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Site recognition and substrate screens for PKN family proteins

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The PRKs [protein kinase C-related kinases; also referred to as PKNs (protein kinase Ns)] are a kinase family important in diverse functions including migration and cytokinesis. In the present study, we have re-evaluated and compared the specificity of PKN1 and PKN3 and assessed the predictive value in substrates. We analysed the phosphorylation consensus motif of PKNs using a peptide library approach and demonstrate that both PKN1 and PKN3 phosphorylate serine residues in sequence contexts that have an arginine residue in position -3. In contrast, PKN1 and PKN3 do not tolerate arginine residues in position $+1$ and -1 respectively. To test the predictive value of this motif, site analysis was performed on the PKN substrate CLIP-170 (cytoplasmic linker protein of 170 kDa); a PKN target site was identified that conformed to the predicted pattern. Using a protein array, we identified 22 further substrates for PKN1, of which 20 were previously undescribed substrates. To evaluate further the recognition signature, the site on one of these hits, EGFR (epidermal growth factor receptor), was identified. This identified Thr^{654} in EGFR as the PKN1 phosphorylation site and this retains an arginine residue at the -3 position. Finally, the constitutive phosphorylation of EGFR on Thr^{654} is shown to be modulated by PKN *in vivo*.

Key words: cytoplasmic linker protein of 170 kDa (CLIP-170), epidermal growth factor receptor (EGFR), kinase substrate, phosphorylation consensus motif, protein kinase C-related kinase (PRK), protein kinase N (PKN).

INTRODUCTION

The PRK [PKC (protein kinase C)-related kinase] family are a subfamily of the serine/threonine kinases with a C-terminal kinase domain closely related to those of PKC, while their N-termini are distinct comprising a conserved repeated domain (HR1a,b,c) followed by a C2-related domain. PKNs (protein kinase Ns) are activated by fatty acids [1] and phospholipids *in vitro* [2], although the *in vivo* significance of this activation remains unclear [3].

The PKN family plays an important role in diverse functions, including regulation of cell cycle [4], receptor trafficking [5], vesicle transport [6] and apoptosis [7]. The PKNs are implicated in signal transduction as effectors of Rho, Rac and PI3K (phosphoinositide 3-kinase) [8–10]. In particular, PKN1 has been linked to stress-induced pathways implicated upstream of c-Jun transcription via p38 activation induced by hyperosmotic stress [11] with associated activation of PKN1 [6]. PKN is also linked to the ligand-dependent transcriptional activation of the androgen receptor [12,13]. PKN1 has been implicated as well in milk secretion by regulating the tight junction in the mammary gland [14]. PKN2 plays a role alongside fyn in controlling cell–cell adhesion in keratinocytes [15] and the maturation of apical junctions [16]. In addition, PKN2 can modulate migration in astrocytes by up-regulating cortactin phosphorylation [8]. PKN3 has been identified as an effector required for malignant cell growth downstream of activated PI3K [9]. More recently, it has been shown that knockdown of PKN3 can decrease the growth of prostate and pancreatic tumours, and prevents lung metastasis in mouse models [17,18].

More than 20 proteins and several peptides have been shown to be phosphorylated by PKN1 and PKN2, including α-actinin, adducin, CDC25C, vimentin and TRAF1 (tumour-necrosis-factor-receptor-associated factor 1) [1,2,13,19–30]. In contrast, there are no known substrates for PKN3. The phosphorylation consensus motif of a protein helps to determine which substrates are phosphorylated by a kinase. Concerning the PKN phosphorylation consensus motifs, Zhu et al. [31] have evaluated the importance of arginine residues in seven N-terminal and three C-terminal positions of serine-directed peptides for several kinases, including PKN1. Very recently, Shiga et al. [32] confirmed these results using a combinatorial peptide library method. These observations provide a theoretical prediction for PKN phosphorylation motifs.

The development of new technologies such as peptide and protein arrays has made it practical to investigate the optimal phosphorylation consensus motif and to identify new substrates and partners not only for one kinase, but for several proteins at the same time [31,33,34]. In the present study, we have investigated the kinase substrate specificity of PKN1 and PKN3 re-evaluated using a peptide array and assessed utility using newly reported substrates for the kinase. We confirm that PKN1 follows the phosphorylation consensus motif by the phosphorylation *in vitro* of a newly identified PKN substrate, CLIP-170 (cytoplasmic linker protein of 170 kDa). Furthermore, novel substrates for

Abbreviations used: CLIP-170, cytoplasmic linker protein of 170 kDa; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione transferase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKN, protein kinase N; PRK, PKC-related kinase; siRNA, small interfering RNA.

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PKN1 were identified using a protein array. These results were validated by identifying the site of EGFR [EGF (epidermal growth factor) receptor] phosphorylation by PKN1 as Thr^{654}; this site conforms to the consensus defined by the peptide array and is shown to be modulated *in vivo* through PKN.

EXPERIMENTAL

Reagents

Arachidonic acid was purchased from Sigma. An antibody against total EGFR (1005) (sc-03) was purchased from Santa Cruz Biotechnology. The anti-phospho-EGFR (Thr^{654}) (3F2) antibody was purchased from Abcam (antibody 78283). The anti-PKN1 antibody (BD 610687) was purchased from BD Biosciences, the anti-PKN2 antibody (catalogue number 2612) was from Cell Signaling Technology, and the anti-PKN3 antibody (NBP1-30102) was from Novus Biologicals. The anti-tubulin α antibody (clone B-5-1-2) was from Sigma. The EGFR L861Q protein (catalogue number PV3873) was purchased from Invitrogen. [γ-^{32}P]ATP was purchased from PerkinElmer. [γ-^{33}P]ATP was purchased from GE Healthcare. EGF was from Calbiochem. The peptide arrays (PepChip Kinomics) were bought from Pepscan. The protoarrays were from Invitrogen. CLIP-170 was generously provided by Dr Franck Perez (Cell Biology Section, Curie Institute, Paris, France) [35].

DNA cloning and protein production

The full-length cDNAs for human PKN1 and PKN3 were subcloned into a modified pFastbac-1 vector (Invitrogen) that encodes an N-terminal GST (glutathione transferase) tag and a thrombin cleavage site. Recombinant baculoviruses were generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Routine baculovirus production of human GST–PKN1 and GST–PKN3 in *Spodoptera frugiperda* cells (Sf9 cells) was achieved. The first 650 amino acids of CLIP-170 or the whole protein was fused to GST into the pGEX-2TK vector. To subclone the first 650 amino acids and the whole CLIP-170, one common forward primer was used: 5′-GGATCCATGAGTATGCTAAAGCCAAGTGGG-3′. A reverse primer, 5′-GAATTCTCACCTCAAGGCTTCCATCTCTTT-3′, was used to subclone the first 650 amino acids and the primer 5′-GAATTCTCAGAAGGTTTCGTCGTCATTGCA-3′ was used to subclone the whole protein. The recombinant proteins were produced and purified from *Escherichia coli*. GST fused to CLIP-170 was removed by incubating the purified protein with thrombin at 4 °C overnight.

Peptide array

A peptide array (PepChip Kinomics) with specific phosphorylation sites was used to study the PKN phosphorylation consensus motif. Each array has 960 peptides of 11-amino-acids-long derived from known phosphorylation sites from human protein sequences. In addition, each peptide comes annotated with reported upstream kinases. Moreover, the peptides that belong to a substrate have a physiological relevance in stress, growth, differentiation and other responses. The glass slides contained 976 peptides (including 16 control peptides) spotted in triplicate.

For array kinase assays, the glass slides containing the peptides were incubated with each PKN in the following buffer: 20 mM Tris/HCl (pH.7.5), 40 μM arachidonic acid, 4 mM $MgCl_2$, 10 % glycerol, 0.01 mg/ml BSA, 0.01 % Brij-35, 10 μM ATP and 300 μCi/ml [γ-^{33}P]ATP. PKN1 and PKN3 were diluted to 26 and 13 μg/ml respectively in a buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA (pH 8), 0.2 % Triton X-100 and 1 mM DTT (dithiothreitol).

PKN1, PKN3 or no kinase were incubated on the PepChip Kinomics arrays for 2 h in a humidified incubator at 37 °C. Subsequently, the arrays were washed in 2 M NaCl, 1 % Triton X-100, PBS, 0.1 % Tween 20 and water, after which slides were dried and exposed to an X-ray film screen for 2 or 24 h for PKN1 and PKN3 respectively in order to visualize in the linear range.

Imaging of the incorporated [γ-^{33}P]ATP was performed using a conventional scanner (Epson Expression 1680). Data analysis was performed using Microsoft Excel and the R Statistical Package (http://www.R-project.org). After image acquisition and quantification using ImageQuantTL software (Amersharm Biosciences), the data from each triplicate was normalized with a median-centring method. Signals with high intensity in the PKN slide but low intensity in the control (no PKN) slide were considered as peptide substrates recognized by PKN1 or PKN3. After performing an ANOVA *F* test, the peptides that had a *P* value higher than 9.7×10^{-4} were eliminated. The peptides were sorted by spot intensity and the top 20 peptides based were chosen to analyse the phosphorylation consensus motif of each kinase using the WebLogo v3 application (http://weblogo.threeplusone.com/) [36].

Protein arrays

Glass slides containing 4700 human proteins from Invitrogen were used to identify PKN1 substrates. The slides were incubated with PKN1 following the manufacturer's instructions with the following modifications. Briefly, the slides were incubated with the kinase in 20 mM Tris/HCl (pH 7.5), 40 μM arachidonic acid, 4 mM $MgCl_2$, 1 % Nonidet P40, 5 mg/ml BSA, 1 μM ATP and 33 nM [γ-^{33}P]ATP. PKN1–GST was diluted in a buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.2 % Triton X-100 and 1 mM DTT (the final concentrations in the protein array assay were: 168 μM EDTA, 0.033 % Triton X-100 and 168 μM DTT). The final PKN1–GST concentration in the assay was 52 nM. The slides were incubated with or without (control) kinase for 1 h at 37 °C. The slides were exposed to an X-ray film for different times, scanned and the spots were analysed using the software provided by Invitrogen in order to identify the hits.

Kinase assay

A total of 3 μg of CLIP-170 or 0.3 μg of EGFR L861Q (PV3873; Invitrogen) were incubated with PKNs in 20 mM Tris/HCl (pH 7.5), 40 μM arachidonic acid, 4 mM $MgCl_2$, 100 μM ATP and 0.025 μCi [γ-^{32}P]ATP. PKN1 and PKN3 were diluted to 310 and 50 nM respectively in a buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.2 % Triton X-100 and 5 mM DTT (the final concentrations in the kinase assay were: 166 μM EDTA, 0.033 % Triton X-100 and 833 μM DTT). The kinases were incubated with the substrates for different times at 30 °C. The reactions were stopped by adding 4× NuPAGE sample buffer (Invitrogen), and the proteins were resolved by SDS/PAGE, Coomassie-stained, dried and exposed to X-ray film.

Western blot analysis

SDS/PAGE was performed using the NuPAGE system (Invitrogen) with 4–12 % Bis-Tris gels. Proteins were transferred on to a PVDF membrane and probed as indicated in the

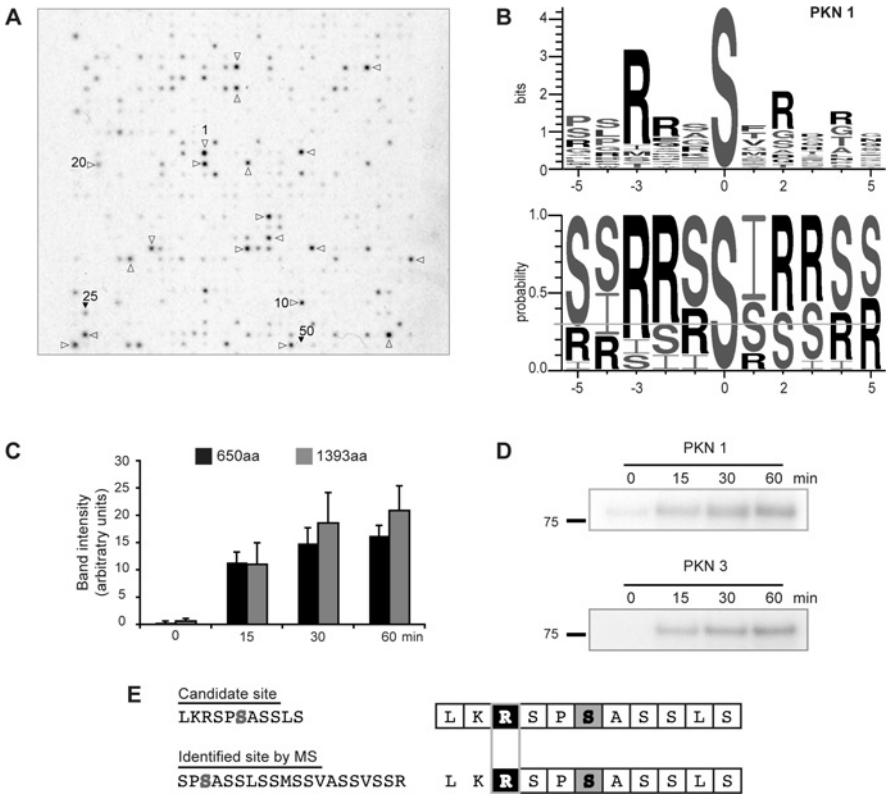

Figure 1 PKN phosphorylation consensus motif

(**A**) Representative image of PepChip Kinomics array (Pepscan) of one of three arrays phosphorylated by PKN1. The open-headed arrows show the top 20 phosphorylated peptides. The 25th and 50th phosphorylated peptides are also shown for reference. (**B**) Phosphorylation consensus motif using WebLogo version 3.0 based on the top 20 phosphorylated peptides. The *x*-axis shows the positions in relation to the serine/threonine (position 0). The *y*-axis in the upper panel shows the bits. The graphs show the amino acid preference near the serine/threonine. The height of the letters is proportional of the frequency in the top 20 peptides. The lower graph shows the grouped amino acid probability: S groups, neutral amino acid (serine, glycine, histidine, threonine, alanine and proline residues); R groups, hydrophilic amino acids (arginine, lysine, aspartic acid, glutamic acid, asparagine and glutamine residues); and I groups, hydrophobic amino acids (isoleucine, methionine, leucine, valine, phenylalanine, tryptophan and tyrosine residues). The line shows the 70% probability. (**C**) Phosphorylation of CLIP-170 by PKN1 in an *in vitro* kinase assay with CLIP-170 as substrate. The first 650 amino acids of the protein or the whole CLIP-170 (1393 amino acids) were tested. The samples were incubated for 0–60 min, resolved by SDS/PAGE, Coomassie-stained and dried. [γ-^{32}P] incorporation was analysed by exposing the gel to an X-ray film. The results are representative of three different experiments. (**D**) Phosphorylation of CLIP-170 (first 650 amino acids) by PKN1 and PKN3 was carried out as in (**C**). The autoradiographs are shown. (**E**) Identification of phosphorylation sites in CLIP-170 by PKN1. The tryptic peptide identified by MS corresponds to a candidate peptide (residues 310–329) that has an arginine residue in position -3.

Figure legends. Levels of PKN1, PKN2 and PKN3 in A431 cells were quantified against recombinant PKN1, PKN2 and PKN3 by Western blot. Quantification was determined using an ImageQuant LAS 4000 mini instrument to obtain a series of exposures of the ECL readout; ImageJ was used to determine the levels of expression relative to recombinant PKN proteins.

Mass spectrometry

In vitro-phosphorylated CLIP-170 was reduced with 10 mM DTT (Sigma), followed by alkylation with 50 mM iodoacetamide (Sigma) prior to digestion in-gel with trypsin (Promega) overnight at 37 °C. Extracted peptides were analysed by online nanoLC-MS/MS using a linear ion trap instrument (Q-TRAP 4000; Applied Biosystems).

siRNA (small interfering RNA) transfection

A431 cells were maintained in DMEM (Dulbecco's modified Eagle's medium)/10% FBS (fetal bovine serum) (Gibco) containing penicillin/streptomycin at 37 °C, in a humidified atmosphere containing 10% CO_2. On day 1, the cells were transfected with 25 nM siRNA luciferase (5′-AATCGAAGTATTCCGCGTACG-3′) or PKNs {8.33 nM each PKN: PKN1 (5′-AAGGGCACGGGAACTGGAGTT-3′), PKN2 (5′-AAGCATGGCATGTGTCTCTATT-3′) and PKN3 (5′-GAGAGCCTGTACTGCGAGAAG-3′); as described elsewhere [9]}. siRNA transfections were carried out with Lullaby (OZ-Biosciences). After 24 h, the medium was replaced by fresh medium, and at 96 h after the siRNA transfection, the medium was replaced by CO_2-independent medium/0.5% FBS (Gibco) for 5 h. The cells were collected in 2× NuPAGE LDS sample buffer (Invitrogen) containing 2 mM EDTA.

RESULTS

PKN phosphorylation consensus motif

We used a peptide library in order to study the influence of the residues up to five amino acids away (C-terminal and N-terminal) from candidate phosphorylation sites for PKN1 and PKN3. On the basis of the top 20 phosphorylated peptides (Figure 1A), PKN1 and PKN3 were shown to have a preference for the phosphorylation of serine residues which have an arginine residue in position -3 (Figure 1B and Supplementary Figure S1 at http://www.BiochemJ.org/bj/438/bj4380535add.htm). In fact

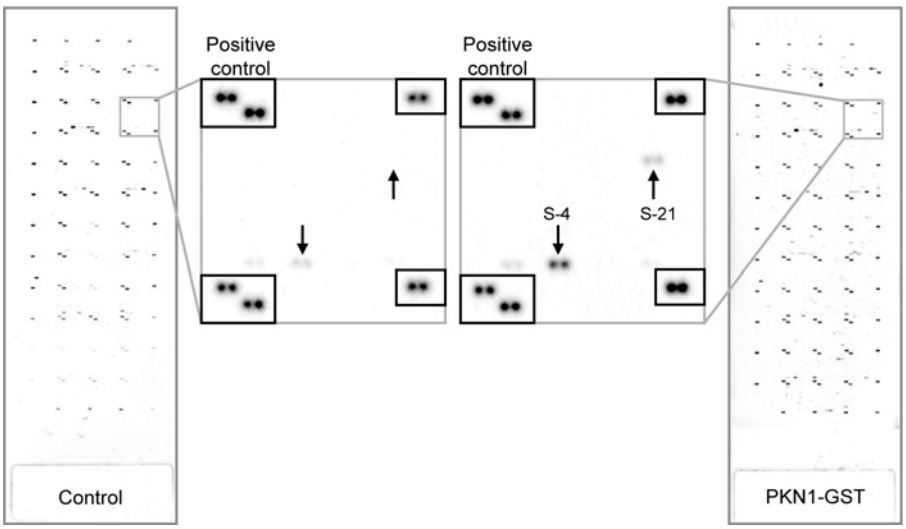

Figure 2 PKN1 protein array

An image of the substrate protein array is shown. Comparison of the control slide (no kinase) and the slide incubated in presence of PKN1–GST. The positive controls (proteins that autophosphorylate) are present in the negative control slide and the slide incubated with PKN1; these are important for alignment and normalization. The 4th and 21st hits are shown in the magnified region.

PKN1 and PKN3 show an 80 and 85 % preference for an arginine residue in position −3 respectively. In contrast, PKN1 and PKN3 do not tolerate an arginine residue in position +1 and −1 respectively. PKN1 shows a 50 and 40 % preference for an arginine residue in positions +2 and −2 respectively. In addition, PKN3 shows a 70 % preference for hydrophobic amino acids (isoleucine, methionine, leucine, valine, phenyalanine, tryptophan and tyrosine residues) in position +1 against a 55 % preference for PKN1. These PKNs show a strong preference (at least 70 %) for neutral amino acids (serine, glycine, histidine, threonine, alanine and proline residues) in position −5 for PKN1 and −1 and +5 for PKN3. Finally, PKN1 shows a 70 % preference of hydrophilic amino acids (arginine, lysine, aspartic acid, glutamic acid, asparagine and glutamine residues) in position −2. If we take a 70 % preference cut-off, the PKN1 phosphorylation consensus motif is Ne-x-Arg-Hi-x-Ser-Z-x-x-x-x and the PKN3 is x-x-Arg-x-Ne/Z-Ser-Ho-x-x-x-Ne (where x denotes a position with no strict amino acid requirements, Z is a position strictly not tolerating arginine, Ne is a neutral amino acid, Hi is a hydrophilic amino acid and Ho is a hydrophobic amino acid). Even though PKN1- and PKN3-phosphorylated peptides were not identical, the kinases share peptide substrates in the array. Of the 20 top hits for each kinase, five peptides were hits for both PKN1 and PKN3; of the top 30, 12 peptides were hits for PKN1 and PKN3. The results indicate that PKN1 and PKN3 share recognition, but retain some specificity.

PKN phosphorylates CLIP-170

The identification of a PKN phosphorylation consensus motif was assessed using the hitherto uninvestigated PKN substrate CLIP-170 (H. Mellor and P.J. Parker, unpublished work). We confirmed that CLIP-170 is phosphorylated by PKN1 and PKN3 *in vitro*. Figure 1(C) shows that PKN1 phosphorylates equally the N-terminal half of CLIP-170 and intact CLIP-170. In addition, PKN3 phosphorylates the N-terminal half of CLIP-170, as shown in Figure 1(D). By MS we identified Ser^{312} as a target for PKN; this is a site identified previously as being occupied in CLIP-170 (http://www.phosphosite.org/). The sequence surrounding Ser^{312} follows the Arg-x-Ne-S-Z-x-x-x-x-Ne pattern, consistent with the specificity analysis derived from the peptide array (Figure 1E).

New PKN substrates and partners

To avoid any bias from the known substrate approach, we performed a protein array in order to identify PKN1 substrates. We found 22 substrates (Figure 2 and Table 1), of which 20 were previously unidentified (the two known substrates validated the screening approach: CDC25C and adducin 2 [20,27]). For the identified substrates, 21 out of 22 retain at least one motif that followed the phosphorylation consensus motif Arg-x-x-[Ser/Thr]-x.

PKNs phosphorylate EGFR at a consensus site, Thr^{654}

In order to validate the protein array results, we analysed the EGFR. To confirm this phosphorylation event, we performed a kinase assay in solution with purified proteins. We compared the sequences of different EGFR constructs spotted on to the Invitrogen protein array and we observed two different EGFR sequences, only one of which was phosphorylated by PKN1 (Figure 3A). Sequence analysis of these two different versions of EGFR indicates two possible sites of phosphorylation that follow the PKN consensus. One site corresponds to Ser^{1071}. However, the peptide that contains this serine residue (Leu-Gln-Arg-Tyr-Ser-Ser-Asp-Pro-Thr-Gly-Ala) was ranked number 235 in the PKN1 peptide array, so it was unlikely to be the PKN1 phosphorylation site. The second site was Thr^{654} and, although it has been shown previously that PKN can phosphorylate *in vitro* a peptide corresponding to this site (Val-Arg-Lys-Arg-Thr-p-Leu-Arg-Arg-Leu) [2,37], it was unknown whether the protein was targeted by PKNs at this site. In order to determine whether PKNs could phosphorylate EGFR at Thr^{654}, we used a site-specific antibody. Figure 3(B) and Supplementary Figure S2 (at http://www.BiochemJ.org/bj/438/bj4380535add.htm) show that Thr^{654} is phosphorylated by PKN1 and PKN3 *in vitro*.

Table 1 Substrates identified for PKN1

The proteins identified as PKN1 substrates in the substrate screen are classified by function. The protein full name and the database ID are also shown.

Family	Subfamily	PKN1 substrate	GenBank® accession number	Full name
Protein kinase	TK	EPH5	NM_004439.4	EPH receptor A5
		PTK2	NM_153831.2	PTK2 protein tyrosine kinase 2
		RET	NM_020975.2	Ret proto-oncogene
		v-erb-b	NM_005228	Epidermal growth factor receptor
	TLK	MAP3K9	NM_033141.2	Mitogen-activated protein kinase kinase kinase 9
	STE	OSR1	NM_005109	Oxidative-stress responsive 1
		STK3	NM_006281	Serine/threonine kinase 3
		STK4	NM_006282.1	Serine/threonine kinase 4
	CK1	–	–	–
	AGC	GRK4	NM_182982.2	G-protein-coupled receptor kinase 4
		PKCζ	NM_002744.2	Protein kinase Cζ
	CAMK	MARKL1	NM_031417.1	MAP/microtubule affinity-regulating kinase like 1
	CMGC	CLK3	NM_001292	CDC-like kinase 3
		PCTAIRE	NM_006201	PCTAIRE protein kinase 1
		STK23	NM_014370.1	Serine/threonine kinase 23
Enzymes	Hydrolases	EXO1	NM_130398.1	Exonuclease 1
	Transferases	BAZ2B	BC012576.1	Bromodomain adjacent to zinc finger domain, 2B
	Phosphatases	CDC25C	NM_001790.2	Cell division cycle 25C
Protein binding	Cytoskeleton	Adducin 2	NM_001617	Adducin 2 (β)
	GTPase	ARHGAP15	NM_018460	Rho GTPase activating protein 15
	Other	MPP7	NM_173496	Membrane protein, palmitoylated 7
Other		PPFIBP2	NM_003621.1	PTPRF-interacting protein-binding protein 2
		FAM64A	NM_019013	Family with sequence similarity 64, member A

PKNs modulate phosphorylation of EGFR on Thr^{654} in cells

Having demonstrated the phosphorylation by PKN *in vitro*, we evaluated whether PKNs contributed to EGFR Thr^{654} phosphorylation *in vivo*. Using siRNAs targeting all three PKN isoforms (all three PKN proteins are expressed in these cells), we observed in A431 cells that the constitutive phosphorylation of Thr^{654} in EGFR was decreased in comparison with the control condition (Figures 3C and 3D). In response to EGF, PKN1/PKN2/PKN3 knockdown did not block the increase in Thr^{654} phosphorylation, although there was a reduced total phosphorylation reflective of the basal effect. This indicates that the PKN effect on Thr^{654} under basal conditions is independent of EGF and is reflective of a transmodulation pathway.

In order to determine which PKN family member(s) is responsible for this effect, we de-convoluted the PKN siRNAs. PKN1 knockdown was found to have a very similar effect on EGFR Thr^{654} phosphorylation as that seen for the knockdown of all PKNs (Figures 3E and 3F). PKN2 had a minor effect and PKN3 none at all, although we observe that, in contrast with PKN1 and PKN2, the knockdown of PKN3 was not efficient in A431 cells. The evidence suggests that the PKN responsible for controlling basal EGFR Thr^{654} phosphorylation in A431 cells is principally PKN1. This result may reflect the fact that PKN1 is the major PKN isoform expressed in these cells; quantitative analysis of the expression in A431 cells shows that PKN1 is expressed at 0.44 μg/mg of cell protein, PKN2 at 0.13 μg/mg of cell protein and PKN3 at 0.03 μg/mg of cell protein.

DISCUSSION

We have analysed the phosphorylation consensus motifs of PKN1 and PKN3 using a peptide array. PKN1 and PKN3 were selected for similar phosphorylation consensus motifs based on residues both in the N-terminal and C-terminal sides of the phosphorylation site. The phosphorylation consensus motifs are consistent with a previously unmapped site we identify in the present study in the PKN substrate CLIP-170. To further assess the predictive capacity of the motif identified, we used an array approach to identify protein substrates for PKN1. This screen identified a number of *in vitro* substrates among which was the EGFR. The arrayed proteins (two different length forms of EGFR) and the determined sequence specificity of PKN predicted that Thr^{654} was targeted by PKN on the EGFR. *In vitro*, we show that EGFR is phosphorylated by PKN1 on Thr^{654}, consistent with this specificity. Finally, we show that PKN depletion by siRNA decreased the constitutive EGFR phosphorylation on Thr^{654} in A431 cells and that this is primarily a function of PKN1. We conclude that PKN specificity has a predictive capacity as applied to both a predetermined substrate (CLIP-170) and a newly identified protein substrate (EGFR). In the case of the latter, the phosphorylation at Thr^{654} has hitherto been ascribed to PKC; the evidence from the present study is that this is more accurately defined as a PKC superfamily target site.

Phosphorylation consensus motif

Previous groups have studied PKN phosphorylation motifs. Zhu et al. [31] evaluated only the importance of an arginine residue in positions near the phosphorylated serine residue in peptides phosphorylated by PKN1 and they did not analyse the importance of other amino acids as the present study does. Zhu et al. [31] also showed that an arginine residue was important in position -3, -2 and $+2$ and that an arginine residue was not allowed in position $+1$ and $+3$. We confirm these findings, except that we did not find a non-allowance of arginine in position $+3$: PKN1 and PKN3 have a preference for arginine in position -3 (80 and 85 % respectively; Figure 1 and Supplementary Figure S1) and a preference in position -2 (40 and 30 % respectively). More recently, Shiga et al. [32], using a combinatorial peptide library method, confirmed the results of Zhu et al. [31] and showed

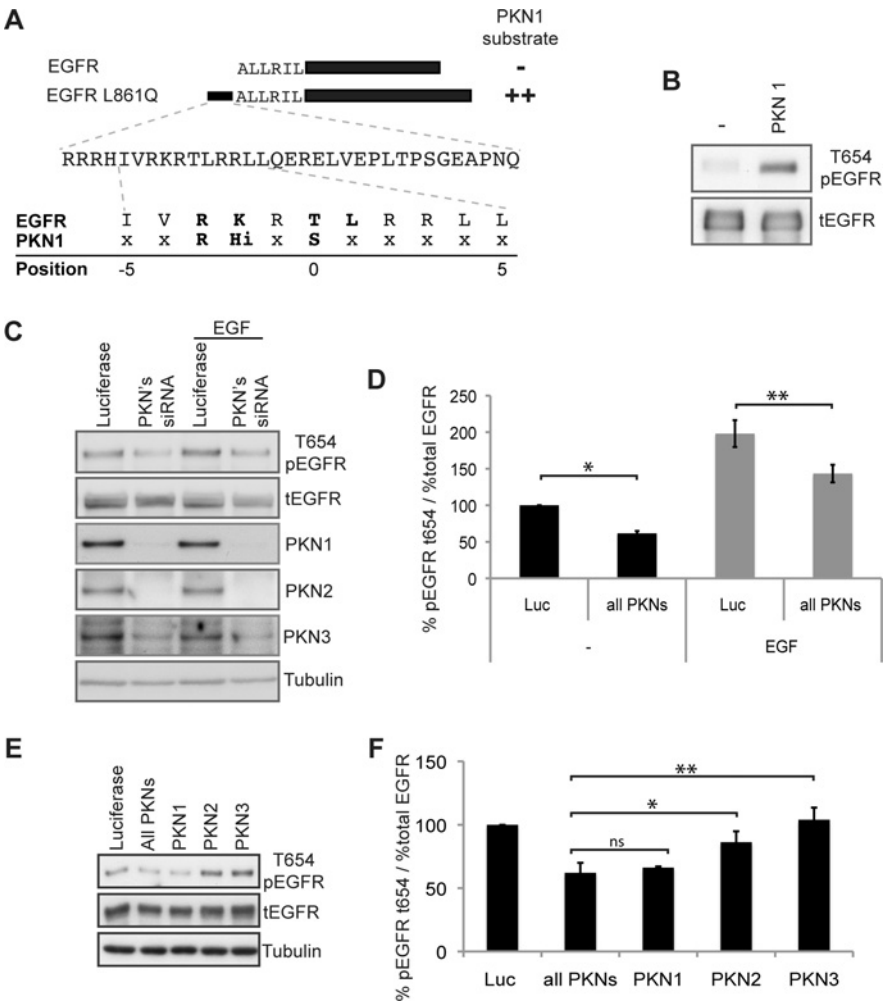

Figure 3 Phosphorylation of EGFR by PKN1

(**A**) The EGFR sequences present in the substrate array; only the second sequence was phosphorylated by PKN1. In the first region of this EGFR substrate, there is a threonine residue that has an arginine in position -3, Thr^{654}. x denotes a position with no strict amino acid requirements and Hi denotes hydrophilic amino acids (arginine, lysine, aspartic acid, glutamic acid, asparagine and glutamine residues). (**B**) *In vitro* kinase assay using the kinase domain of EGFR L861Q as a substrate. The samples were incubated for 15 min, resolved by SDS/PAGE, analysed by Western blotting and probed with the anti-phospho-EGFR (Thr^{654}) antibody and anti-total EGFR antibody as indicated. (**C**) A431 cells were transfected with 20 nM luciferase or 20 nM PKNs siRNA. After 96 h, the cells were depleted of serum for 5 h in a CO_2-independent medium containing 0.5 % FBS. The cells were treated or not with 100 ng/ml EGF for 30 min. The cells were collected and probed for phospho-EGFR (Thr^{654}) (T654 pEGFR), total EGFR (tEGFR), PKN1, PKN2 and PKN3, with tubulin α as a loading control. The blot is representative of at least four different experiments. (**D**) Quantification of four different experiments. (**E**) A431 cells were treated as in (**D**) and with de-convoluted PKNs siRNA. After 96 h, the cells were depleted of serum for 5 h in a CO_2-independent medium containing 0.5 % FBS. The cells were treated or not with 100 ng/ml EGF for 30 min. The cells were collected and probed for phospho-EGFR (Thr^{654}), total EGFR, with tubulin α as a loading control. (**F**) Quantification of two different experiments. $^*P < 0.05$ and $^{**}P < 0.01$. ns, not significant.

that both PKN1 and PKN3 preferentially phosphorylate serine residues with an arginine residue in position -3. Shiga et al. [32] also showed that these kinases have a preference for hydrophobic amino acids in position $+1$ and basic amino acids downstream of the phosphorylation site, concluding that the best phosphorylation consensus motif for PKN1 and PKN3 is Lys-Arg-Arg-Lys-Pro-Ser-Phe-Arg-Asn-Pro. Shiga et al. [32] found a preference for a lysine residue in positions -5 and -2. In general, we observed a very similar pattern of recognition in the present study (albeit basic residues rather than any lysine-specific preferences). However, Shiga et al. [32] noted a preference for a proline residue in position -1, an asparagine residue in position $+3$ and a proline residue in position $+5$; we did not find these preferences. These differences between the three studies probably reflect the nature of the arrays: Zhu et al. [31] used degenerate peptides where only arginine is changed, Shiga et al. [32] used degenerate peptides where one amino acid is kept constant, and our present study used a large array of synthetic peptides based on known target sites. The key feature is, however, consistent between all three studies and that is the very strong preference for an arginine residue at the -3 position.

Since PKN belongs to the AGC family, we expected that the PKN phosphorylation consensus motif would follow a pattern similar to that of other AGC family members. If we compare with other kinases that belong to distinct branches of kinases, it is evident that the pattern is very different. For example, CDC2 phosphorylates peptides with the pattern x-Ser-Pro-x-Lys, whereas CK2 targets the consensus [Ser/Thr]-[Asp/Glu]-x-[Asp/Glu] [38,39]. These motifs are very different from the PKN motif Arg-x-x-[Ser/Thr]-x-x-x. The presence of an arginine residue in position -3 is similar to other AGC family (basophilic) kinases {PKC, [Lys/Arg]-x-x-[Ser/Thr]-x-[Lys/Arg];

and PKA (protein kinase A), Arg-Arg-x-[Ser/Thr]-Phe} [39]. It is possible that the presence of peptides phosphorylated by members of the AGC family could influence the PKN results; however, if we analyse the whole collection of peptides spotted in the slide (960 peptides), only 20 % present an arginine residue in position −3 (Supplementary Figure S3 at http://www.BiochemJ.org/bj/438/bj4380535add.htm). Therefore it is not the case that the preference of an arginine residue in position −3 by PKN1 and PKN3 only reflects a bias in the array composition, since peptide substrates identified were enriched to ∼80 % probability of arginine at the −3 site for PKN1.

PKN and PKC are close phylogenetic neighbours. As expected, they share some similarities in the phosphorylation consensus motif (Supplementary Figure S4 at http://www.BiochemJ.org/bj/438/bj4380535add.htm). The preference of an arginine residue in position −3 and hydrophobic amino acid in position +1 are significant similarities. If we analyse more closely, PKN1 and PKN3 have a preference for an arginine residue in position −2 (40 and 30 % respectively; Figure 1 and Supplementary Figure S1) and many PKC substrate peptides have a lysine or arginine residue in this position as well. In addition, PKN1 presents a preference for an arginine residue (50 %) in position +2 as do PKCα and PKCη, whereas PKCβ and PKCγ prefer a lysine residue here. Concerning PKN3, there is a preference for an arginine residue in position −5 (>30 %), as for PKCα, PKCγ, PKCδ and PKCη. Thus there is a substantial overlap in specificity indicative of shared targets *in vivo* where phosphorylation is triggered under different circumstances by regulatory inputs particular to individual members of this family of proteins [40], controlling the same downstream events. Evidence for such properties have come from the use of a *Drosophila* PKN–*Drosophila* PKC chimaera to rescue *Drosophila* PKN loss [41,42].

It was proposed that PKN has a pseudosubstrate in the ACC1 region, overlapping the Rho-binding domain (Supplementary Figure S4). This region corresponds to amino acids 39–53. The replacement of Ile^{46} with a serine residue converts a peptide based on this sequence into a potent substrate for PKN, whereas the authentic isoleucine counterpart inhibits the activity of PKN [43]. However, deletion of this region does not activate PKN [44]. If we compare this region with the phosphorylation consensus motif, it has an arginine residue for PKN1 and PKN2 and a lysine residue for PKN3 in position −3. However, there are some discrepancies between the sequences. For example, the PKN1 region has an arginine residue in position +1, whereas the phosphorylation consensus motif does not permit this amino acid in this position. This could explain why this region (amino acid 39–53) does not operate as a pseudosubstrate site. However, as for other proteins, the presence of a consensus phosphorylation site in a protein does not guarantee that the protein is a substrate *in vivo* and authentic phosphorylation sites do not always conform to the consensus [45]. Evidently this is the case as well for PKN. Some peptides that have been shown to be phosphorylated by PKN1 do not follow the phosphorylation consensus pattern (Supplementary Figure S4).

PKN substrates and EGFR

We found 22 substrates *in vitro* of which 15 are kinases. This is a high proportion, reflective of their representation in the protein array. However, these are not false positives lighting up because they are protein kinases, since the software is designed to eliminate the kinases that autophosphorylate; in order to define a protein as a hit, the mean signal of the replicates (in the PKN1 slide) needs to be at least 1.5 times that of the mean signals from the corresponding proteins on the negative control (no kinase). Thus we are confident that these kinases are PKN substrates *in vitro*.

We have shown that PKN1 phosphorylates EGFR on Thr^{654} *in vitro* and *in vivo*. The Thr^{654} site is a known phosphorylation site for PKC *in vivo* [46,47]. Thr^{654} is located on the cytoplasmic side of the plasma membrane nine residues distant from the transmembrane domain. It has been shown that the phosphorylation of EGFR at Thr^{654} by PKC reduces the tyrosine kinase activity of the receptor [47], inhibits lysosomal degradation to promote recycling back to the cell surface [48] and positively mediates drug inhibition of prostate tumour growth *in vivo* [49].

We found that the PKN effect on Thr^{654} phosphorylation was only observed under basal conditions and had little influence on the response to EGF. This suggests that the effect of PKN in Thr^{654} phosphorylation under basal conditions is EGF-independent. By contrast, the EGFR phosphorylation on Thr^{654} by PKC can be the product of EGF binding [50]. That indicates that PKC and PKN can control the same substrate; however, the input that regulates this phosphorylation event is different. EGFR can be transmodulated by other growth factors [51] and it is anticipated that PKN lies on one of these pathways as reflected in its requirement for basal Thr^{654} phosphorylation in A431 cells.

In summary, the peptide and protein library approach has provided information about substrate specificity for PKN1 and PKN3 and new substrates for PKN1. In addition, we have defined CLIP-170 and EGFR as substrates for PKN1 and PKN3 and determined the sites involved. The predictive nature of the specificities defined and the protein substrates identified *in vitro* should serve to help determine the signalling pathways for these kinases.

AUTHOR CONTRIBUTION

Alejandra Collazos made the CLIP-170 constructs and the experiments presented in the Figures of the paper. Nicholas Michael and Nick Totty performed the MS experiments. Richard Whelan helped with the preliminary EGFR experiments. Gavin Kelly analysed the peptide array data. Harry Mellor identified CLIP-170 as a PKN substrate. Leon Pang made the PKN1 and PKN3 recombinant baculovirus constructs. Alejandra Collazos and Peter Parker planned the experiments, analysed the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Site recognition and substrate screens for PKN family proteins

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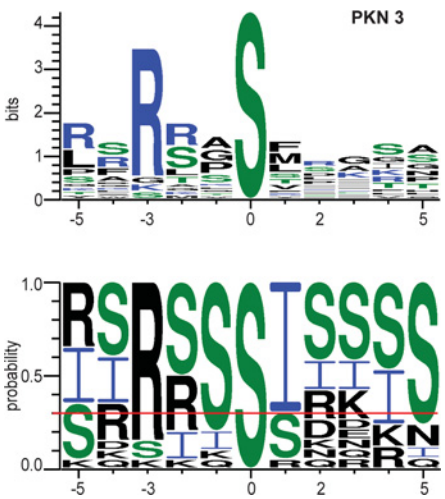

Figure S1 PKN3 phosphorylation consensus motif

A phosphorylation motif illustrated using WebLogo version 3.0 based on the top 20 phosphorylated peptides phosphorylated by PKN3 is shown. The *x*-axis shows the positions in relation to the serine/threonine (position 0). The *y*-axis in the upper panel show the bits. The graphs show the amino acid preference near the serine/threonine. The height of the letter is proportional of the frequency in the top 20 peptides. The lower panel shows the grouped amino acid probability: S groups, neutral amino acids (serine, glycine, histidine, threonine, alanine and proline residues); R groups, hydrophilic amino acids (arginine, lysine, aspartic acid, glutamic acid, asparagine and glutamine residues); I groups, hydrophobic amino acids (isoleucine, methionine, leucine, valine, phenyalanine, tryptophan and tyrosine residues). The red line shows 70 % probability.

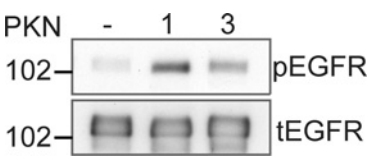

Figure S2 Phosphorylation of EGFR by PKN1 and PKN3

In vitro kinase assay using the kinase domain of EGFR L861Q as a substrate. The samples were incubated for 15 min, resolved by SDS/PAGE, analysed by Western blotting and probed with the anti-phospho-EGFR (Thr^{654}) antibody and anti-(total EGFR) antibody as indicated.

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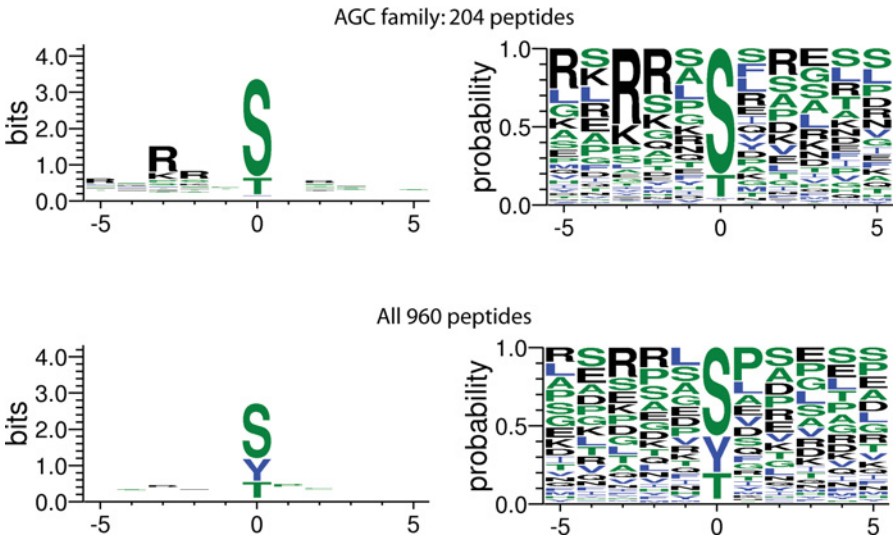

Figure S3 Phosphorylation motif in the peptide library

Upper panel: the phosphorylation motif of peptides phosphorylated by proteins members of the AGC family. A total of 204 peptides printed on the array were analysed using WebLogo version 3.0. The left and right graphs show the bits and the probability respectively, as in Figure 1(B) of the main text. Lower panel: the phosphorylation motif of all of the peptides present on the PepChip Kinomics array. A total of 960 peptides printed on the peptide slide were analysed using WebLogo version 3.0.

A

Position	-5	-4	-3	-2	-1	0	1	2	3	4	5
PKN1	Ne	X	**R**	**Hi**	X	**S**	**Z**	X	X	X	X
PKN3	X	X	**R**	X	Ne/Z	**S**	**Ho**	X	X	X	Ne
PKCα	R	R	**R**	K	G	**S**	**F**	R	R	K	A
PKCβI	L	K	**R**	K	G	**S**	**F**	K	K	F	A
PKCβII	L	K	**R**	K	G	**S**	**F**	K	K	F	A
PKCγ	R	R	**R**	K	G	**S**	**F**	K	R	K	A
PKCδ	R	K	**R**	K	G	**S**	**F**	F	Y	G	G
PKCε	X	K	**R**	K	M	**S**	**F**	F	E	F	F
PKCη	R	R	**R**	R	R	**S**	**F**	R	R	X	R
PKCζ	F	K	**R**	Q	G	**S**	**F**	F	Y	F	F
Pseudosubstrate											
PKN1	R	L	**R**	R	E	I	R	K	E	L	K
PKN2	R	I	**K**	R	E	I	R	K	E	L	K
PKN3	V	I	**R**	R	A	I	Q	K	E	L	K

B

Position	-5	-4	-3	-2	-1	0	1	2	3	4	5
PKN1	Ne	X	R	Hi	X	**S**	Z	X	X	X	X
PKN3	X	X	R	X	Ne/Z	**S**	Ho	X	X	X	Ne
PKN 1 substrates											
HDAC5 [o]	G	T	V	I	S	**T**	F	K	K	R	A
HDAC9 [o]	G	N	V	V	T	**S**	F	K	K	R	M
MARCKS [a]	K	K	K	R	F	**S**	F	K	K	S	F
MARCKS [a]	F	S	F	K	K	**S**	F	K	L	S	G
MARCKS [a]	K	L	S	G	F	**S**	F	K	K	S	K
PKCα [b,*]	F	A	R	K	G	**S**	L	R	Q	K	N
PKCε [b,*]	R	K	R	Q	G	**S**	V	R	R	R	V
PKCδ [b,*]	M	N	R	R	G	**S**	I	K	Q	A	K
PKCζ [b,*]	I	Y	R	R	G	**S**	R	R	W	R	K
S6 [b]	K	R	R	R	L	**S**	S	L	R	A	
TAU [c]	K	D	R	V	Q	**S**	K	I	F	S	L
TAU [c]	L	K	N	V	K	**S**	K	I	G	S	T
TAU [c]	L	S	K	V	T	**S**	K	C	G	S	L
TRAF1[e]	L	E	R	N	L	**S**	E	L	Q	L	Q
PKN 2 substrates											
EGFR [d]		V	R	K	R	**T**	L	R	R	L	
S6 [d]	K	R	R	R	L	**S**	S	L	R	A	
GS [d]	L	S	R	T	L	**S**	V	A	A	K	K

*Ala in the normal sequences were substituted by Ser

C

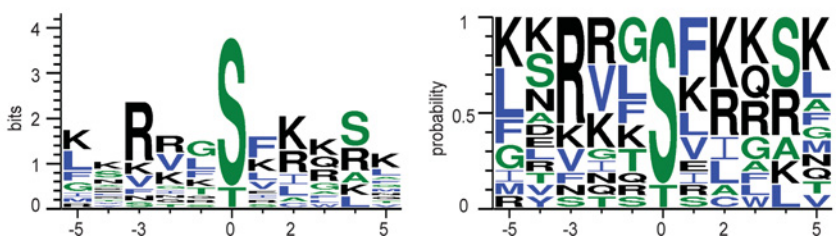

Figure S4 PKN and PKC phosphorylation consensus motif comparison

(**A**) PKN1, PKN3 and PKC phosphorylation consensus motif and PKN pseudosubstrates [1,2]. The serine residue in position 0, arginine/lysine residue in position −3 and phenylalanine residue preference in position +1 are highlighted in a box. X denotes a position with no strict amino acid requirements, Z a position strictly not tolerating arginine, Ne a neutral amino acid (serine, glycine, histidine, threonine, alanine and proline residues), Hi a hydrophilic amino acid (arginine, lysine, aspartic acid, glutamic acid, asparagine and glutamine residues) and Ho a hydrophobic amino acid (isoleucine, methionine, leucine, valine, phenyalanine, tryptophan and tyrosine residues). (**B**) Alignment of known peptides phosphorylated by PKN1 and PKN2. Serine residues in position 0 and position −3 are highlighted in a box. [o] from [3], [a] from [4], [b] from [5], [c] from [6], [d] from [7], and [e] from [8]. GS, glycogen synthase; HDAC, histone deacetylase; MARCKS, myristoylated alanine-rich C kinase substrate; TRAF1, tumour-necrosis-factor-receptor-associated factor 1. (**C**) Phosphorylation motif illustrated using WebLogo version 3.0 based on PKN1 substrates from (**B**). The *x*-axis shows the positions in relation to the serine/threonine (position 0). The *y*-axis in the left-hand graph show the bits. The graphs show the amino acid preference near the serine/threonine. The height of the letter is proportional to the frequency in the top 20 peptides. The right-hand graph shows the amino acid probability.

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