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Site recognition and substrate screens for PKN family proteins.

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Synopsis

The protein kinase C related kinases (PRKs also referred to as PKNs) are a kinase family important in diverse functions including migration and cytokinesis. Here, we have re-evaluated and compared specificity for PKN1 and PKN3, and assessed predictive value in substrates. We analysed the phosphorylation consensus motif of PKNs using a peptide library approach and demonstrate that both PKN1 and 3 phosphorylate serine residues in sequence contexts that have an arginine at position -3. In contrast, PKN1 and 3 do not tolerate Arg in position +1 and -1 respectively. To test the predictive value of this motif, site analysis was performed on the PKN substrate CLIP170; 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, was identified. This identified threonine 654 in EGFR as the PKN1 phosphorylation site and this retains an arginine at the -3 position. Finally, the constitutive phosphorylation of EGFR on Thr-654 is shown to be modulated by PKN *in vivo*.

Short title: PKN sites and substrates

Keywords: Protein Kinase N; substrates; phosphorylation consensus motif.

Abbreviations used: AA, arachidonic acid; PKC, protein kinase C, PKN, protein kinase N, EGFR epithelial growth factor receptor; PI3K, phosphoinositide3-kinase.

INTRODUCTION

The PKC-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 amino termini are distinct comprising a conserved repeated domain (HR1a,b,c) followed by a C2-related domain. PKNs 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 play an important role in diverse functions including regulation of cell cycle [4], receptor traffic [5], vesicle transport [6] and apoptosis [7]. The PKNs are implicated in signal transduction as effectors of Rho, Rac and PI3Kinase [8-10]. In particular, PKN1 has been linked to stress-induced pathways implicated upstream of c-Jun transcription via p38 activation induced on 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]. PKN2 can modulate as well 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 knock-down 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 [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 PKN phosphorylation consensus motifs, Zhu et al. had evaluated the importance of Arg in 7 N-terminal and 3 C-terminal positions of serine directed peptides for several kinases, including PKN1. [31]. Very recently, Shiga et al confirmed these results using a combinatorial peptide library method. [32]. These observations provide a theoretical prediction for PKN phosphorylation motifs.

The developing of new technologies such as peptide and protein arrays has made it practical to investigate the optimal phosphorylation consensus motif and identify new substrates and partners, not only for one kinase, but for several proteins at the same time [31, 33, 34]. Here we have investigated 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. Furthermore, novel substrates for PKN1 were identified using a protein array. These results were validated by identifying the site of EGFR phosphorylation by PKN1 as Thr-654; this site conforms to the consensus defined by peptide array and is shown to be modulated *in vivo* through PKN.

EXPERIMENTAL

Reagents

Arachidonic acid was purchased from Sigma. Total EGFR (1005) sc-03 Antibody was purchased from Santa Cruz Biotechnology, Inc. EGFR Thr-654 [3F2] Antibody was purchased from Abcam (antibody 78283). PKN1 antibody (BD 610687) was purchased from BD biosciences, PKN2 antibody (Cat 2612) was from Cell Signalling and PKN3 antibody (NBP1-30102) from Novus biologicals. Tubulin α clone B-5-1-2 antibody was from Sigma. EGFR L861Q protein (Cat 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 Presto BV, Lelystad, The Netherlands. The protoarrays were from Invitrogen. CLIP-170 was a kind gift from Frank Perez [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 glutathione S-transferase (GST) 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'ggatccatgagtatgctaaagccaagtggg3'. A reverse primer 5'gaatttcacctcaaggttcctctcttt3' was used to subclone the first 650 amino acids and the primer 5'gaatttcagaaggttcgtcgtcattgca3' was used to subclone the whole protein. The recombinant proteins were produced and purified from *E. coli*. The 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 PKNs 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 contain 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 pH 7.5, 40 μ M Arachidonic acid, 4 mM $MgCl_2$, 10% Glycerol, 0.01 mg/ml BSA, 0.01% V/V Brij-35, 10 μ M ATP and 300 μ Ci/ml [γ - 33 P]-ATP. PKN1 and PKN3 were diluted to 26 μ g/ml and 13 μ g/ml respectively in a buffer containing 20 mM Tris pH 7.5, 1 mM EDTA pH 8, 0.2% Triton X-100 and 1 mM DTT.

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 2h or 24h for PKN1 and 3 respectively, in order to visualise in the linear range the reaction.

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 (Amersham, Biosciences), the data of 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 recognised 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 pH 7.5, 40 μ M Arachidonic Acid, 4 mM $MgCl_2$, 1% NP40, 5 mg/ml BSA, 1 μ M ATP, and 33 nM [γ - ^{33}P]-ATP. PKN1-GST was diluted in a buffer containing 20 mM Tris pH 7.5, 1 mM EDTA pH 8, 0.2% Triton X-100 and 1 mM DTT (the final concentrations in the protein array assay were: 168 μ M EDTA, 0.033% Triton X-100 and 168 μ M DTT). The final PKN1-GST concentration in the assay was 52 nM. The slides were incubated with or without (control) kinase for 1h 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

Three μ g of CLIP-170 or 0.3 μ g of EGFR L861Q (Invitrogen, PV3873) were incubated with PKNs in 20 mM Tris pH 7.5, 40 μ M Arachidonic Acid, 4 mM $MgCl_2$, 100 μ M ATP, and 0.025 μ Ci [γ - ^{32}P]-ATP. PKN1 and PK3 were diluted at 310 nM and 50 nM respectively in a buffer containing 20 mM Tris pH 7.5, 1 mM EDTA pH 8, 0.2% Triton X-100 and 5 mM DTT (the final concentrations in the kinase assay were: 166 μ M EDTA, 0.033% Triton X-100 and 833 μ M DTT). The kinases were incubated with the substrates for different times at 30°C. The reactions were stopped by adding Nupage sample buffer 4x (Invitrogen), resolved by SDS-PAGE, Coomassie stained, dried and exposed to X-ray film.

Western blot

SDS-PAGE was performed using the NuPAGE system (Invitrogen) with 4-12% Bis-Tris gels. Proteins were transferred to a PVDF membrane and probed as indicated in the Figure legends. Levels of PKN1, 2 and 3 in A431 were quantified against recombinants PKN1, 2 and 3 by westerns blot. Quantitation was derived 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 dithiothreitol (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 on-line nanoLC-MS/MS using a linear ion trap instrument (Applied Biosystems Q-TRAP 4000).

siRNA Transfection

A431 cells were maintained in DMEM/10% FBS (Gibco) + penicillin/streptomycin at 37°C, in a humidified atmosphere containing 10% CO₂. On the first day, the cells were transfected with 25 nM siRNA Luciferase (AATCGAAGTATTCCGCGTACG) or PKNs (8.33 nM each PKNs: PKN1 (AAGGGCACGGGAAGTGGAGTT), PKN2 (AAGCATGGCATGTGTCTCTATT) and PKN3 (GAGAGCCTGTACTGCGAGAAG; as described elsewhere [9]). siRNA transfections were carried out with Lullaby (OZ-Biosciences). 24 h later, the medium was replaced by fresh medium. 96 h after the siRNA transfection, the media was replaced by CO₂ independent media/0.5% FBS (Gibco) for 5h. The cells were collected on Nupage LDS sample buffer 2x (Invitrogen) + 2 mM EDTA.

RESULTS

PKN phosphorylation consensus motif

We used a peptide library in order to study the influence of the residues up to 5 amino acids away (C-terminal and N-terminal) from candidate phosphorylation sites for PKN1 and PKN3. Based on the top 20 phosphorylated peptides (Fig. 1A), PKN1 and PKN3 were shown to have a preference for the phosphorylation of Serines, which have an Arg in position -3 (Fig. 1B and Fig. S1). In fact PKN1 and 3 show an 80 and 85% preference for Arg in position -3, respectively. In contrast, PKN1 and 3 do not tolerate Arg in position +1 and -1 respectively. PKN1 shows a 50% and 40% of preference for Arg in positions +2 and -2 respectively. In addition, PKN3 shows a 70% preference for hydrophobic amino acids (Ile, Met, Leu, Val, Phe, Trp, Tyr) in position +1 against a 55% preference for PKN1. These PKNs show a strong preference (at least 70%) for neutral amino acids (Ser, Gly, His, Thr, Ala, Pro) in position -5 for PKN1 and -1 and +5 for PKN3. Finally, PKN1 shows a 70% preference of hydrophilic amino acids (Arg, Lys, Asp, Glu, Asn, Glu) 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 a position strictly not tolerating Arg, Ne a neutral amino acids, Hi a hydrophilic amino acid and Ho a hydrophobic amino acid). Even though, PKN1 and 3 phosphorylated peptides were not identical, the kinases share peptide substrates in the array. Of the 20 top hits for each kinase, 5 peptides were hits for both PKN1 and 3; of the top 30, 12 peptides were hits for PKN1 and 3. 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. Parker unpublished data). We confirmed that CLIP-170 is phosphorylated by PKN1 and 3 *in vitro*. Figure 1C shows that PKN1 phosphorylates equally the N-terminal half of CLIP170 and intact CLIP-170. PKN3 phosphorylates as well the N-terminal half of CLIP-170 as shown in Fig. 1D. By mass spectrometry we identified S312 as a target for PKN, this is a site identified previously as being occupied in CLIP-170 (<http://www.phosphosite.org/>). Sequence surrounding S312 follows the Arg-x-Ne-S-Z-x-x-x-x-Ne pattern, consistent with the specificity analysis derived from the peptide array (Fig. 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 (Fig. 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 of 22 retain at least one motif that follows 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 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 the Invitrogen protein array and we observed 2 different EGFR sequences, only one of which was phosphorylated by PKN1 (Fig. 3A). Sequence analysis of these 2 different versions of EGFR indicates 2 possible sites of phosphorylation that follow the PKN consensus. One site corresponds to Ser 1071. However, the peptide that contains this Ser (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 it although it had previously been shown 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 if the protein was targeted by PKNs at this site. In order to determine if PKNs could phosphorylate EGFR at Thr-654, we used a site-specific antibody. Figure 3B and Figure S4 shows that Thr-654 is phosphorylated by PKN1 and PKN3 *in vitro*.

PKNs modulate phosphorylation of EGFR on Thr-654 in cells

Having demonstrated the phosphorylation by PKN *in vitro*, we evaluated if 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 (Fig. 3C and 3D). In response to EGF, PKN1/2/3 knock-down 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 deconvoluted the PKN siRNAs. PKN1 knock-down was found to have a very similar effect on EGFR Thr 654 phosphorylation as that seen for the knock-down of all PKNs (Fig. 3E,F). PKN2 had a minor effect and PKN3 none at all, although we note that in contrast to PKN1 and PKN2, the knock-down of PKN3 is not efficient in A431 cells. The evidence suggests that the PKN responsible for controlling basal EGFR Thr654 phosphorylation in A431 cells is principally PKN1. This result may reflect the fact that the PKN1 is the major PKN isoform expressed in these cells; quantitative analysis of expression in A431 cells shows that PKN1 is expressed at 0.44 µg/mg of cell protein, PKN2 0.13 µg/mg of cell protein and PKN3 0.03 µg/mg of cell protein.

DISCUSSION

We have analysed the phosphorylation consensus motifs of PKN1 and 3 using a peptide array. PKN1 and 3 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 here 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 amongst 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 predictive capacity as applied to both a predetermined substrate (CLIP170) 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 here 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. evaluated only the importance of Arg in positions near the phosphorylated serine in peptides phosphorylated by PKN1 and they did not analyse the importance of other amino acids as the study does here.

Zhu et al. showed that Arg was important in position -3, -2 and +2 and Arg was not allowed in position +1 and +3 [31]. We confirm these findings, except that we did not find a non-allowance of Arg in position +3: PKN1 and 3 have a preference for Arg in position -3 (80 and 85% respectively, Fig. 1 and Fig. S1) and a preference in position -2 (40 and 30% respectively). More recently, Shiga et al. using a combinatorial peptide library method, confirmed the results of Zhu et al. and showed that both PKN1 and PKN3 phosphorylate preferentially serines with an Arg in position -3. Shiga et al. also showed that these kinases have a preference for hydrophobic amino acids in position +1 and basic amino acids downstream of the phosphorylation site [32], 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. found a preference for lysine in position -5 and -2. In general, we see a very similar pattern of recognition here (albeit basic residues rather than any lysine specific preferences). However Shiga et al. noted a preference for proline in position -1, asparagine in position +3 and proline in position +5; we did not find these preferences. These differences between the three studies likely reflects the nature of the arrays: Zhu et al. used degenerate peptides where only Arg is changed, Shiga et al. used degenerate peptides where one amino acid is kept constant and our 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 Arg 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 while 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 Arg in position -3 is similar to other AGC family (basophilic) kinases. (PKC: [Lys/Arg]-x-x-[Ser/Thr]-x-[Lys/Arg]; PKA: 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 Arg in position -3 (Fig. S2). Therefore it is not the case that the preference of Arg in position -3 by PKN1 and 3 only reflects a bias in the array composition since peptides substrates identified were enriched to ~80% probability of Arg at the -3 site for PKN1.

PKN and PKC are close phylogenetic neighbours. As expected, they share some similarities in the phosphorylation consensus motif (Fig. S3). The preference of Arg in position -3 and the hydrophobic amino acid in position +1 are significant similarities. If we analyse more closely, PKN1 and 3 have a preference for Arg in position -2 (40 and 30% respectively, Fig. 1 and Fig. S1) and many PKC substrate peptides have Lys or Arg in this position as well. In addition, PKN1 presents a preference for Arg (50%) in position +2 as do PKC α , and η ; PKC β and γ prefer a Lys residue here. Concerning PKN3, there is a preference for Arg in position -5 (>30%), as for PKC α , γ , δ and η . So, 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 this family of proteins [40], controlling the same downstream events. Evidence for such properties have come from the use of a dPKN-dPKC chimera to rescue dPKN loss [41, 42].

It was proposed that PKN has a pseudosubstrate in the ACC1 region, overlapping the Rho binding domain (Fig. S3). This region corresponds to amino acids 39 to 53. The substitution of Ile-46 with a Ser converts a peptide based on this sequence into a potent substrate for PKN, while the authentic Ile counterpart inhibits activity of PKN [43]. However, the deletion

of this region does not activate PKN [44]. If we compare this region with the phosphorylation consensus motif, it has an Arg for PKN1, and PKN2 and a Lys for PKN3 in position -3. However, there are some discrepancies between the sequences. For example, the PKN1 region has an Arg in position +1, while the phosphorylation consensus motif does not permit this amino acid in this position. This could explain why this region (amino acid 39 to 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 (Fig. S3).

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). So, 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 reflected in its requirement for the 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 mass spectrometry experiments. Richard, D. H. Whelan helped with the preliminary EGFR experiments. Gavin Kelly analysed the peptide array data. Harry Mellor identified Clip-170 as a PKN substrate. Leon C. H. Pang made the PKN1 and PKN3 recombinant baculoviruses constructs. Alejandra

Collazos and Peter J. Parker planned the experiments, analysed the data and wrote the manuscript.

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Table and figure legends

Table 1

Family	Subfamily	PKN1 Substrate	Database ID	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
	CAMK		PKCζ	NM_002744.2	Protein kinase C, zeta
		CMGC	MARKL1	NM_031417.1	MAP/microtubule affinity-regulating kinase like 1
			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 (beta)	
		ARHGAP 15	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	

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.

Figure 1. PKN phosphorylation consensus motif. (A) Representative image of pepchip 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 WebLog version 3.0 based on the top 20 phosphorylated peptides. The x axis shows the positions in relation to the Ser/Thr (position 0). The y axis in the upper panel shows the bits. The graphs show the amino acid preference near the Ser/Thr. The height of the letter is proportional of the frequency in the top 20 peptides. The lower graph shows the grouped amino acid probability: S groups neutral amino acid (Ser, Gly, His, Thr, Ala, Pro); R groups hydrophilic amino acids (Arg, Lys, Glu, Asp, Asn, Gln); I groups hydrophobic amino acids (Ile, Met, Leu, Val, Phe, Trp, Tyr). The red line shows 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 or the whole CLIP-170 (1393 amino acids) were tested. The samples were incubated for 0-60 min, resolved by SDS-PAGE, stained using Coomassie, and dried. γ -[³²P]-incorporation was analysed by exposing the gel to a X-film. The data is representative of 3 different experiments. (D) Phosphorylation of CLIP 170 (first 650 amino acids) by PKN1 and PKN3 was carried out as in 1C. The autoradiographs are shown. E, Identification of phosphorylation

sites in CLIP-170 by PKN1. The tryptic peptide identified by mass spectrometry corresponds to a candidate peptide (residues 310-329) that has an Arg in position -3.

Figure 2. PKN1 protein array. An image of 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 normalisation. The 4th and 21st hits are shown in the magnified region.

Figure 3. Phosphorylation of EGFR by PKN1. (A) 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 Thr that has an Arg in position -3, Thr-654. X denotes a position with no strict amino acid requirements, Z a position strictly not tolerating Arg, Ne a neutral amino acids (Ser, Gly, His, Thr, Ala, Pro), Hi a hydrophilic amino acids (Arg, Lys, Glu, Asp, Asn, Gln) and Ho a hydrophobic amino acids (Ile, Met, Leu, Val, Phe, Trp, Tyr). (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, western blotted and probed with the EGFR Thr-654 antibody and total EGFR antibody as indicated. (C) A431 cells were transfected with 20 nM Luciferase or 20 nM PKNs siRNA. After 96h, the cells were depleted of serum for 5 h in a CO₂ independent medium containing 0.5% FBS. The cells were treated or not with EGF 100ng/ml for 30 min. The cells were collected and probed for EGFR Thr-654, total EGFR, PKN1, PKN2, PKN3, and tubulin- α as a loading control. This blot is representative of at least 4 different experiments. (* $p < 0.05$) (D) Quantification of 4 different experiments. (E) A431 cells were treated as in Fig. 3D and with de-convoluted PKNs siRNA. After 96h, the cells were depleted of serum for 5 h in a CO₂ independent medium containing 0.5% FBS. The cells were treated or not with EGF 100ng/ml for 30 min. The cells were collected and probed for EGFR Thr-654, total EGFR, PKN1, PKN2 and tubulin- α as a loading control. (F) Quantification of 2 different experiments.

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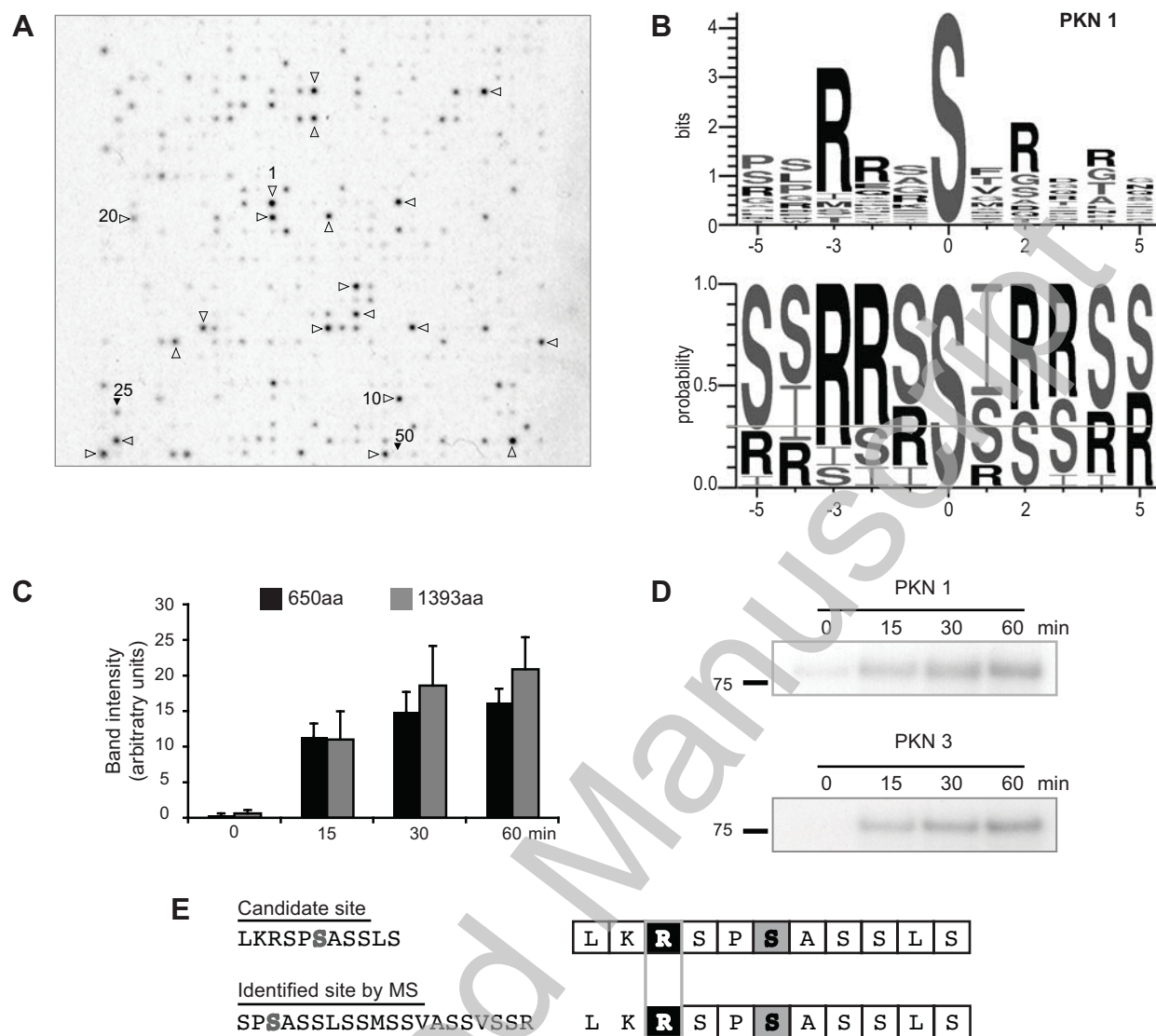


Fig 1

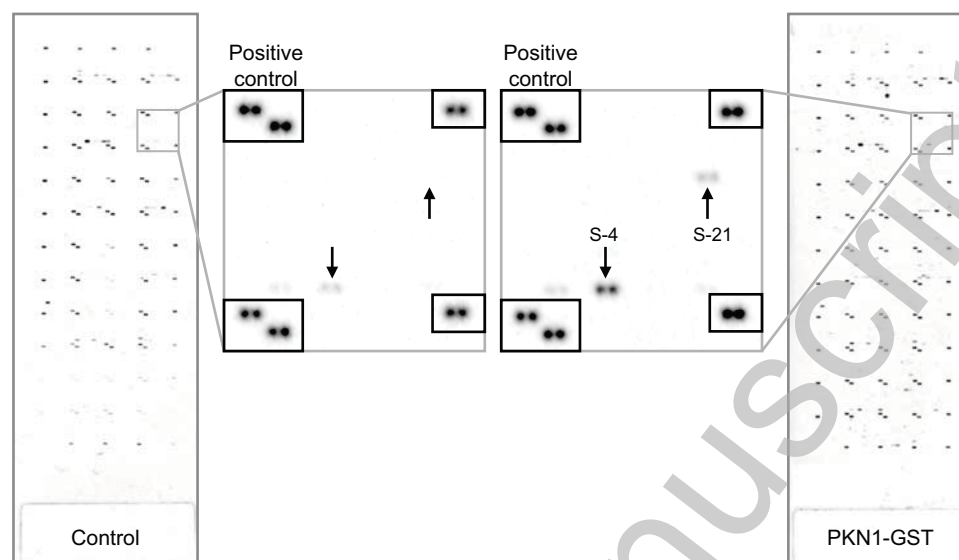


Fig 2

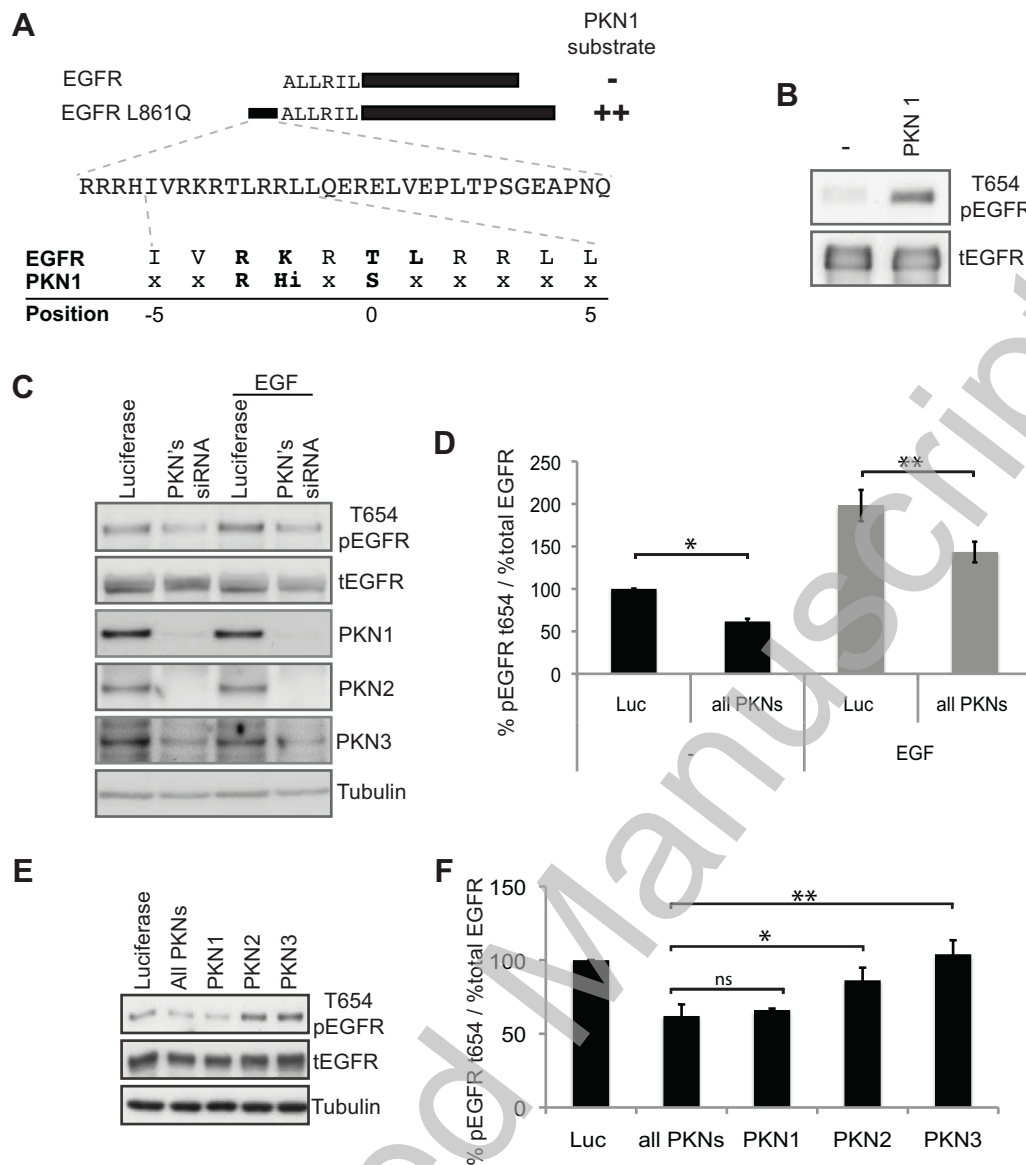


Fig 3