.1 mM non-essential amino acids, antibiotics (100 units of penicillin G and 0.1 mg/ml streptomycin), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol and 25 ng/ml murine leukaemia inhibitory factor as described previously [10]. Prior to stimulation, HEK-293 and ES cells were deprived of serum for 18 or 4 h, respectively. Inhibitors were dissolved in DMSO at a 1000-fold higher concentration than they were used. These inhibitors, or the equivalent volume of DMSO as a control, were added to the tissue culture medium 30 min prior to stimulation unless indicated otherwise. The cells were stimulated with the indicated agonists, lysed in 0.5 ml of ice-cold lysis buffer and centrifuged at 4 °C for 15 min at 13000 ***g***. The supernatants were frozen in liquid nitrogen and stored at − 80 °C. Protein concentrations were determined using the Bradford method and BSA was employed as the standard.

Generation of HEK-293 stable cell lines

HEK-293 cells were cultured to 50 % confluence on 10-cm-diameter dishes and transfected with 2.5 μg of the pEGFP-C2 vector encoding FLAG-epitope-tagged WNK1 employing the

FuGENE-6™ transfection reagent, following the manufacturer's protocol. After 48 h, G-418 was added to the medium to a final concentration of 3 mg/ml, and the medium was changed every 48 h maintaining G-418. After 10 days, colonies of fluorescing cells were isolated and expanded.

Phosphorylation of WNK1 by AGC kinases

PKA was from Sigma; GST–SGK1 [29], GST–S6K1 [30] and GST–PKBα [31] were expressed in HEK-293 cells which were stimulated with 20 ng/ml IGF1 for 15 min (SGK1), 40 min (S6K1) or 10 min (PKBα) prior to cell lysis. GST–MSK1 and GST–RSK1 [9] were also expressed in HEK-293 cells which were stimulated with PMA (400 ng/ml) for 15 min before lysis. These active GST-tagged AGC kinase members were then affinity-purified on glutathione–Sepharose as described previously for PDK1 [27]. The phosphorylation assays using the GST–WNK1(1–667/KD) (where KD means kinase dead), GST–WNK1(1–667/KD/T60V), GST–CREB, GST–BAD or histone 2B as substrates were set up in a total vol of 20 μl containing 2 μg of substrate, 2 m-units of AGC kinase, 10 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP (500 c.p.m./pmol). After incubation for 30 min at 30 °C, incorporation of phosphate was determined following electrophoresis of samples on a NuPAGE Bis-Tris 4–12 % gels and autoradiography of the Coomassie Blue-stained gels. The AGC kinases were also assayed using Crosstide (GRPRTSSFAEG, 30 μM) or Kemptide (LRRASLG, 30 μM) in parallel assays and incorporation of ^{32}P radioactivity into these peptides was determined using P81 phosphocellulose [32].

Mapping the site on WNK1 labelled by PKBα and MSK1

In order to map the site on WNK1 phosphorylated by PKBα and MSK1, GST–WNK1(1–667/KD) was phosphorylated as described above, except that the reaction was performed for 60 min and a 10-fold higher specific activity of ATP was employed. The reactions were terminated by adding 1 % (by mass) SDS and 10 mM dithiothreitol and heating at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 1 % (by vol), the sample was agitated for 30 min at room temperature and subjected to electrophoresis on a 4–12 % NuPAGE Bis-Tris gel. The ^{32}P-labelled band corresponding to WNK1(1–667/KD) was excised, cut into smaller pieces that were washed sequentially for 15 min on a vibrating platform with 0.5 ml of the following: water, a 1:1 mixture of water/acetonitrile, 0.1 M ammonium bicarbonate, a 1:1 mixture of 0.1 M ammonium bicarbonate/acetonitrile and finally acetonitrile. The gel pieces were dried by rotary evaporation and incubated in 80 μl of 20 mM ammonium bicarbonate, 0.1 % (by mass) *N*-octylglucoside containing 1 μg of Asp-N endoprotease. After 16 h the supernatant was removed, the gel pieces washed for 10 min in a further 0.1 ml of acetonitrile, and the combined supernatants containing >65 % of the ^{32}P radioactivity were chromatographed on a Vydac C_{18} column as described in the legend to Figure 2 (see below).

Phosphopeptide sequence analysis

Peptides were analysed by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS on a PerSeptive Biosystems Elite-STR mass spectrometer using α-cyanocinnamic acid as the matrix. Spectra were obtained in both the linear and reflector modes.

Immunoblotting of WNK1 immunoprecipitates

The Total-WNK1 antibody (1 mg) was covalently coupled to Protein G–Sepharose (1 ml) using the dimethyl pimelidate method [33]. For each immunoprecipitation, 5 μl of the WNK1–Protein G–Sepahrose conjugate was incubated with HEK-293 (0.5 mg) or ES (0.75 mg) cell lysates for 60 min at 4 °C. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, and washed once either with buffer A and resuspended to a final volume of 80 μl in SDS sample buffer. For the T60-P immunoblots, 10 μl, and for the Total-WNK1 immunoblots, 5 μl, of the WNK1 immunoprecipitates were subjected to SDS/PAGE and transferred to nitrocellulose (3–8 % Tris/acetate gels and a transfer of 3 h at 40 V). The nitrocellulose membranes were incubated in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl and 0.2 % (by vol.) Tween containing 10 % (by mass) skimmed milk and then were immunoblotted at 4 °C for 16 or 2 h at room temperature using either 2 μg/ml T60-P antibody in the presence of 5 μg/ml dephospho-EYRRRRHTMDKDSRGA peptide or 0.5 μg/ml Total-WNK1 antibody.

Immunoblotting of cell lysates

For blots of total cell lysates, 20 μg of protein was immunoblotted. Membranes were incubated in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl and 0.2 % (by vol) Tween containing 10 % (by mass) skimmed milk and then were immunoblotted at 4 °C for 16 or 2 h at room temperature using the indicated antibodies (1–2 μg/ml for the sheep antibodies or 1000-fold dilution of commercial antibodies). For sheep antibodies and phospho-CREB, blotting with the primary antibody was carried out in 10 % (by mass) skimmed milk, whereas for the remainder of the commercial antibodies, blotting was performed in 5 % (by mass) BSA. In addition, for all of the phosphospecific antibodies raised in sheep, blotting was performed in the presence of 5 μg/ml of the dephosphopeptide antigen used to raise the antibody. Detection was performed using horseradish-peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (ECL®; Amersham Biosciences).

Immunoprecipitation and assay of p90RSK and MSK1

HEK-293 cell lysates was used to immunoprecipitate MSK1 (500 μg of protein) and p90RSK (250 μg of protein). The lysates were incubated at 4 °C for 1 h on a shaking platform with 5 μg of each antibody coupled to 5 μl of Protein G–Sepharose. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, and twice with 1 ml of buffer A. The assay (50 μl) contained washed Protein G–Sepharose immunoprecipitate, 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP ($\approx$500 c.p.m./pmol) and Crosstide (GRPRTSSFAEG, 30 μM). The assays were carried out for 30 min at 30 °C, with the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described previously [32]. A unit of activity was defined as the amount of enzyme that catalysed the phosphorylation of 1 nmol of peptide substrate in 1 min.

WNK1 autophosphorylation and phosphorylation of MBP (myelin basic protein)

We incubated 0.2 μg of GST–WNK1(1–667), GST–WNK1 (1–667/KD), GST–WNK1(1–667/T60V), GST–WNK1(1–667/T60E), expressed in HEK-293 cells, or WNK1 immunoprecipitated from 250 μg of HEK-293 cells, with 5 μl of WNK1–Protein

G–Sepahrose conjugate antibodies as above. The 20 μl reaction mixture contained 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EGTA, 0.03 % (by vol) Brij-35, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (500 c.p.m./pmol) in the presence of 10 μM MBP. After incubation for the indicated times at 30 °C, the reactions were terminated by the addition of SDS sample buffer and the samples electrophoresed on a NuPAGE Bis-Tris 4–12 % gel, using Mes running buffer, and analysed by autoradiography. Brij-35 was found to significantly stabilize WNK1 activity in the kinase assay (results not shown) and was therefore included in the assay.

Immunofluorescence

HEK-293 cells were cultured on coverslips to 50 % confluence on six-well cluster plates and transfected with 1 μg of a construct per well encoding wild-type GFP-WNK1 with the FuGENE-6™ transfection reagent following the manufacturer's protocol. 24 h post-transfection, cells were serum-starved for 18 h. Cells were left unstimulated or were stimulated with 100 ng/ml IGF1 in the presence or absence of 100 μM LY294002 fixed in 4 % (w/v) paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgSO_4$, pH 7.0). The cells were washed three times with PBS/0.1 % (v/v) Tween, once with water and mounted on slides with Mowiol. The cells were imaged using Zeiss LSM 510 META confocal microscope.

RESULTS

Phosphorylation of WNK1 at Thr-60 by PKBα and MSK1

As mentioned in the Introduction, Thr-60 of WNK1 lies in an Arg-Arg-Arg-Arg-His-Thr-Met sequence (Figure 1A), which is a potential AGC kinase phosphorylation motif, and this sequence is conserved in all characterized mammalian WNK1 sequences. As we were unable to express and purify sufficient amounts of full-length WNK1 for *in vitro* phosphorylation and peptide mapping studies, we expressed a catalytically inactive N-terminal fragment of WNK1 comprising residues 1–667, in which Asp-368 in the catalytic domain was mutated to Ala to prevent WNK1 autophosphorylation. This fragment was expressed in HEK-293 cells as a fusion protein with GST [hereafter termed GST–WNK1(1–667/KD)] and affinity-purified on glutathione–Sepharose. PKBα and MSK1, but not PKA, SGK1, S6K1 or RSK1 (all at $\approx$0.1 unit/ml using peptide substrates) phosphorylated GST–WNK1(1–667/KD) (Figure 1B). Control experiments showed that under the same conditions, as expected, the pro-apoptotic protein BAD was phosphorylated with similar efficiency by PKA, SGK1, PKBα, MSK1 and RSK1 [24], whereas the transcription factor CREB was phosphorylated to a similar extent by MSK1 and PKA, but at a vastly lower rate by RSK1 [9] (Figure 1B). Histone 2B was also similarly phosphorylated by PKA, S6K1, PKBα and MSK1, and to lower extent by RSK1 and SGK1.

Under the conditions employed, PKBα and MSK1 (at $\approx$0.1 units/ml) phosphorylated GST–WNK1(1–667/KD) to 0.16 (PKBα) and 0.11 (MSK1) mol of phosphate/mol of protein after 60 min. Digestion of the labelled GST–WNK1(1–667/KD) with Asp-N, followed by chromatography on a C_{18} column revealed that both PKBα and MSK1 had phosphorylated GST–WNK1(1–667/KD) at one major phosphopeptide termed P1, eluting at 14.3 % acetonitrile (Figure 2A). Phosphoamino acid analysis revealed that peptide P1 contained only phosphothreonine (Figure 2B) and MALDI-TOF MS established the identity of P1 as the WNK1 phosphopeptide comprising residues 52–61 phosphorylated at Thr-60 (Figure 2C). Moreover, when Thr-60 on GST–WNK1(1–667/KD) was mutated to Ala, the resulting mutant was no longer phosphorylated by PKBα and weakly by MSK1 (Figure 2D), further confirming that Thr-60 is the major site of phosphorylation on this fragment of WNK1 by these kinases.

Generation of a phosphospecific antibody that recognizes WNK1 phosphorylated at Thr-60

As described in the Experimental section, we raised a phospho-specific antibody against a phospho-peptide that encompasses the Thr-60 phosphorylation site on WNK1 (T60-P antibody). In order to establish the specificity of this antibody, full-length GST–WNK1 was expressed in HEK-293 cells, which were stimulated with IGF1 in the presence or absence of the PI 3-kinase inhibitor LY294002. The T60-P phosphospecific antibody recognized wild-type GST–WNK1 and stimulation of cells with IGF1 increased recognition by the T60-P phosphospecific antibody, which was markedly suppressed by LY294002 (Figure 3). In contrast, a mutant GST–WNK1(T60V) in which Thr-60 was mutated to valine was not recognized by the T60-P phosphospecific antibody (Figure 3). These data indicated that the T60-P antibody was specific for WNK1 phosphorylated at Thr-60 and that IGF1 stimulation of cells is promoting the phosphorylation of WNK1 at this residue in a PI 3-kinase-dependent manner. A catalytically inactive full-length GST–WNK1(D368A) mutant was similarly phosphorylated at Thr-60 as wild-type GST–WNK1 when expressed in HEK-293 cells (Figure 3), indicating that this phosphorylation of GST–WNK1 at Thr-60 is not mediated by autophosphorylation. Control experiments established that, as expected, IGF1 induced phosphorylation of PKBα at Thr-308 and phosphorylation of the PKB substrate GSK3α/GSK3β at Ser-21/Ser-9 and that these phosphorylations were inhibited by treatment of cells with LY294002 (Figure 3).

IGF1 induces phosphorylation of endogenous WNK1 at Thr-60

When analysing the results presented in Figure 3, we noticed that a protein with a molecular mass corresponding to that of endogenous WNK1 (251 kDa) was detected in the IGF1-stimulated cells and not in the control or LY294002-treated cells. This protein ran at the same molecular mass as endogenous WNK1 protein detected with an anti-WNK1 total antibody (Figure 3). In order to confirm that endogenously expressed WNK1 was phosphorylated at Thr-60, we immunoprecipitated endogenous WNK1 from unstimulated and IGF1-stimulated HEK-293 cells and immunoblotted the WNK1 immunoprecipitates with the T60-P WNK1 phosphospecific antibody (Figure 4A, panel 1). This analysis revealed, in unstimulated cells, that endogenous WNK1 was not significantly phosphorylated but within 2 min of IGF1 stimulation WNK1 was markedly phosphorylated at Thr-60 and this was sustained for at least 40 min.

Pharmacological evidence that PKB mediates phosphorylation of WNK1 at Thr-60

The rapid IGF1-induced phosphorylation of WNK1 at Thr-60 indicated that this could be induced by PKB, which is activated within 1–2 min. Monitoring phosphorylation of PKB at Thr-308 and Ser-473 (Figure 4A, panel 2) as well as phosphorylation of its substrates GSK3α and GSK3β (Figure 4A, panel 3) confirmed that PKB was significantly activated within 2 min of IGF1 stimulation and phosphorylation was also maintained for 40 min. In contrast, S6K was activated much more slowly, with no significant activation of S6K (Figure 4A, panel 4) or phosphorylation

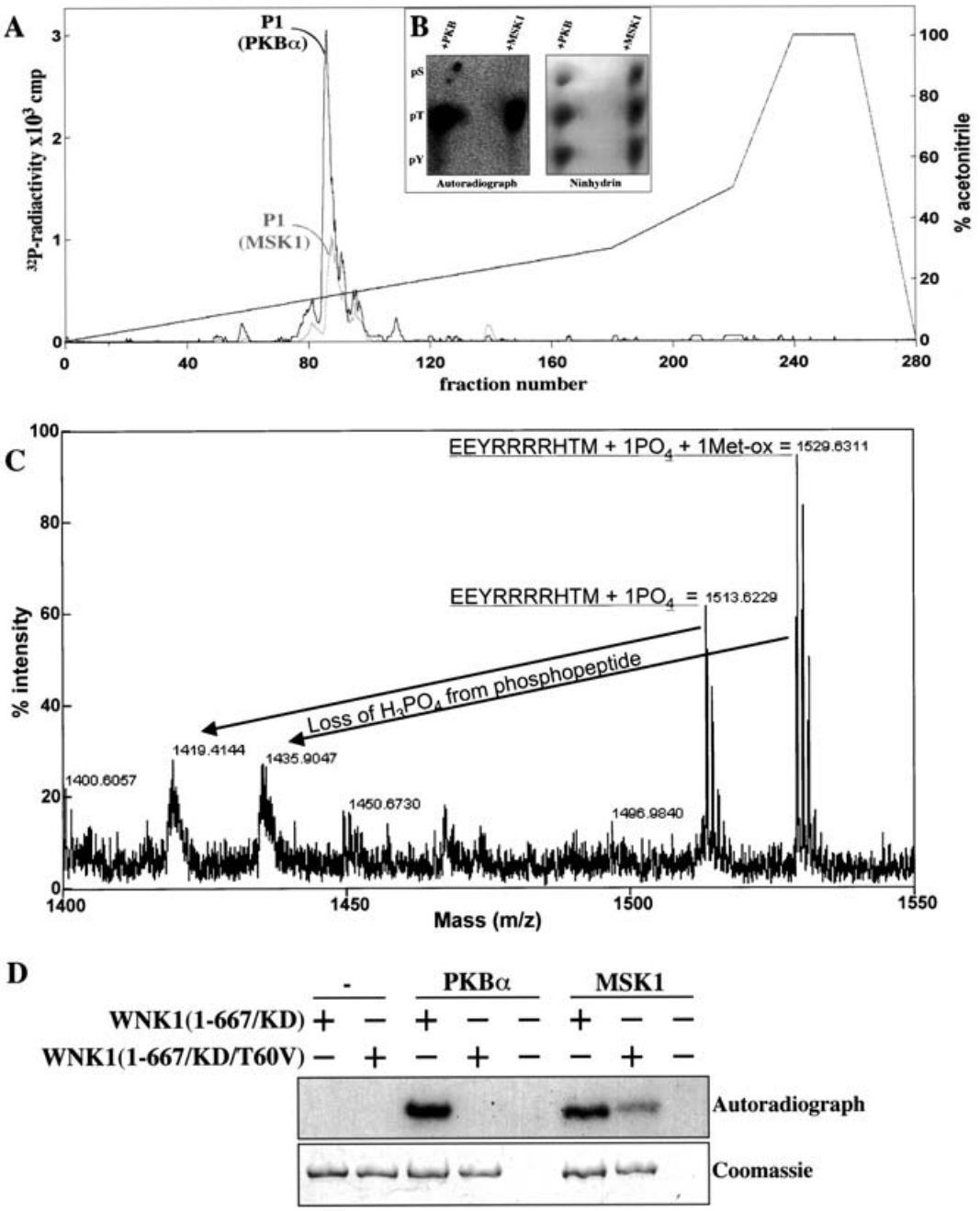

Figure 2 Phosphorylation of WNK1 at Thr-60 by PKBα and MSK1

(**A**) GST–WNK1(1–667/KD) that had been phosphorylated with PKBα or MSK1 was digested with Asp-N endoprotease and chromatographed on a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1 % (by vol.) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. The major ^{32}P-containing peptide, termed P1, was eluted at 14.3 % acetonitrile. The HPLC profile for PKBα phosphorylation is shown with a black line and that for MSK1 phosphorylation by a grey line. (**B**) The ^{32}P-labelled peptide P1 was hydrolysed for 90 min at 110 °C in 6 M HCl and subjected to phosphoamino acid analysis. An autoradiograph of the ninhydrin-stained separation on thin-layer cellulose is shown. pS, pT and pY show the positions of phosphoserine, phosphothreonine and phosphotyrosine respectively. The phosphoamino acid analysis established that the P1 peptide is phosphorylated on threonine. (**C**) The MALDI-TOF MS profile of the fraction corresponding to the major radioactive peak P1 derived following phosphorylation with PKBα, revealed the P1 phosphorylated peptide encompassed residues 52–61 of WNK1 phosphorylated at a single residues. As this peptide contains only a single Thr at a residue equivalent to Thr-60 of WNK1, this is defined as the site of phosphorylation. A similar spectrum was obtained for P1 derived following MSK1 phosphorylation (results not shown). (**D**) GST–WNK1(1–667/KD) or GST–WNK1(1–667/KD/T60V) was phosphorylated with the indicated AGC kinases under conditions described in the legend to Figure 1(B). Similar results were obtained in two separate experiments.

of the S6 protein (Figure 4A, panel 5), occurring before 5 min. Consistent with PKB mediating phosphorylation of WNK1, two structurally distinct PI 3-kinase inhibitors, namely LY294002 (Figure 4B, panel 1) and wortmannin (Figure 4C, panel 1), inhibited IGF1-induced phosphorylation of WNK1 at Thr-60. As expected, these inhibitors also prevented phosphorylation of PKB, GSK3α/GSK3β, S6K and S6 protein (Figure 4B and 4C, panels 2–5). Treatment of cells with the mTOR (mammalian target of

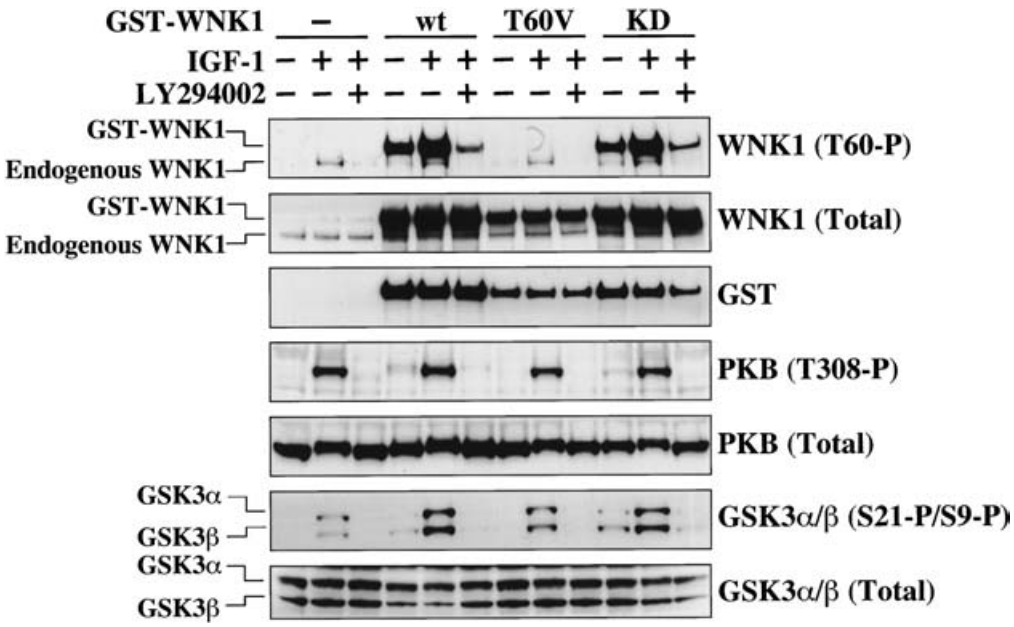

Figure 3 Generation and characterization of a WNK1 Thr-60 phosphospecific antibody

HEK-293 cells were left untransfected or transfected with plasmids encoding the expression of full-length wild-type (wt) or indicated mutants of GST–WNK1. At 24 h post-transfection, cells were deprived of serum for 18 h, incubated in the presence or absence of 100 μM LY294002 for 30 min and either left unstimulated or stimulated with 20 ng/ml IGF1 for 20 min as indicated. Cells were lysed and lysates (20 μg of protein) were immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments.

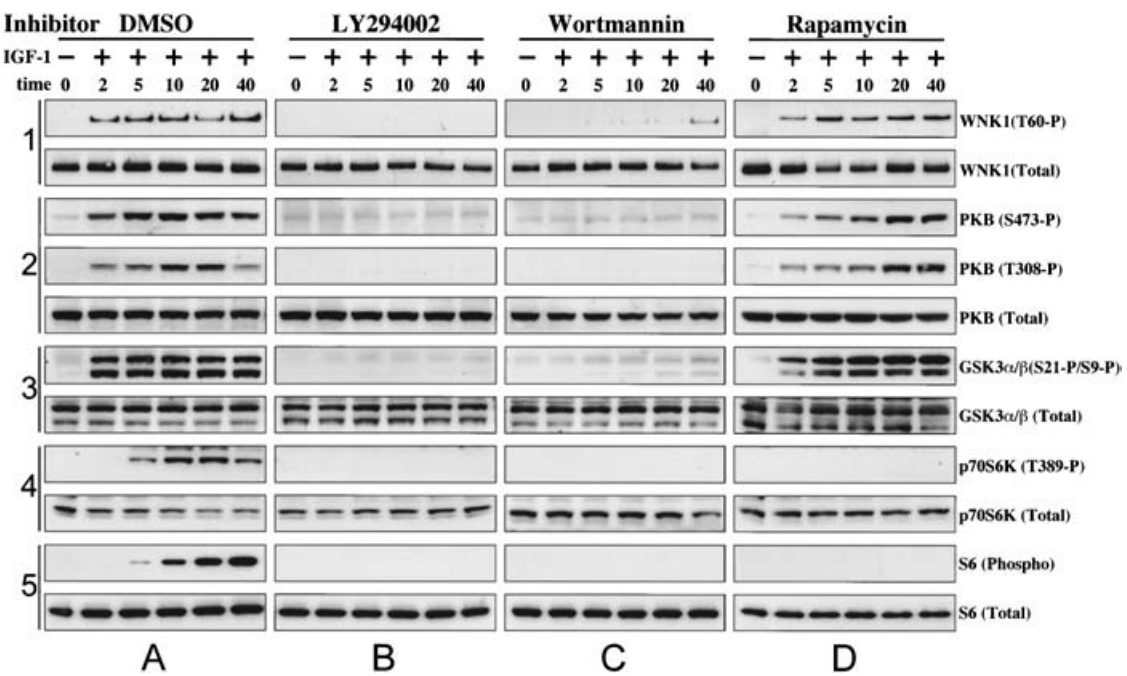

Figure 4 Pharmacological evidence that PKB phosphorylates WNK1

HEK-293 cells were deprived of serum for 18 h, treated for 30 min in the presence or absence of 100 μM LY294002, 100 nM wortmannin or 100 nM rapamycin prior to stimulation with 20 ng/ml IGF1 for the times indicated. The cells were lysed, WNK1 was immunoprecipitated, subjected to electrophoresis on a 3–8 % gradient polyacrylamide gel, transferred to nitrocellulose and immunoblotted with either the T60-P antibody or the total antibody raised against the WNK1(61–667) protein. In addition, cell lysates (20 μg of protein) were immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments.

rapamycin) inhibitor rapamycin, which inhibits S6K activation and hence S6 protein phosphorylation, without affecting PKB activation or phosphorylation of GSK3α/GSK3β (Figure 4D, panels 2–5), had no effect on IGF1-induced phosphorylation of WNK1 (Figure 4D, panel 1), confirming that S6K does not mediate this phosphorylation.

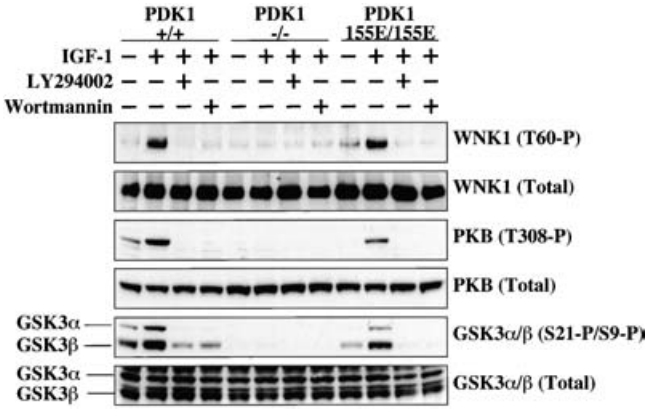

Figure 5 Genetic evidence that PKB phosphorylates WNK1

The indicated mouse ES cell lines were deprived of serum for 4 h, incubated in the presence or absence of 100 μM LY294002 for 30 min, 100 nM wortmannin for 10 min and then either left unstimulated or stimulated with 20 ng/ml IGF1 for 20 min. The cells were lysed, WNK1 was immunoprecipitated, subjected to electrophoresis on a 3–8 % gradient polyacrylamide gel, transferred to nitrocellulose and immunoblotted with either the T60-P antibody or the total antibody. Cell lysates (20 μg of protein) were also immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments.

Genetic evidence that PKB mediates phosphorylation of WNK1 at Thr-60

PKB is activated by phosphorylation of its activation loop (Thr-308) by PDK1 [5]. We have previously generated mouse ES cells deficient in the expression of PDK1 (termed PDK1$^{-/-}$ cells) and, as expected, these cells possess no detectable PKB activity or phosphorylation of Thr-308 after IGF1-stimulation [10]. We have also recently generated PDK1$^{L155E/L155E}$ knock-in ES cells in which a substrate-docking site on PDK1 is disrupted in a way that prevents activation of S6K, SGK and RSK substrates without affecting PKB activation [28]. We employed these cells to investigate whether IGF1 induced the phosphorylation of endogenous WNK1 in PDK1$^{-/-}$ and PDK1$^{L155E/L155E}$ knock-in ES cells (Figure 5). IGF1 induced a marked phosphorylation of WNK1 at Thr-60 in both wild-type PDK1$^{+/+}$ and PDK1$^{L155E/L155E}$ knock-in ES cells, in which PKB is fully activated and GSK3α/GSK3β is phosphorylated. As in HEK-293 cells, phosphorylation of WNK1 in response to IGF1 was inhibited by treatment of cells with LY294002 and wortmannin (Figure 5). In contrast, in PDK1$^{-/-}$ ES cells, IGF1 failed to induce detectable WNK1 phosphorylation under conditions in which PKB is not activated and GSK3α/GSK3β are not phosphorylated. These results strongly support the notion that PKB mediates the phosphorylation of WNK1 at Thr-60.

Verification of whether RSK, MSK and PKA phosphorylate WNK1 *in vivo*

In order to study whether isoforms of RSK or MSK could mediate phosphorylation of WNK1, we stimulated HEK-293 cells with PMA. This induces marked activation of these kinases through the ERK1/ERK2 signalling pathways without activating PKB. PMA treatment of HEK-293 cells induced a very weak phosphorylation of WNK1 at Thr-60 compared with that observed with IGF1 in parallel experiments (Figure 6A). As expected under these conditions, PMA greatly stimulated ERK1/ERK2 phosphorylation (Figure 6A) as well as RSK and MSK1 activation (Figure 6B). In addition, PMA also induced phosphorylation of the GSK3α/GSK3β and the transcription factor CREB (Figure 6A), which are phosphorylated by RSK [34] and MSK isoforms [35] respectively under these conditions. PMA does not detectably induce phosphorylation of PKB at Thr-308, consistent with previous findings showing that phorbol esters do not activate PKB in HEK-293 cells. In order to verify whether the weak phosphorylation of WNK1 at Thr-60 induced by PMA was mediated by RSK or MSK, we tested whether this phosphorylation was affected by the PD184352 inhibitor of MEK (mitogen-activated protein kinase/ERK kinase) activation [36]. We found that the PMA-induced phosphorylation of WNK1 was not affected by PD184352, under conditions where this drug prevented ERK phosphorylation and phosphorylation of GSK3α/GSK3β and CREB (Figure 6D). This suggests that a PMA-induced kinase, perhaps a PKC isoform not regulated by ERK, may weakly phosphorylate WNK1 at Thr-60.

To investigate whether PKA could phosphorylate WNK1 *in vivo*, HEK-293 cells were stimulated with the adenylate cyclase agonist forskolin for periods of up to 80 min. Under conditions where forskolin induced phosphorylation of the PKA substrates CREB [37] and GSK3α/GSK3β [38], WNK1 was only weakly phosphorylated at Thr-60 compared with phosphorylation induced by IGF1 in parallel experiments. To investigate whether PKA mediated the weak phosphorylation of WNK1 induced by forskolin, we verified whether this phosphorylation was sensitive to H89, an inhibitor of PKA [39]. We found that treatment of cells with 10 μM H89 conditions that prevent forskolin-induced phosphorylation of CREB and GSK3α/GSK3β, also inhibited the weak phosphorylation of WNK1 at Thr-60 (Figure 6D). These findings indicate that PKA may weakly phosphorylate WNK1 in cells.

Evidence that phosphorylation of WNK1 does not affect catalytic activity

No physiological substrates for WNK1 have thus far been identified and, to date, WNK1 catalytic activity has been assessed by monitoring autophosphorylation and phosphorylation of the non-specific kinase substrate MBP, which WNK1 phosphorylates poorly [18]. We first compared the autophosphorylation and MBP kinase activities of wild-type GST–WNK1 (1–667) and mutants of GST–WNK1(1–667) in which Thr-60 was mutated to either Val to prevent phosphorylation or Glu to mimic phosphorylation. As a further control we employed catalytically inactive GST–WNK1(1–667/KD). In Figure 7(A) we show that wild-type and both the Thr-60 mutants autophosphorylated and phosphorylated MBP at similar rates, providing evidence that phosphorylation of WNK1 does not affect the activity of this N-terminal fragment of WNK1. A catalytically inactive GST–WNK1(1–667/KD) mutant did not autophosphorylate or phosphorylate MBP (Figure 7A), indicating the MBP phosphorylation was indeed being catalysed by WNK1 and not by another kinase present as a trace contaminant in the preparation.

We also immunoprecipitated endogenous WNK1 from HEK-293 cells that had been left unstimulated or stimulated with IGF1 in the presence or absence of LY294002. As a control, immunoprecipitations were performed with preimmune antibody from the same lysates and phosphorylation of MBP was measured in a standard kinase assay employing Mg[γ-^{32}P]ATP. The results showed that significantly higher levels of MBP kinase activity were observed in the immunoprecipitations performed with the WNK1 antibody compared with the pre-immune antibody. However, no significant differences in MBP kinase activity were observed from the WNK1 MBP kinase activity derived from control or IGF1- or LY294002-treated cells (Figure 7B). It was not possible to measure autophosphorylation of endogenous WNK1 reliably in this assay (results not shown).

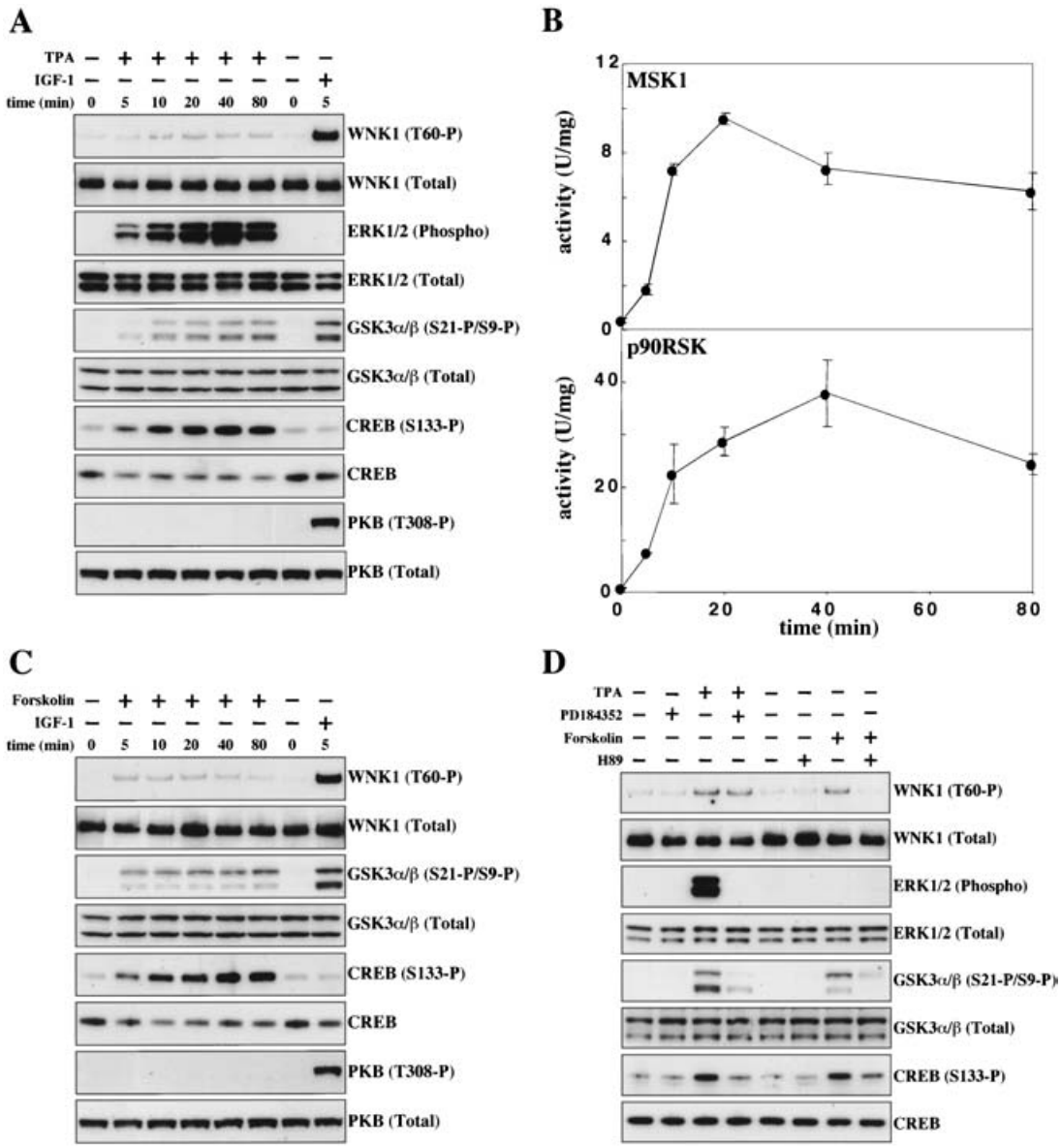

Figure 6 Weak phosphorylation of WNK1 induced by PMA and forskolin

(**A**) HEK-293 cells were deprived of serum for 18 h, prior to stimulation with 400 ng/ml PMA (TPA) or 20 ng/ml IGF1 for the times indicated. The cells were lysed, WNK1 was immunoprecipitated, subjected to electrophoresis on a 3–8 % gradient polyacrylamide gel, transferred to nitrocellulose and immunoblotted with either the T60-P antibody or the total antibody. Cell lysates (20 μg of protein) were also immunoblotted with the indicated antibodies. (**B**) As for (**A**) except that MSK1 and p90RSK were immunoprecipitated from the lysates of PMA-stimulated cells and assayed as described in the Experimental section. The results shown are the average kinase activity $\pm$ S.D. for each determination performed in duplicate. (**C**) HEK-293 cells were treated as described in (**A**) except that the cells were stimulated with 20 μM forskolin for the indicated times. (**D**) HEK-293 cells were deprived of serum for 18 h, treated for 30 min in the presence or absence of 2 μM PD184352 or 10 μM H89 prior to stimulation with 400 ng/ml PMA or 20 μM forskolin for 20 min. The cells were lysed and analysed as described in (**A**).

Evidence that phosphorylation of WNK1 does not affect cellular localization

In order to examine the cellular localization of WNK1, we generated a HEK-293 cell line that stably expresses GFP-tagged full-length WNK1 at the same level as the endogenous WNK1 protein (Figure 8A). Employing confocal microscopy, we found that GFP–WNK1 was largely cytoplasmic and excluded from the nucleus (Figure 8B). Stimulation of cells with IGF1 in the absence or presence of LY294002 did not detectably affect localization of WNK1 (Figure 8B). Control studies demonstrated that IGF1 induced phosphorylation of GFP–WNK1 at Thr-60 to the same extent as the endogenous WNK1 protein (Figure 8C). We also co-transfected the HEK-293 cells stably expressing GFP–WNK1 with a constitutively active PKB mutant and found that it did not alter WNK1 cellular localization (results not shown).

DISCUSSION

In this study we provide the first evidence that endogenously expressed WNK1 is phosphorylated physiologically by PKB in response to a stimulus that activates PI 3-kinase. In addition to employing solely the standard pharmacological approaches to validate the phosphorylation of WNK1 at Thr-60 (Figure 4), we have also made use of the recently developed PDK1$^{L155E/L155E}$

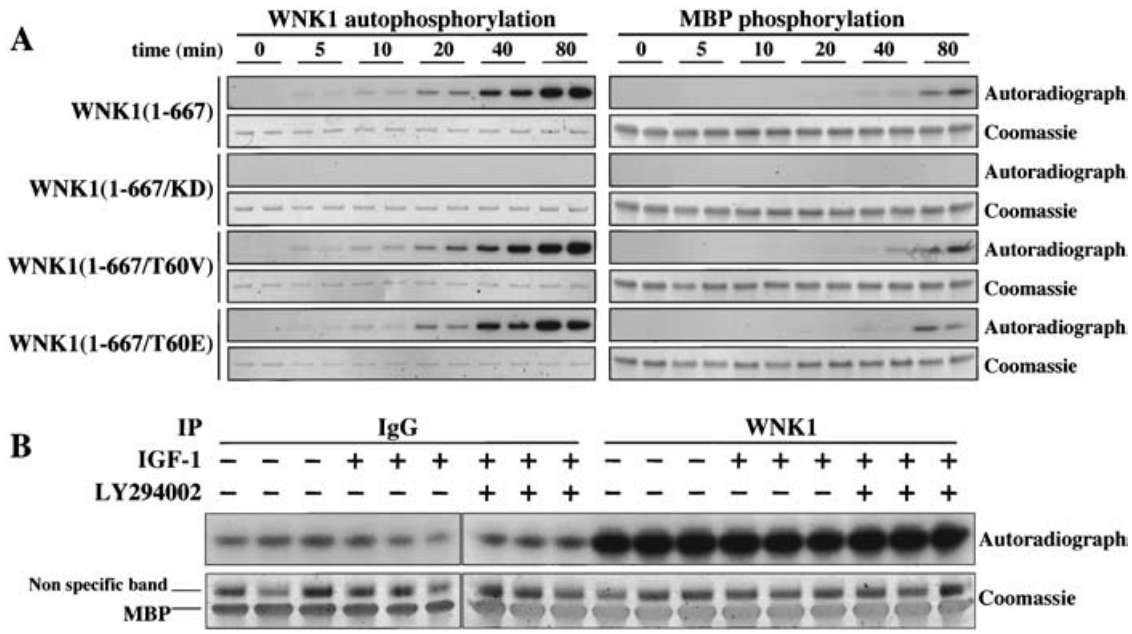

Figure 7 Evidence that phosphorylation of WNK1 at Thr-60 does not affect its catalytic activity

(**A**) The same amounts of purified wild-type GST–WNK1(1–667) or the indicated mutants of GST–WNK1(1–667) were incubated for the times indicated with Mg[γ-^{32}P]ATP in the presence of MBP as described in the Experimental section. Reactions were terminated by the addition of SDS sample buffer, subjected to electrophoresis on a 4–12 % gradient polyacrylamide gel, which was stained and autoradiographed. (**B**) HEK-293 cells were deprived of serum for 18 h, treated for 30 min in the presence or absence of 100 μM LY294002 prior to stimulation with 20 ng/ml IGF1 for 30 min. The cells were lysed, WNK1 immunoprecipitated and WNK1 assayed by incubation for 30 min with Mg[γ-^{32}P]ATP in the presence of MBP as described in the Experimental section. As a control immunoprecipitations were also performed form each of the cell lysates in parallel experiments employing preimmune IgG antibody. Similar results were obtained in two separate experiments.

knock-in ES cells. In these cells, IGF1 induces activation of PKB but not other AGC kinase members that lack a pleckstrin homology domain. The finding that endogenously expressed WNK1 in ES cells is phosphorylated at Thr-60 in the wild-type PDK1$^{+/+}$ and PDK1$^{L155E/L155E}$, but not the PDK1$^{-/-}$ ES cells, provides strong genetic evidence that PKB mediates this phosphorylation and illustrates the utility of these knock-out and knock-in cell lines in validating PKB substrates (Figure 5). Thus we have been able to establish that WNK1 is a physiological substrate for PKB, without having to rely on the overexpression of constitutively active or dominant-negative mutants of PKB, PDK1 or PI 3-kinase, which are prone to many artefacts. It is our belief that, in the absence of a potent and specific PKB inhibitor, the combination of the pharmacological and genetic approach employed in this study is the most reliable way to validate a PKB substrate. Lienhard and colleagues [40], using a pan-PKB-substrate-specific antibody, identified a $\approx$250 kDa protein in adipocyte cells that became phosphorylated in an insulin-dependent and wortmannin-sensitive manner. The authors were unable to identify this protein. However, as WNK1 is of similar size, it would be of interest to establish whether this protein corresponded to WNK1.

MSK1 appears to phosphorylate WNK1 efficiently *in vitro*. However, following MSK1 activation by PMA stimulation, we failed to observe a PD184352-dependent inhibition of the weak phosphorylation of WNK1 that occurs under these conditions (Figure 6). This suggests that MSK1 does not phosphorylate WNK1 *in vivo*. As PMA stimulates PKC isoforms, it is possible that one of these may weakly phosphorylate WNK1. Consistent with this notion, epidermal growth factor stimulation of HEK-293 cells, which might not be expected to activate PKC isoforms, did not induce a detectable phosphorylation of WNK1 (results not shown).

PKA normally phosphorylates proteins preferably at a Ser residue. A notable exception is inhibitor-1, which PKA phosphorylates at a Thr-30 that lies in a RRRRPTP sequence [41], highly similar to that of WNK1 that also possesses four consecutive Arg residues in equivalent positions. *In vitro*, under conditions in which known PKA substrates were efficiently phosphorylated, WNK1 was not detectably phosphorylated (Figure 1). *In vivo* however, forskolin did induce a minor phosphorylation of WNK1 that was inhibited by H89. These data may indicate that PKA weakly phosphorylates WNK1 in cells.

In mammals there are three other closely related WNK isoforms (WNK2, WNK3, WNK4) [19], but only WNK4 possesses a conserved Ser or Thr residue lying in an AGC kinase consensus sequence at the region equivalent to Thr-60 in WNK1. The sequence is RARRFSG in which the underlined Ser-47 corresponds to the putative site of AGC kinase phosphorylation. In future work, it would be of interest to establish whether PKB or another AGC kinase might phosphorylate this residue on WNK4. Although the two Arg residues located at the -3 and -5 positions relative Ser-47, which are required for phosphorylation of most PKB substrates, are conserved, studies with synthetic peptides indicate that a small Gly residue in the $+1$ position, might be unfavourable for PKB phosphorylation [15,42]. Interestingly, mutations in WNK4 have also been linked to the development of PHAII in humans [20]. Thus far there have been no reports that WNK2 and WNK3 are involved in regulating hypertension.

The only functional assay developed to date for the WNK family kinases involves measuring the effect that overexpression of these enzyme in *Xenopus* oocytes has on the activity of the co-expressed Na–Cl co-transporter. In this assay, WNK4, but not WNK1, markedly inhibited the Na–Cl co-transporter [22,43]. Whether this involves phosphorylation of the ion channel by WNK4 is not known. However, WNK4, but not WNK1, can

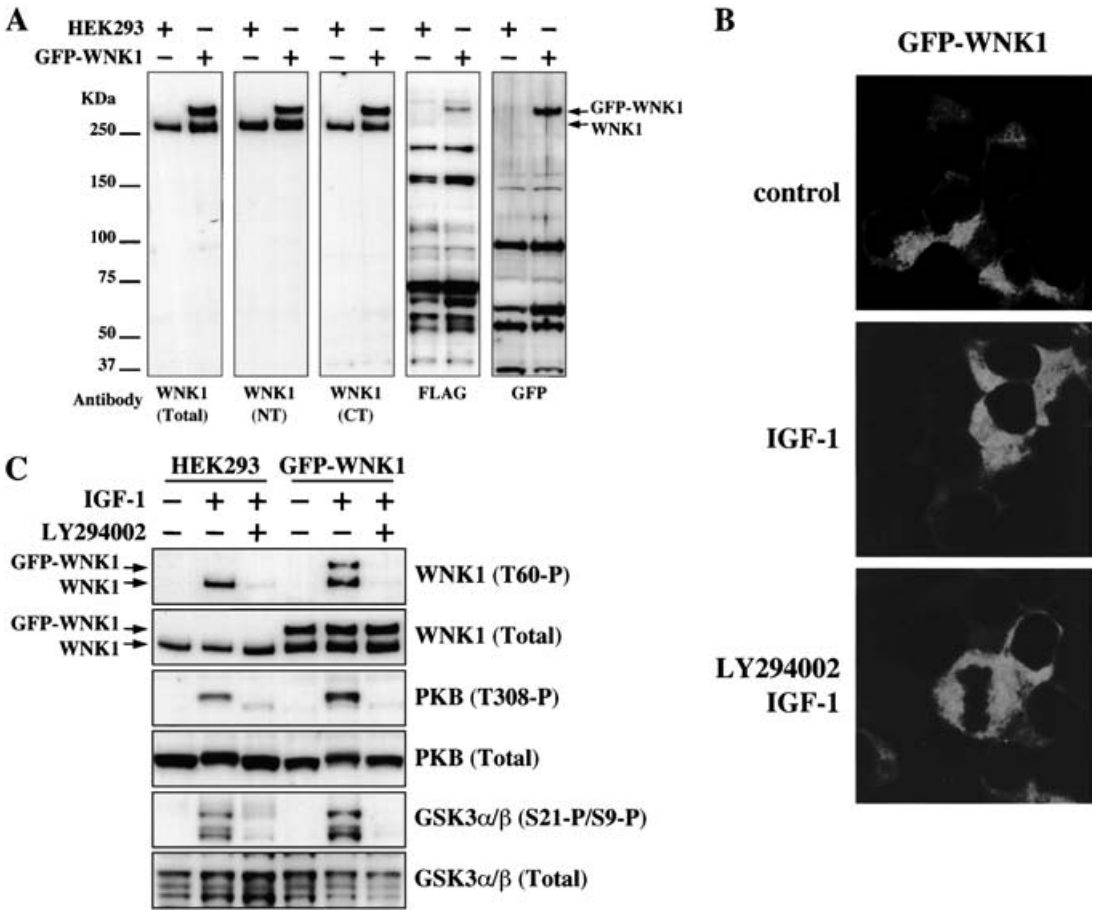

Figure 8 Evidence that phosphorylation of Thr-60 does not effect GFP-WNK1 localization

(**A**) Analysis of control HEK-293 cells and HEK-293 cells stably expressing GFP–FLAG–WNK1 generated as described in the Experimental section; 20 μg of cell lysate protein was immunoblotted with the indicated antibodies. (**B**) HEK-293 cells stably expressing GFP–WNK1 were deprived of serum for 18 h, incubated in the presence or absence of 100 μM LY294002 for 30 min and then either left unstimulated or stimulated with 100 ng/ml IGF1 for 30 min. The cells were fixed and the localization of GFP–WNK1 visualized by confocal microscopy as described in the Experimental section. Similar results were obtained in three separate experiments. (**C**) As in (**B**) except that cells were lysed following the stimulation and 20 μg of protein of cell lysates was immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments.

apparently associate with the Na–Cl co-transporter. The studies we have thus far performed do not establish a role for WNK1 phosphorylation at Thr-60. This has been difficult to assess due to our lack of knowledge on the physiological function of WNK1. Regarding the lack of effect PKB phosphorylation on WNK1 catalytic activity, it should be noted that MBP is a very poor substrate for WNK1 and autophosphorylation of a kinase does not always correlate with its intrinsic catalytic activity. It is possible that in the presence of a physiological WNK1 substrate, phosphorylation of WNK1 at Thr-60 might affect phosphorylation of this substrate.

Many PKB substrates interact with 14-3-3 proteins following their phosphorylation by PKB. We have tested whether endogenous WNK1 immunoprecipitated from HEK-293 cells stimulated with IGF1 is associated with 14-3-3 proteins or could be captured by 14-3-3–Separose [44]. These results provided no evidence that WNK1 could associate with 14-3-3 proteins in IGF1 stimulated cells (C. MacKintosh and A. C. Vitari, unpublished work). It is possible that phosphorylation of Thr-60 could enable WNK1 to interact with other proteins. Once a robust assay for WNK1 is developed, it will be very interesting to assess how phosphorylation of WNK1 at Thr-60 affects WNK1 function. Probably the best approach to address this problem would be to generate a knock-in mutation in the mouse gene encoding WNK1, converting this to a non-phosphorylatable and/or acidic residue to assess the effect that this has on regulating responses relevant to blood-pressure control in mice.

Interestingly, SGK1 has also been implicated in regulating certain potassium, sodium and chloride channels and sustained high levels of SGK1 protein and activity may contribute to conditions such as hypertension and diabetic nephropathy [4,45]. This prompted us to test whether SGK could phosphorylate WNK1. However, SGK1 only phosphorylated WNK1 weakly (Figure 1). Also, the finding that WNK1 is phosphorylated in the PDK1$^{L155E/L155E}$ ES cells, where SGK isoforms would not be activated, also indicates that PKB rather than SGK is the crucial mediator of WNK1 phosphorylation. Nevertheless, we cannot exclude that in other cells or tissues that we have not investigated, SGK isoforms might phosphorylate WNK1. In future work it would be important to explore whether this link between WNK1 and the PI 3-kinase/PKB signalling pathway plays a role in regulating blood pressure.

We thank Moustapha Aoubala for preparation of antibodies as well as the School of Life Sciences DNA Sequencing Service. A. C. V. is the recipient of a Pfizer-sponsored studentship. D. R. A. is supported by the Association for International Cancer Research, Diabetes UK and the Medical Research Council.

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Received 5 November 2003; accepted 11 November 2003
Published as BJ Immediate Publication 11 November 2003, DOI 10.1042/BJ20031692