

Protein kinase D1 (PKD1) phosphorylation on Ser²⁰³ by type I p21-activated kinase (PAK) regulates PKD1 localization

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ABSTRACT

While PKC-mediated phosphorylation of protein kinase D1 (PKD1) has been extensively characterized, little is known about PKD1 regulation by other upstream kinases. Here, we report that stimulation of epithelial or fibroblastic cells with G protein-coupled receptor (GPCR) agonists, including angiotensin II or bombesin induced rapid and persistent PKD1 phosphorylation at Ser²⁰³, a highly conserved residue located within the PKD1 N-terminal domain. Exposure to PKD or PKC family inhibitors did not prevent PKD1 phosphorylation at Ser²⁰³, indicating that it is not mediated by autophosphorylation. In contrast, several lines of evidence indicated that the phosphorylation of PKD1 at Ser²⁰³ is mediated by kinases of the class I PAK subfamily. Specifically: 1) exposing cells to four structurally unrelated PAK inhibitors (PF-3758309, FRAX486, FRAX597 and

IPA-3) that act via different mechanisms abrogated PKD1 phosphorylation at Ser²⁰³; 2) siRNA-mediated knockdown of PAK1 and PAK2 in IEC-18 and Swiss 3T3 cells blunted PKD1 phosphorylation at Ser²⁰³; 3) Phosphorylation of Ser²⁰³ was markedly increased *in vitro* when recombinant PKD1 was incubated with either PAK1 or PAK2 in the presence of ATP. PAK inhibitors did not interfere with GPCR activation-induced rapid translocation of PKD1 to the plasma membrane but strikingly prevented the dissociation of PKD1 from the plasma membrane and blunted the phosphorylation of nuclear targets, including class IIa histone deacetylases (HDACs). We conclude that PAK-mediated phosphorylation of PKD1 at Ser²⁰³ triggers its membrane dissociation and subsequent entry into the nucleus, thereby regulating the phosphorylation of PKD1 nuclear targets, including class IIa HDACs.

INTRODUCTION

Protein kinase D (PKD) is an evolutionarily conserved protein kinase family with structural, enzymological, and regulatory properties different from the PKC family members. Accumulating evidence implicates PKD1, the founding and most studied member of the family, in the regulation of multiple biological responses, including signal transduction (1-6), chromatin organization (7), Golgi function, gene expression (5,6,8-10) and cell survival, polarity, adhesion, motility, differentiation, DNA synthesis and proliferation, [reviewed in Refs. (11,12)]. In fibroblasts, PKD1 overexpression potently enhances ERK activation, DNA synthesis and cell proliferation induced by Gq-coupled receptor agonists (1,13,14). In intestinal epithelial cells, PKD1 promotes proliferative and migratory responses *in vitro* and in crypt intestinal epithelial cells *in vivo* (3,15). Furthermore, PKD family members are increasingly implicated in inflammation, T cell development, angiogenesis, cardiac hypertrophy and cancer (11,12,16-18). Recently, *PRKDI* hotspot mutations have been identified in adenocarcinomas of the salivary gland tumors (19). The involvement of PKD1 in mediating such a diverse array of normal and abnormal biological functions depends on dynamic changes in its spatial localization, combined with its distinct substrate specificity. Consequently, the mechanisms that coordinate and modulate PKD multi-site phosphorylation with its subcellular localization are of importance and attracting intense interest.

We proposed a model of PKD1 activation that integrates the spatial and temporal changes in PKD1 localization with its multi-site phosphorylation (11). In the framework of this model, PKD1 is kept in an inactive state in un-stimulated cells through N-terminal domain repression of its catalytic domain activity (11). PKD1 can be activated

within intact cells by a remarkable array of stimuli acting through receptor-mediated pathways. Our own studies demonstrated rapid, protein kinase C (PKC)-dependent, PKD1 activation in response to phorbol esters (13,20,21), G protein-coupled receptor (GPCR) agonists (1,10,13,22-29) that act through G_q, G₁₂, G_i, and Rho (24,28-32), growth factors that signal via tyrosine kinase receptors (22,33), cross-linking of B-cell receptor and T-cell receptor in B and T lymphocytes, respectively (34-36) and oxidative stress (37,38). The phosphorylation of Ser⁷⁴⁴ and Ser⁷⁴⁸ in the PKD1 activation loop, also referred as activation segment or T-loop, is critical for PKD1 activation (11,27,30,39,40). Rapid PKC-dependent PKD1 activation is followed by a late, PKC-independent phase of activation induced by G_q-coupled receptor agonists (3,14,41). PKD1 catalytic activation within cells leads to its autophosphorylation at Ser⁹¹⁶ and Ser⁷⁴⁸ (1,3,14,36,41).

Additional studies demonstrated that PKD family members undergo rapid subcellular re-distributions in response to stimulation by GPCR agonists and growth factors. Specifically, PKD1 translocates from the cytosol to the plasma membrane followed by its reverse translocation from the plasma membrane to the cytosol and Golgi followed by subsequent accumulation in the nucleus after activation (3,26,38,42-44). Despite the importance of the N-terminal region of PKD1 in mediating auto-inhibition, membrane translocation, nuclear import, interaction with other proteins and Golgi localization, surprisingly little is known about its regulation by post-translational modifications. In this context, the highly conserved Ser²⁰³ in the N-terminal region of PKD1 (equivalent to Ser²⁰⁵ in the human PKD1) is of interest because it is highly represented in phosphoproteomic databases (45), but neither its signal-dependent

regulation nor the kinase responsible for its phosphorylation has been identified.

The p21-activated kinase (PAK) family, which are effectors of Rac and/or Cdc42 in their GTP-bound state, regulate fundamental cellular processes, including motility, proliferation, apoptosis, and gene transcription (46). PAKs are subdivided into two groups: type I PAKs (PAK1, PAK2, and PAK3) and type II PAKs (PAK4, PAK5, and PAK6) which have distinct modes of catalytic activation and both unique and common substrates (47). The PAKs are over-expressed or mutated in many cancer cells (47), including cancers of the gastrointestinal tract (48-51) and promote pro-oncogenic signaling in these cells (52). Although several pathways, including Raf/MEK/ERK and Wnt/ β -catenin, have been implicated in PAK signaling, it is recognized that downstream targets in PAK-initiated cascades remain to be identified (53). It is not known whether the PAKs can regulate the phosphorylation and/or the dynamic sub-cellular distribution of the PKDs during cell activation.

Here, we demonstrate that agonist-mediated activation of GPCRs in multiple cellular model systems, including epithelial and fibroblastic cells, induces rapid and striking phosphorylation of PKD1 on Ser²⁰³, revealing a novel input in PKD1 regulation. Based on pharmacological, biochemical and genetic evidence, we identify PAK family I as the upstream protein kinases that phosphorylates PKD1 on Ser²⁰³ in response to GPCR agonists. The phosphorylation of this residue regulates the time of residence in the membrane of activated PKD1 via accelerating its reverse translocation from the plasma membrane to the cytosol. The inhibition of PAK1/2 retains PKD1 in the membrane and prevents PKD-mediated phosphorylation and nuclear extrusion of histone deacetylase (HDAC)5, a member of the class IIa HDACs characterized by

nuclear/cytoplasmic shuttling. Thus, our results uncover a new regulatory crosstalk between PKD1 and PAK1/2 that stimulates PKD1 phosphorylation at a site that regulates its subcellular localization and identify a new PAK/PKD1/HDAC signaling pathway in intact cells.

RESULTS

Rapid phosphorylation of PKD1 on Ser²⁰³ in response to GPCR agonists in multiple cell model systems

In order to examine regulation of PKD1 phosphorylation in its N-terminal region, we used intestinal epithelial IEC-18 cells (54,55), which endogenously express G α q/11-coupled receptors for angiotensin II (ANG II) and vasopressin (24,25,56-60). These cells have been extensively used as a model system to examine signal transduction pathways in response to GPCR activation (3,15,24,25,56,58,59,61). Lysates of IEC-18 cells stimulated with ANG II for various times (0.5-300 min) were analyzed by Western blotting with an antibody that detects the phosphorylated state of PKD1 at Ser²⁰³, a highly conserved residue located between the cysteine-rich motifs (cys1 and cys2) which comprise the cysteine-rich domain (CRD) in the N-terminal region of PKD1 (**Fig. 1 A**). ANG II induced a rapid (detectable within 30 s), sustained (persisted for at least 5 h) and dramatic increase in PKD1 phosphorylation at Ser²⁰³ in intestinal epithelial IEC-18 cells (**Fig. 1 B**). A similar increase in PKD1 phosphorylation at Ser²⁰³ was induced when these cells were challenged with vasopressin (50 nM) instead of ANG II (Data not shown).

We next determined whether PKD1 phosphorylation at Ser²⁰³ in response to Gq-coupled receptor activation can be demonstrated in other cellular model systems. As shown in **Fig.1 C**, bombesin induced rapid, persistent and striking increase in PKD1 phosphorylation at Ser²⁰³

in Swiss 3T3-PKD.GFP cells which endogenously express GPCRs for the GI peptides of the bombesin/GRP family (13,60,62-76). We also detected phosphorylation of ectopically expressed PKD1 at Ser²⁰³ in COS-7 cells in response to bombesin receptor activation (**Fig.1, D**) and in HEK 293 cells stimulated with carbachol via endogenously expressed muscarinic receptors (**Fig.1, E**).

To verify the specificity of the antibody used for detecting the phosphorylated state of PKD1 at Ser²⁰³, we assessed its reactivity against GFP-tagged wild type PKD1 (GFP-PKD1) or a GFP-PKD1 mutant with Ser²⁰³ replaced by non-phosphorylatable alanine (S203A). COS-7 cells were co-transfected with constructs encoding the bombesin/GRP receptor and either GFP-PKD1 or GFP-PKDS203A. The phospho-antibody reacted strongly with GFP-PKD1 isolated from bombesin-stimulated cells but did not recognize the PKD1 mutant in which Ser²⁰³ was replaced by alanine, i.e. GFP-PKDS203A (**Fig. 1, D**). A similar result was obtained when GFP-PKD1 and GFP-PKDS203A were transfected into HEK 293 cells stimulated with carbachol (**Fig. 1, E**) or IEC-18 cells challenged with ANG II (**Fig. 1, F**). These results confirmed the specificity of the antibody used to detect the phosphorylated state of PKD at Ser²⁰³. Collectively, the results presented in **Fig.1** demonstrate that agonist-induced activation of multiple GPCRs induced robust phosphorylation of PKD1 at Ser²⁰³ in a variety of model cell systems.

In order to determine whether PKD1 phosphorylation at Ser²⁰³ can be demonstrated *in vivo*, we used epithelial cells eluted from the ileum of transgenic mice over-expressing PKD1 (3). As shown in **Fig. 1, G**, PKD1 phosphorylation at Ser²⁰³ was also detected in lysates of epithelial cells isolated from transgenic mice overexpressing PKD1. Collectively, the results in **Fig. 1**

identify a novel signal-responsive phosphorylation in the N-terminal regulatory region of PKD1.

Dissociation of PKD1 phosphorylation at Ser²⁰³ from PKD1 catalytic activity

As a first step to identify the protein kinases responsible for PKD1 phosphorylation at Ser²⁰³, we considered at least two alternative mechanisms involving autophosphorylation or transphosphorylation. In the first mechanism, PKD1 activation via membrane translocation and activation loop phosphorylation would lead to its autophosphorylation at Ser²⁰³. In the second mechanism, PKD1 phosphorylation would result from transphosphorylation by a different upstream protein kinase.

If PKD1 phosphorylation at Ser²⁰³ is mediated by autophosphorylation, we expected that this phosphorylation should be prevented by PKD family inhibitors, including kb NB 142-70 (77) and CRT0066101 (78) and abolished in kinase-inactive PKD1 mutants. To test these predictions, IEC-18 cells were treated with increasing concentrations of either CRT0066101 (**Fig. 2, A**) or kb NB 142-70 (**Fig. 2, B**) prior to stimulation with ANG II. As expected, exposure of intact cells to increasing concentrations of the PKD family inhibitors suppressed PKD1 activation in a dose-dependent manner, as scored by autophosphorylation at Ser⁹¹⁶. In contrast, CRT0066101 (**Fig. 2, A**) or kb NB 142-70 (**Fig. 2, B**) did not prevent Ser²⁰³ phosphorylation in response to ANGII stimulation, even at 10 μ M, the highest concentration tested of each inhibitor (quantification in **Fig. 2, C**).

In consonance with the results obtained with IEC-18 cells, treatment of Swiss 3T3-PKD1.GFP cells with increasing concentrations of CRT0066101 (**Fig. 2, D**) or kb NB 142-70 (**Fig. 2, E**) blocked PKD1 autophosphorylation at Ser⁹¹⁶ but did not

prevent Ser²⁰³ phosphorylation in response to bombesin stimulation (quantification in **Fig. 2, F**). Furthermore, bombesin also induced Ser²⁰³ phosphorylation in Swiss 3T3 cells expressing a kinase-inactive PKD1 mutant (**Fig. 2, G**). These results indicate that PKD1 phosphorylation at Ser²⁰³ can be dissociated from its catalytic activity implying that the modification of this site is mediated by transphosphorylation through a different upstream protein kinase(s).

Dissociation of PKD1 phosphorylation at Ser²⁰³ from PKC family catalytic activity and other basophilic protein kinases

In order to pursue the transphosphorylation hypothesis, we attempted to identify the protein kinase(s) that phosphorylates PKD1 at Ser²⁰³. The multiple basic residues in the sequence surrounding Ser²⁰³ (**Fig. 1 A**) indicated that the upstream kinase must be a basophilic protein kinase. As a first step to identify endogenous PKD1 Ser²⁰³ kinase(s), we tested multiple small-molecule inhibitors targeting a variety of protein kinases, especially basophilic kinases.

Given the important role of PKCs in PKD family regulation (11,12,79) and the fact that the sequence surrounding Ser²⁰³ was recognized as a PKC-putative phosphorylation site by motif-based profile-scanning programs, we initially determined the effect of PKC inhibitors, including GF 109203X, also known as GF I (72,80,81) and Go6983 (82) on GPCR-induced PKD Ser²⁰³ phosphorylation. Suppression of PKCs with Go6983 or GF I did not prevent PKD1 phosphorylation at Ser²⁰³ in response to either ANG II in IEC-18 (**Fig. 2, H**) or bombesin in Swiss 3T3-PKD.GFP (**Fig. 2, J**). The PKC-inhibitory activity of Go6983 or GF I in the same cell lysates was verified by abrogation of PKD1 phosphorylation at Ser⁷⁴⁴ (**Fig. 2 H, J**), a well-established target of PKCs in the activation loop of PKD1

(11,83). Since Go6983 inhibits all isoforms of the PKC family, these results indicate that PKD1 phosphorylation at Ser²⁰³ is not mediated by any of the PKCs.

The absence of proline at the P+1 position in the sequence surrounding Ser²⁰³ (see above) excludes proline-directed kinases, including MAPK families, Cdks and mTOR (84,85). Accordingly, the MEK inhibitors U0126 and PD98059 or the active-site mTOR inhibitor KU0063794 did not prevent ANG II-induced phosphorylation of PKD1 at Ser²⁰³ in IEC-18 cells (**Table 1**). Treatment of these cells with multiple chemical inhibitors known to suppress the activity of other basophilic protein kinases, including Akt, AMPK, Aurora A, CaMKII, Clk family, MSK1, PKA, Pim1, ROCK, RSK and S6K or tyrosine kinases that function upstream of phosphorylation cascades in GPCR signaling (79), including EGFR, Src and FAK did not prevent ANGII-induced PKD1 phosphorylation at Ser²⁰³ (**Table 1**).

ATP-competitive inhibitors of PAK prevent PKD1 phosphorylation at Ser²⁰³

In striking contrast to the lack of inhibitory effects on PKD1 phosphorylation at Ser²⁰³ by multiple inhibitors, the pyrrolopyrazole p21-activated kinase (PAK) inhibitor PF-3758309 potently abrogated GPCR-induced PKD1 phosphorylation at Ser²⁰³. PF-3758309 is an ATP-competitive inhibitor of both PAK type I and II and also inhibits a number of other protein kinases at higher concentrations (86,87). Treatment of IEC-18 cells with increasing concentrations of PF-3758309 suppressed GPCR-induced PKD1 Ser²⁰³ phosphorylation in a concentration-dependent manner (**Fig. 3 A**). PAK activation leads to autophosphorylation of several sites, including Ser¹⁴⁴ in PAK1 (equivalent to Ser¹⁴¹ in PAK2). We verified that treatment with PF-3758309 also blunted PAK1 autophosphorylation, scored by phosphorylation at Ser¹⁴⁴. Indeed, treatment

with PF-3758309 suppressed GPCR-induced PKD1 Ser²⁰³ phosphorylation and PAK1 phosphorylation at Ser¹⁴⁴ at similar concentrations in IEC-18 cells (**Fig. 3 A**; quantification in **Fig. 3B**).

The results obtained with PF-3758309 prompted us to test other structurally unrelated inhibitors of the PAKs. FRAX597 and FRAX486 are preferential ATP-competitive inhibitors of type I PAKs (87,88). As shown in **Fig. 3 C**, FRAX597 potently inhibited phosphorylation of PKD1 at Ser²⁰³ and PAK1/2 on Ser^{144/141} induced by ANG II in IEC-18 cells at similar concentrations (quantification in **Fig. 3D**), suggesting that group I PAKs mediate PKD1 phosphorylation at Ser²⁰³.

It is plausible that different upstream kinases phosphorylate PKD at Ser²⁰³ at different times of GPCR stimulation. Consequently, we determined whether PAK inhibitors prevented PKD1 Ser²⁰³ phosphorylation at various times after stimulation with ANG II in IEC-18 cells. Exposure to PF-3758309 (**Fig. 3 E**) or FRAX486 (**Fig. 3 F**) inhibited phosphorylation of PKD1 Ser²⁰³ in response to GPCR stimulation at all times tested. We verified that PF-3758309 and FRAX486 also suppressed GPCR-induced PAK1/2 autophosphorylation at Ser^{144/141} in IEC-18 at all times tested. The doublet detected in IEC-18 with the PAK1/2 phospho-antibody corresponded to PAK1 pSer¹⁴⁴ (upper band) and PAK2 pSer¹⁴¹ (lower band) as shown by using siRNA-mediated knockdown of each of these PAK isoforms (Data not shown).

In agreement with the results obtained with IEC-18 cells, treatment of Swiss 3T3-PKD1.GFP with PF-3758309 prior to bombesin stimulation prevented phosphorylation of PKD1 at Ser²⁰³ and PAK2 (the major isoform expressed in these cells) at Ser¹⁴¹ at similar concentrations (**Fig. 3, G**; quantification in **Fig. 3 H**). We also verified that exposure to PF-3758309 or

FRAX 486 inhibited PKD1 Ser²⁰³ and PAK2 Ser¹⁴¹ phosphorylation induced by bombesin in Swiss 3T3-PKD.GFP cells at all times tested (**Fig. 3 I, J**). Although both FRAX597 and PF-3758309 inhibit other kinases, many of these off-target effects are non-overlapping (87,89). Therefore, these results imply that type I PAKs mediate PKD1 phosphorylation at Ser²⁰³ in response to GPCR stimulation in different cell systems.

IPA-3, an allosteric inhibitor of group I PAKs also prevented PKD1 phosphorylation at Ser²⁰³.

In order to substantiate the results obtained with the ATP-competitive inhibitors, we also tested the compound IPA-3 (90,91), a specific allosteric inhibitor that binds to the N-terminal regulatory domain of PAK1/2/3 rather than to their kinase catalytic domain. Treatment with IPA-3 inhibited the increase in PKD1 phosphorylation at Ser²⁰³ in a dose-dependent manner in IEC-18 cells challenged with ANG II (**Fig. 3, L**; quantification in **Fig. 3 M**). In contrast, a structural isomer of IPA-3 displaying no inhibitory effect toward the PAKs (PIRS), tested at 30 μ M, did not interfere with GPCR-induced PKD1 Ser²⁰³ phosphorylation in these cells (**Fig. 3, L**; quantification in **Fig. 3 M**).

Treatment with IPA-3 also abolished PKD1 phosphorylation at Ser²⁰³ in Swiss 3T3-PKD1.GFP cells stimulated with bombesin (**Fig. 3, N**; quantification in **Fig. 3 O**). Furthermore, IPA-3 also prevented PKD1 phosphorylation at Ser²⁰³ in Swiss 3T3 cells expressing a kinase-dead PKD1 mutant (Data not shown). Together, the results presented in **Fig. 3** obtained with three structurally unrelated inhibitors that act via different mechanisms (i.e. PF-3758309, FRAX597 and IPA-3) indicate that group I PAK mediates PKD1 phosphorylation on Ser²⁰³ in intact epithelial and fibroblast cells.

Identification of PAK1 and PAK2 as the upstream protein kinases that phosphorylate PKD1 at Ser²⁰³

In order to corroborate the results obtained with chemical inhibitors, we silenced the expression of PAK1 and PAK2 in IEC-18 cells using RNAi (PAK3, the other member of group I is expressed primarily in neuronal cells). In line with the notion that the type I PAKs mediate PKD1 phosphorylation at Ser²⁰³, knockdown of PAK1 and PAK2 prevented the increase in PKD1 phosphorylation at Ser²⁰³ in response to ANG II in IEC-18 cells (**Fig. 4, A**; quantification in **Fig. 4 B**). Similarly, knockdown of PAK1 and PAK2 blocked the increase in PKD1 phosphorylation at Ser²⁰³ in response to bombesin in Swiss 3T3-PKD.GFP (**Fig. 4, C**; quantification in **Fig. 4 D**).

Further evidence for a role of type I PAKs in PKD1 phosphorylation at Ser²⁰³ was obtained by transient co-transfection of COS-7 cells with constructs encoding bombesin receptor, PKD1 and either wild type or constitutively activated PAK1 in which Thr⁴²³ was replaced by Glu (92). Our results demonstrate that expression of PAK1^{T423E} (but not wild type PAK1) markedly increased the phosphorylation of PKD1 at Ser²⁰³ in unstimulated cells to a degree comparable to that produced by agonist stimulation (**Fig. 4, E**; quantification in **Fig. 4 F**). Similar results were obtained when the transient co-transfections were performed using HEK 293 cells instead of COS-7 cells (Data not shown). We also used a dominant-negative strategy to test the role of the PAKs in PKD1 phosphorylation at Ser²⁰³. We expressed a highly conserved fragment corresponding to the auto-inhibitory domain (AID) of PAK 1 (residues 83–149) that inhibits type I PAK activity (93). The expression of GST-AID prevented agonist-stimulated increase in PKD1 Ser²⁰³ phosphorylation (**Fig. 4, E**; quantification in

Fig. 4 F). On the basis of multiple approaches, we can conclude that PKD1 phosphorylation at Ser²⁰³ is mediated through a PAK1/2-dependent pathway.

We next determined whether Ser²⁰³ of PKD1 can serve as a direct substrate for activated PAK1 and PAK2. To test this possibility, recombinant PKD1 was incubated in the absence or presence of activated recombinant PAK1 or PAK2. After 5 min, the reactants were analyzed by Western blotting using the antibody that detects the phosphorylated state of PKD1 at Ser²⁰³. We found that the phosphorylation of Ser²⁰³ was markedly increased *in vitro* when PKD1 was incubated with either PAK1 (**Fig. 4 G**) or PAK2 (**Fig. 4 H**) in the presence of ATP (quantification in **Fig. 4 I**).

Based on evidence generated via pharmacological, biochemical and genetic approaches, we conclude that the group I PAKs phosphorylate the N-terminal domain of PKD1 at Ser²⁰³ in a stimulus-dependent manner. The identification of PAK-mediated PKD1 phosphorylation reveals a new point of integration in the signal transduction pathways initiated by GPCR agonists and raises important questions concerning its function in cell regulation.

PKD1 phosphorylation at Ser²⁰³ regulates its intracellular redistribution in response to GPCR agonists.

The CRD domain of PKD1, comprised of a tandem repeat of cysteine-rich motifs, mediates its translocation from the cytosol to the plasma membrane in response to GPCR-induced generation of DAG (11). Since Ser²⁰³ lies between the cysteine-rich motifs of the CRD (**Fig. 1A**), we considered the possibility that the phosphorylation of this residue regulates the intracellular localization of PKD1 in response to GPCR stimulation. To explore this mechanism with high resolution, we generated IEC-18 cell lines that express PKD1-EGFP in an inducible manner. After exposure to

doxycycline for 24 h to induce the expression of PKD1-EGFP, cells were treated in the absence or presence of PF-3758309, FRAX597 or IPA-3 and subsequently stimulated with ANG II for various times. In line with previous results, stimulation of control IEC-18 cells with ANG II induced a rapid (3 min) and striking translocation of PKD1 from the cytosol to the plasma membrane, causing a localized fluorescence at the cell periphery (**Fig. 5 A**). The translocation of PKD1 was transient as it quickly dissociated from the plasma membrane, returned to the cytoplasm and accumulated in the nucleus of most cells after 30 min of stimulation (**Fig. 5 A**; quantification in **Fig. 5 B**). In sharp contrast, cells treated with PF-3758309 (**Fig. 5 C, D**), FRAX597 (**Fig. 5 E, F**) or IPA-3 (**Fig. 5 G, H**) displayed PKD1-EGFP at the plasma membrane but not in the nucleus, even after 60 min of ANG II stimulation. As shown in **Fig. 5, I, J** (quantification in **Fig. 5 K**), the results obtained with chemical inhibitors were corroborated by knockdown of the expression of PAK1 and PAK2 in IEC-18 cells using siRNAs (**Fig. 5, L**). Specifically, cells depleted of PAK1 and PAK2 exhibited PKD1-EGFP at the plasma membrane but not in the nucleus, even after 60 min of ANG II stimulation (**Fig. 5, I, J** (quantification in **Fig. 5 K**). Collectively, the results implied that PAK-mediated phosphorylation of PKD1 facilitates membrane dissociation and nuclear localization of PKD1 in GPCR-stimulated cells.

To rule out possible interference with the intracellular distribution of PKD1 due to the presence of the fluorescent tag, we analyzed the localization of endogenous PKD1. As illustrated by representative cells displayed in **Fig. 6**, the distribution of endogenous PKD1 was very similar to that detected with EGFP-PKD1. Specifically, endogenous PKD1 was located primarily in the cytoplasm of unstimulated IEC-18 cells.

Stimulation of these cells with ANG II induced rapid (3 min) PKD1 translocation to the plasma membrane. Subsequently, PKD1 underwent reverse translocation returning to the cytosol and nucleus 60 min after stimulation. Treatment of the cells with chemical inhibitors of group I PAKs, including PF-3758309 (**Fig. 6 B**), FRAX597 (**Fig. 6 C**) and IPA-3 (**Fig. 6 D**) prevented the dissociation of endogenous PKD1 from the plasma membrane in agonist-stimulated cells. Similarly, siRNA-mediated knockdown of PAK1 and PAK2 also impeded reverse translocation of endogenous PKD1 from the plasma membrane to the cytoplasm and nucleus in cells stimulated with ANG II (**Fig. 6 E and F**). We verified that transfection of siRNAs targeting PAK1 and PAK 2 resulted in the extensive knockdown of their protein expression (similar to results shown in **Fig. 4 A and Fig. 5 L**). The images obtained were quantified and the results (bars in **Fig. 6**) substantiated that chemical inhibitors of group I PAKs and siRNA-mediated knockdown of PAK1 and PAK2 prevent the dissociation of endogenous PKD1 from the plasma membrane.

Having established that PAK1 and PAK2 regulate the intracellular distribution of PKD1 in agonist-stimulated cells, we next determined the role of Ser²⁰³ phosphorylation in this process. Specifically, cultures of IEC-18 cells were transiently transfected with wild type GFP-PKD1 or a mutant GFP-PKD1 with Ser²⁰³ substituted by alanine (PKD1S203A) and then the localization of these PKD1 forms was examined after various times of ANG II stimulation. In unstimulated cells, GFP-PKD1 and GFP-PKD1(S203A) were predominantly in the cytoplasm. As expected, stimulation of IEC-18 cells with ANG II induced a rapid but transient translocation of wild type GFP-PKD1 to the plasma membrane (**Fig. 7 A**). ANG II also induced rapid translocation of

GFP-PKD1(S203A) from the cytosol to the plasma membrane but this mutant PKD1 remained at the membrane for much longer with significant fluorescent signal localized to the plasma membrane even after 60 min of stimulation (**Fig. 7 A**; quantification in **Fig. 7 B**). These results substantiated the notion that PAK1/2-mediated phosphorylation of PKD1 at Ser²⁰³ accelerates its reverse translocation from the membrane to the interior of the cell. Taken together, the results show, for the first time, that the PAKs of the group I regulate the intracellular localization of PKD1. In this manner, it was plausible that the PAKs control the access to specific substrates phosphorylated by activated PKD1.

Identification of a novel PAK/PKD/HDAC signaling pathway.

Class IIa histone deacetylases (HDACs), including HDAC4, HDAC5 and HDAC7, and HDAC9, are unique among the HDAC family, in that they shuttle between the nucleus and the cytoplasm in response to extracellular signals. Phosphorylation of specific conserved residues in HDAC4, HDAC5, HDAC7, and HDAC9 leads to their nuclear extrusion and sequestration in the cytoplasm. PKD1 has been identified as one of the upstream kinases that mediate the phosphorylation and subcellular localization of class IIa HDACs in a variety of cell types, including intestinal epithelial cells (94). In view of our preceding findings, we hypothesized that PAK-mediated reverse translocation of activated PKD1 and its subsequent entry into the nucleus is necessary for the phosphorylation of nuclear targets, including class IIa HDACs.

In order to determine whether the PAKs control the access of PKD1 to specific substrates in intact cells, cultures of IEC-18 cells were transfected with epitope (FLAG)-tagged HDAC5. After 48 h, the cells were treated without or with PF-3758309, FRAX597 or IPA-3 to inhibit PAK1/2 and, as a control, with CRT0066101 to inhibit

PKDs. Subsequently, the cultures were challenged without or with ANG II for 60 min and fixed. As shown in **Fig. 7 C**, FLAG-tagged HDAC5 was present in the nucleus of unstimulated cells and strikingly translocated to the cytosol in response to ANG II stimulation. Treatment with CRT0066101 prevented the nuclear extrusion of FLAG-tagged HDAC5, in agreement with previous results (94). A salient feature of our current results is that exposure to the PAK inhibitors PF-3758309, FRAX597 or IPA-3 strikingly inhibited the nuclear extrusion of HDAC5 (**Fig. 7 C**; quantification in **Fig. 7 D**). In line with the notion that nuclear extrusion of HDAC5 is mediated by its phosphorylation, we verified that PF-3758309, FRAX597 and IPA-3 inhibited HDAC5 phosphorylation at Ser⁴⁹⁸, a site phosphorylated by PKD1. PF-3758309 (**Fig. 7 F**) and FRAX597 (**Fig. 7 G**) inhibited HDAC5 phosphorylation at Ser⁴⁹⁸ in a concentration-dependent manner. Furthermore, knockdown of PAK1 and PAK2 prevented the increase in HDAC5 phosphorylation at Ser⁴⁹⁸ in response to ANG II in IEC-18 cells (**Fig. 7 H**). Similar results were obtained when we examined agonist-induced intracellular shuttling of HDAC7 in IEC-18 cells. Since cell treatment with PF-3758309, FRAX597 and IPA-3 or knockdown of PAK1/2 did not interfere with PKD1 activation, as scored by autophosphorylation at Ser⁹¹⁶ (results shown in **Fig. 3** and **Fig. 4**) or activation loop phosphorylation at Ser⁷⁴⁴ (Data not shown), we conclude that PAK1/2 regulate HDAC5 localization and phosphorylation by facilitating the dissociation of active PKD1 from the plasma membrane and its subsequent entry into the nucleus (**Fig. 7 J**).

DISCUSSION

While PKC-mediated phosphorylation of PKD has been extensively studied as a critical on/off switch of kinase catalytic activity, little is known about the regulation

and function of PKD1 phosphorylation by other upstream kinases. Analysis of phosphoproteome databases indicated that Ser²⁰³ of PKD1 has been detected in many studies but nothing was known about its regulation and function and the upstream kinase involved had not been identified. Here, we report that stimulation of a variety of cell types with GPCR agonists induced rapid and sustained PKD1 phosphorylation at Ser²⁰³, thus identifying a novel signal-dependent phosphorylation site in the N-terminal domain of PKD1.

At least two alternative mechanisms involving autophosphorylation or transphosphorylation could explain PKD1 Ser²⁰³ phosphorylation in response to GPCR agonists. Treatment with PKD family inhibitors, at concentrations that extinguished PKD autophosphorylation at Ser⁹¹⁶, as well as the use of cells transfected with a PKD1 kinase-inactive mutant indicated that the phosphorylation of Ser²⁰³ is not mediated by autophosphorylation. Consequently, we attempted to identify the protein kinase(s) that transphosphorylate PKD1 at Ser²⁰³. The multiple basic residues in the sequence surrounding Ser²⁰³ (GVRRRRLS²⁰³NVSL, **Fig.1 A**) indicated that the upstream kinase must be a basophilic protein kinase whereas the absence of proline at the P+1 position excluded proline-directed kinases, including MAPK families, Cdk's and mTOR (84,85). Furthermore, multiple small-molecule inhibitors of basophilic protein kinases targeting AKT, AMPK, Aurora A, CaMKII, Clk (Cdc2-like kinase), MSK1, PKA, Pim1, ROCK, RSK and S6K did not prevent ANG II-induced PKD1 phosphorylation at Ser²⁰³. In contrast, several lines of evidence indicated that the phosphorylation of PKD1 at Ser²⁰³ is mediated by kinases of the type I PAK subfamily: **1)** The pyrrolopyrazole PAK inhibitor PF-3758309 prevented PKD1 phosphorylation at Ser²⁰³ induced by either

ANG II in epithelial IEC-18 cells or bombesin in Swiss 3T3 fibroblasts. PF-3758309, an ATP-competitive inhibitor of both PAK group I and II (86,87), impaired PKD1 phosphorylation at Ser²⁰³ in a concentration-dependent manner and after various times of stimulation; **2)** The results obtained with PF-3758309 were corroborated by results using other structurally unrelated inhibitors. FRAX597 is a novel and potent, ATP-competitive inhibitor of group I PAKs (87,88). FRAX597 inhibited PKD1 Ser²⁰³ phosphorylation in epithelial and fibroblastic cells in a concentration-dependent manner and after various times of stimulation; **3)** We substantiated the results obtained with the ATP-competitive inhibitors PF-3758309 and FRAX597, using the compound IPA-3 (90,91), a highly specific allosteric inhibitor that binds covalently to the N-terminal regulatory domain of the PAK group I rather than to the kinase catalytic domain of these enzymes and thereby inhibits PAK activation by GTP-loaded Rac1 and Cdc42. Treatment with IPA-3 blunted PKD1 phosphorylation at Ser²⁰³ in intestinal epithelial IEC-18 cells stimulated with ANG II or in Swiss 3T3 fibroblasts challenged with bombesin. Collectively, the results obtained with three structurally unrelated inhibitors that act via different mechanisms (PF-3758309, FRAX597 and IPA-3) indicate that group I PAK mediates PKD1 phosphorylation at Ser²⁰³ in intact cells; **4)** This conclusion was substantiated by siRNA-mediated knockdown of PAK1 and PAK2 in both IEC-18 and Swiss 3T3 cells and by expression of a dominant negative auto-inhibitory fragment of PAK1 in COS-7 cells; **5)** Phosphorylation of Ser²⁰³ was markedly increased in cells by over-expression of a constitutively activated form of PAK1 (PAK1^{T423E}) and *in vitro* when recombinant PKD1 was incubated with either PAK1 or PAK2 in the presence of ATP; **6)** In kinetic

studies, we verified that PAK1/2 activation in intact cells (scored by autophosphorylation at Ser^{144/141}) in response to GPCR stimulation occurs as rapidly as expected for an upstream kinase in a PAK/PKD cascade (Data not shown). Thus, based on pharmacological, biochemical and genetic evidence, our results indicate that PAK1/2 directly phosphorylate the N-terminal domain of PKD1 at Ser²⁰³ and thus reveal a novel input in PKD1 regulation.

We next explored the possible functional consequence(s) of Ser²⁰³ phosphorylation in PKD1. The CRD domain in the N-terminal region of PKD1 is comprised of a tandem repeat of cysteine-rich motifs (cys1 and cys2) that mediate rapid PKD1 translocation from the cytosol to the plasma membrane in response to GPCR-induced generation of DAG (**Fig. 1 A**). Interestingly, activated PKD1 quickly dissociates from the plasma membrane, returns to the cytoplasm and accumulates in the nucleus. Since phosphorylation has been implicated in the regulation of PKD1 intracellular localization (43,95) and PKD Ser²⁰³ lies between cys1 and cys2 of the CRD, we considered the possibility that the PAK-mediated phosphorylation of his residue could play a role in triggering membrane dissociation of PKD1 after GPCR stimulation. In line with this notion, we show that treatment of intact cells with different PAK inhibitors, including PF-3758309, FRAX486, FRAX597 or IPA-3 or knockdown of PAK1 and PAK2 expression strikingly prevented the dissociation of PKD1-EGFP from the plasma membrane. These results were corroborated by detecting the intracellular distribution of endogenous PKD1. The critical role of Ser²⁰³ phosphorylation in the regulation of PKD1 reverse translocation was confirmed by using wild type GFP-PKD1 or a mutant GFP-PKD1 with Ser²⁰³ substituted by alanine. We found that the mutation S203A impaired PKD1 dissociation from the plasma

membrane after various times of ANG II stimulation. Collectively, the results indicate that PAK-mediated phosphorylation of PKD1 at Ser²⁰³ facilitates rapid membrane dissociation of PKD1 and subsequent accumulation in the nucleus in GPCR-stimulated intact intestinal epithelial cells (**Fig. 7, J**).

It is recognized that the translocation of kinases to specific subcellular locations plays a critical role in the selection of physiological substrates for their phosphorylation. Previous studies demonstrated that active PKD1 acts as a direct class IIa HDAC kinase in a variety of biological systems, inducing their nuclear extrusion and thereby influencing chromatin organization as well as non-canonical functions of these enzymes (96-98). PKD1 also mediates endogenous class IIa HDAC phosphorylation and cytoplasmic localization in response to GPCR activation in intestinal epithelial cells (94). In addition to PKD1, a number of other protein kinases including Ca²⁺/calmodulin-dependent protein kinases (CaMKs) induce class IIa HDAC cytoplasmic accumulation but none of the previous studies implicated the PAKs in the regulation of the subcellular localization of the class IIa HDACs (98). Indeed, the phosphorylation sites in these HDACs that control their subcellular distribution do not conform to the consensus sequence phosphorylated by PAKs. However, we posited that PAK-mediated dissociation of activated PKD1 from the plasma membrane is necessary for its ability to act as a class IIa HDAC kinase. In line with this hypothesis, we found that treatment of intact IEC-18 cells with different PAK inhibitors, including PF-3758309, FRAX 486 or IPA-3 or knockdown of PAK1 and PAK2 abrogated the cytoplasmic accumulation and phosphorylation of HDAC5 and HDAC7 induced by ANG II as effectively as inhibiting directly PKD1

activity with the preferential PKD inhibitor CRT0066101. Consequently, our findings support the novel notion that the PAKs play a role in the regulation of class IIa HDAC phosphorylation and localization through phosphorylation of PKD1 on Ser²⁰³ thereby triggering PKD1 dissociation from the plasma membrane. Thus, our results identify a novel PAK/PKD/HDAC5 pathway in signal transduction (**Fig. 7, J**).

EXPERIMENTAL PROCEDURES

Cell Culture

The non-transformed rat intestinal epithelial IEC-18 cells (54,55), originated from intestinal crypt cells, were purchased from ATCC. These cells express G_q-coupled receptors for ANG II and vasopressin (24,25,56-60) and have been extensively used as a model system to examine signal transduction pathways in response to GPCR activation. Cultures of IEC-18 cells were maintained as described previously (3,4). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 10% CO₂ and 90% air. Stock cultures were sub-cultured every 3-4 days. For experimental purposes, IEC-18 cells were seeded in 35-mm dishes at a density of 2×10^5 cells/dish.

Stock cultures of Swiss 3T3-PKD.GFP cells, which overexpress wild type PKD1 and Swiss 3T3-PKDK618N.GFP cells, that overexpress a kinase-deficient PKD were generated as previously described (1,13). These derivatives of Swiss 3T3 cells which express endogenously receptors for the peptides of the bombesin family (65), were maintained and transferred for experimental purposes as described previously (14). Human embryonic kidney HEK 293 cells and

COS-7 cells were also cultured as described previously (99,100).

Generation of IEC-18 cells with inducible PKD1-EGFP.

An all-in-one Tet-ON inducible system was developed for the expression of PKD1-EGFP. We used the pCD550A-1 lentiviral vector (SystemBio) as a platform and modified it to express the reverse tetracycline-controlled transactivator (rtTA) under the control of the constitutive PGK promoter. The rtTA cDNA is co-expressed with the cDNA encoding DsRed-Fluorescent Protein (Clontech). The two coding sequences are separated by a 2A-like sequence. The “self-cleaving” 2A peptides are used to mediate protein cleavage from a single open reading frame allowing the expression of the two proteins from a single mRNA. We also replaced the original EF1 promoter by an inducible promoter composed of 6 Tetracycline Responsive Elements (TRE) followed by the minimal CMV promoter. The resulting lentiviral vector was designated pCD550A1-TRE-rtTA-Dsred and was used to clone PKD1-EGFP or EGFP under the control of the TRE promoter. Lentiviral infections were performed according to standard procedures. Briefly, 293T cells were co-transfected with pMD2.G and psPAX2 (Addgene Inc.) along with either pCD550A1-TRE-EGFP-rtTA-Dsred or pCD550A1-TRE-PKD1-EGFP-rtTA-Dsred vector using JetPrime transfection reagent (Polyplus) according to the manufacturer protocol. The transfection medium was replaced after 12 h with fresh DMEM medium with 10% FBS and 48 h later the viral supernatants were collected and concentrated by using Centricon Plus-70 filter units (Millipore). IEC-18 cells (passage 2) were infected overnight with viral supernatants supplemented with 8 µg/ml of Polybrene (Sigma). After 72 h, stably transduced IEC-18 cells were separated by

fluorescence-activated cell sorting (FACS) resulting in a homogeneous population of EGFP-labeled cells. Addition of doxycycline to these cells induced PKD1-EGFP expression in a dose-dependent manner, with maximal effect at 1 $\mu\text{g}/\text{ml}$ (Data not shown).

Imaging of PKD1-EGFP fluorescence

Single cell imaging of the EGFP tagged PKD1 was achieved with a fluorescence microscope. The microscope used was an epifluorescence Zeiss Axioskop and a Zeiss water objective (Achromplan 40/.75W Carl Zeiss, Inc.). Images were captured as uncompressed 24-bit TIFF files with a cooled (-12°C) single CCD color digital camera (Pursuit, Diagnostic Instruments) driven by SPOT version 4.7 software. Quantitative analysis of the relative change in plasma membrane and cytosol fluorescence intensity of individual cells was performed by importing the TIF images into Zeiss LSM 510 software and performing profile scans with the largest line width. Five equally spaced line profiles were taken for each cell or cell pair. Intensities were background corrected, and the intensities at the membrane were divided by those in the immediately surrounding cytoplasm. We analyzed 30 to 45 cells in each experiment, and each experiment was performed in duplicate. The selected cells displayed in the figures were representative of 90% of the population of positive cells.

Immunofluorescence

Immunofluorescence of IEC18 cells was performed by fixing the cultures with 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100. After extensive PBS washing, fixed cells were incubated for 2h at 25°C in blocking buffer (BB) consisting of PBS -5% FBS and 2% bovine serum albumin and then stained at 4°C overnight with a polyclonal antibody anti PKD1 (1:500) or a mouse monoclonal

antibody anti-FLAG (1:500) diluted in BB. Subsequently, the cells were washed with PBS at 25°C and stained at 25°C for 60 min with either Alexafluor 546 or Alexafluor 488 - conjugated goat-anti rabbit diluted in BB (1:100) and washed again with PBS. Nuclei were stained using Hoechst 33342 (1:10,000). The samples were imaged with an epifluorescence Zeiss Axioskop and a Zeiss water objective (Achromplan 40/.75W Carl Zeiss, Inc.). Images were captured as uncompressed 24-bit TIFF files with a cooled (-12°C) single CCD color digital camera (Pursuit, Diagnostic Instruments) driven by SPOT version 4.7 software. Alexafluor 546 signals were observed with a HI Q filter set for Rhodamine/TRITC and Alexafluor 488 were observed with a HI Q filter set 41001 (Chroma Technology). The selected cells displayed in the appropriate figures were representative of 90% of the population.

Immunoblotting and detection of PKD1, PAK1/2 and HDAC 5 phosphorylation

Confluent intestinal epithelial IEC-18 cells or Swiss 3T3 fibroblasts, treated as specified in the corresponding Figures, were lysed in 2xSDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (20 mM Tris/HCl, pH 6.8, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol) and boiled for 10 min. After SDS-PAGE, proteins were transferred to Immobilon-P membranes. The transfer was carried out at 100 V, 0.4 A at 4°C for 4 h using a Bio-Rad transfer apparatus. The transfer buffer consisted of 200 mM glycine, 25 mM Tris, 0.01% SDS, and 20% CH_3OH . For detection of proteins, membranes were blocked using 5% nonfat dried milk in PBS (pH 7.2) and then incubated for at least 2 h with the desired antibodies diluted in PBS containing 0.1% Tween. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish

peroxidase-conjugated anti-mouse, anti-rabbit antibody and a FUJI LAS-4000 Mini Luminescent Image Analyzer.

Knockdown of PAK1/2 via siRNA transfection

Silencer Select siRNA duplexes were purchased from Ambion, Life Technologies. The siRNAs were designed to target the mRNA of mouse/rat PAK1 and PAK2. GenBank mRNA sequences; AF082077.1 (PAK1), U19967.1 (PAK2). The sequences of the siRNAs were as follows; PAK1 sense: UGACACGCAAGGCTTATGGac, anti-sense: GUCCAUAAGCCUUGCGUGUca and PAK2 sense: TTAACCTTCTTGACAGTTAcc anti-sense: GGUAACUGUCCAAGAAGUUaa. For siRNA transfection the reverse transfection method was used. The siRNA pool was mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and added to 35mm dishes. IEC-18 cells were then plated on top of the siRNA/Lipofectamine RNAiMAX complex at a density of 2×10^5 cells/35 mm dish. Control transfections were carried out with Stealth siRNA negative control (Invitrogen, Carlsbad, CA). Five days after transfection, cells were used for experiments and subsequent Western blot analysis.

In vitro Kinase Assays

For the assays of PAK1 or PAK2 transphosphorylation of PKD1 on Ser²⁰³, 200 ng of recombinant PKD1 (Sigma-Aldrich # K4268 or Millipore # 14-508) was incubated in the absence or in the presence of 50 ng of recombinant active PAK1 (Sigma-Aldrich, # K2893) or 50 ng of recombinant active PAK2 (Millipore #14-481) in 25 μ l of kinase buffer (100 μ M ATP, 25 mM MOPS, pH 7.2, 12.5 mM, glycerol 2-phosphate, 25 mM MgCl₂, 5 mM EGTA, and 2 mM EDTA and 0.25 mM DTT) for 5 min at 30°C. Reactions were

terminated by addition of 2 \times SDS-PAGE sample buffer and resolved by SDS-PAGE. PKD1 phosphorylation was determined by Western blot analysis using the antibody that detects its phosphorylated state on Ser²⁰³. The active PAK1 was generated by incubating a mixture PAK1/CDC42 in 10 μ l kinase buffer with 5 μ l of 12.5 mM MnCl₂/0.5 mM GTP solution and incubated for 20 min at 30°C prior to the addition of 10 μ l of PKD1 (200 ng).

Transient Cell transfection and Mutagenesis

The single point mutant S203A in the pcDNA3-GFP-PKD construct was made by Retrogen Inc, San Diego, CA. Cultures of IEC-18 cells were transfected with the plasmids containing a cDNA encoding either a GFP PKD WT or a GFP PKD S203A or an epitope (FLAG) tagged-HDAC5 wild type from Addgene (cat #32216) by using Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. Analysis of the cells transiently transfected were performed 48 h after transfection. COS-7 cells were transfected with GFP-PKD WT and bombesin receptor without or with pCMV6M-Pak (Addgene plasmid # 12209) or pCMV6M-PAK1 T423E (Addgene plasmid # 12208) or GST tagged pEBG-Pak1 83-149 (Addgene plasmid # 12214) by using Lipofectamine 3000 (Invitrogen), as suggested by the manufacturer.

PKD Ser²⁰³ phosphorylation in intestinal epithelial cells in vivo.

To verify that PKD1 Ser²⁰³ phosphorylation occurs *in vivo*, we used transgenic mice that express elevated PKD1 protein in the ileal epithelium and control non-transgenic littermates. The generation of PKD1 transgenic mice was described elsewhere (3). To perform anatomical dissection and tissue collection, mice were euthanized in a CO₂ chamber. Epithelial cells were isolated

sequentially along the crypt-villus axis by timed incubations in EDTA-PBS solutions (3). To measure active PKD1 expression and PKD1 Ser²⁰³ phosphorylation, lysates of intestinal cells isolated from gender- and age-matched mice were subjected to immunoblotting, as described above. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Research Committee of the University of California, Los Angeles (Protocol Number: 2014-070-01).

Materials

DMEM, FBS, goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 546 and Silencer Select siRNAs were obtained from Invitrogen (Carlsbad, CA). ANG II, bombesin and carbachol were obtained from Sigma-Aldrich (St. Louis, MO). The kinase inhibitors kb NB 142-70, CRT0066101, Gö6983, GF 109203X, and IPA-3 were from R&D Systems (Minneapolis, MN), FRAX597 and FRAX486 were from Selleckchem (Boston, MA). PF-3758309 was purchased from EMD Millipore (Billerica, MA). Primary antibodies used were: phospho-PKD Ser²⁰³

(ab59413, final dilution 1:1000) and phospho-PAK1/2 on Ser^{144/141} (ab40795, final dilution 1:1000) from Abcam, PKD C-20 (sc-639; final dilutions 1:400), β Tubulin Antibody (sc-36579, final dilution 1:400) and GAPDH (sc-365062; final dilution 1:400) were from Santa Cruz Biotechnology, Inc. (Dallas, Texas), phospho-PKD Ser⁹¹⁶ (#2051; final dilution 1:1000), phospho-PKD Ser^{744/748} (#2054, final dilution 1:1000), PKD1 (#2052; final dilution 1:1000 for Western blotting and 1:500 for immunofluorescence), phospho-HDAC5 Ser⁴⁹⁸ (#3424, final dilution 1:1000) and DYKDDDDK (FLAG) Tag (#8146, final dilution 1:500 for immunofluorescence) were all from Cell Signaling Technology (Danvers, MA). The inhibitors used in Table 1 were purchased either from R&D Systems (Minneapolis, MN) or EMD Millipore (Billerica, MA) or Sigma-Aldrich (St. Louis, MO). FLAG HDAC5 and FLAG HDAC7 were gifts from Reuben Shaw (Addgene plasmid # 32213 and), pCMV6M-PAK1 (Addgene plasmid # 12209), pCMV6M-PAK1 T423E (Addgene plasmid # 12208) and pEBG-PAK1 83-149 (Addgene plasmid # 12214) were all gifts from Jonathan Chernoff. Bombesin receptor (pcDNA3-GRPR) was purchased from cDNA Resource Center (Rolla, MO). All other reagents were of the highest grade available.

Conflict of interest - The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions - ER and JSS are responsible for conception and design of the research; JKC, YN, LH, JSS, RJ, OR and SHY performed the experiments; JKC, JSS, SHY, and ER analyzed the data; ER and JSS interpreted the results of the experiments; JSS and JKC prepared the figures; ER drafted the manuscript; JSS, JKC, RJ, OR and ER edited and revised the manuscript; ER approved the final version of the manuscript.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. PKD1 is rapidly phosphorylated on Ser²⁰³ in response to GPCR agonists in multiple cell model systems and in intestinal cells *in vivo*. **A**, Schematic representation of PKD1 domains and phosphorylation sites. The Ser²⁰³ is located between the cysteine-rich motifs (cys1 and cys2) which comprise the cysteine-rich domain (CRD) in the N-terminal region. The phosphorylation sites within the activation loop of the catalytic domain (Ser⁷⁴⁴ and Ser⁷⁴⁸) as well as the autophosphorylation site (Ser⁹¹⁶) in the C-terminal region of PKD1 are also indicated. **B**, Confluent cultures of IEC-18 cells were incubated without (-) or with 10 nM ANG II for the indicated times. **C**, Confluent cultures of Swiss 3T3 cells were incubated without (-) or with 10 nM bombesin for the indicated times. **D**, Cultures of COS-7 cells, transiently co-transfected with bombesin receptor and GFP-PKD1 either wild type (PKD1 WT) or with Ser²⁰³ mutated to Ala (PKD1S203A), were stimulated without (-) or with 10 nM bombesin (Bom) for 10 min. **E**, Cultures of HEK-293 cells were transiently transfected with either PKD1WT or with PKD1S203A and stimulated with 100 μ M carbachol (arb) for 10 min. **F**, Cultures of IEC-18 cells were transiently transfected with either PKD1WT or PKD1S203A and stimulated without (-) or with 10 nM ANG II for 10 min. In panels **B-F**, the cultures were lysed with 2 \times SDS-PAGE sample buffer and the extracts analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³). Immunoblotting for β -tubulin (**B**, **C**) or GFP (**D**, **E**, **F**) was also included. **G**, Epithelial cells from the ileum of PKD1 transgenic (Tg) mice and non-transgenic (NTg) littermates were isolated sequentially by timed incubations in a EDTA-PBS solution. The lysates of these cells were used to analyze for PKD1 phosphorylated at either Ser⁹¹⁶ (pSer⁹¹⁶) or Ser²⁰³ (pSer²⁰³). Equivalent loading was verified by immunoblotting for β -tubulin. Image editing in **D**: irrelevant lanes were removed (indicated by a thin, vertical dotted line) from the acquired digital images and flanking lanes were juxtaposed using Adobe Photoshop.

Figure 2. PKD1 phosphorylation at Ser²⁰³ can be dissociated from PKD or PKC family catalytic activity. **A**, **B**, Confluent cultures of IEC-18 cells were incubated in the absence (0) or in the presence of increasing concentrations of CRT0066101 (**A**) or kb NB 142-70 (**B**) for 1 h and then stimulated without (-) or with 10 nM ANG II for 10 min. Then, the cultures were lysed with 2 \times SDS-PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at either Ser²⁰³ (pSer²⁰³) or Ser⁹¹⁶ (pSer⁹¹⁶). Lysates were also analyzed for total PKD1 (PKD). **C**, Quantification of 3 independent experiments similar to those shown in **A** and **B** are included: open circles represent pSer²⁰³ while closed circles signify pSer⁹¹⁶. **D**, **E**, Confluent cultures of Swiss 3T3.PKD1 cells were incubated in the absence (0) or in the presence of increasing

concentrations of CRT0066101 (**D**) or kb NB 142-70 (**E**) for 1 h and then stimulated without (-) or with 10 nM bombesin for 10 min. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at either Ser²⁰³ (pSer²⁰³) or Ser⁹¹⁶ (pSer⁹¹⁶). Lysates were also analyzed for total PKD1 (PKD). **F**, Quantification of 3 independent experiments similar to those shown in D and E are included: open circles represent pSer²⁰³ while closed circles signify pSer⁹¹⁶. **G**, Swiss 3T3.PKD1618N cells were stimulated with bombesin (Bom) for the indicated times. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) and GAPDH. **H, J**, Confluent cultures of IEC-18 cells (**H**) or Swiss 3T3 cells (**J**) were incubated in the absence (0) or in the presence of increasing concentrations of Go6983 or 3.5 μM GFI (GF) for 1 h, as indicated, and then stimulated without (-) or with 10 nM ANG II (**H**) or 10 nM bombesin (**J**) for 10 min. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at either Ser²⁰³ (pSer²⁰³) or Ser⁷⁴⁴ (pSer⁷⁴⁴), as a marker of PKC activity. Lysates were also analyzed for total PKD1 (PKD).

Figure 3. The increase in PKD1 phosphorylation at Ser²⁰³ is prevented by ATP-competitive and allosteric inhibitors of the PAKs. A-D, Confluent cultures of IEC-18 cells were incubated in the absence (0) or in the presence of increasing concentrations of PF-3758309 (**A**) or FRAX597 (**C**) for 1 h and stimulated without (-) or with 10 nM ANGII for 15 min. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) or Ser⁹¹⁶ (pSer⁹¹⁶) and PAK1/2 at Ser^{144/141} (PAKpS^{144/141}). Lysates were also analyzed for total PKD1 (PKD). **B** and **D**, Quantification of 4 independent experiments similar to those shown in A and C are included. **E** and **F**, Confluent cultures of IEC-18 cells were incubated in the absence or in the presence of 5 μM PF-3758309 (**E**) or 5 μM FRAX 486 (**F**) for 1 h and then stimulated with 10 nM ANGII for various times, as indicated. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and the extracts analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) and PAK1/2 phosphorylated at Ser^{144/141} (PAKpS^{144/141}). **G**, Confluent cultures of Swiss 3T3.PKD1 cells were incubated in the absence (0) or in the presence of increasing concentrations of PF-3758309 for 1 h and stimulated without (-) or with 10 nM bombesin for 15 min. Then, the cultures were lysed and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) or Ser⁹¹⁶ (pSer⁹¹⁶) and PAK1/2 at Ser^{144/141} (PAKpS¹⁴¹). Lysates were also analyzed for total PKD1 (PKD). **H**, Quantification of 3 independent experiments similar to those shown in G are included. **I, J** Confluent cultures of Swiss 3T3.PKD1 cells were incubated in the absence or in the presence of 5 μM PF-3758309 (**I**) or 5 μM FRAX 486 (**J**) for 1 h and then stimulated with 10 nM bombesin for various times, as indicated. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) and PAK1/2 at Ser^{144/141} (PAKpS¹⁴¹). Lysates were also analyzed for total PKD1 (PKD). **L**, Confluent cultures of IEC-18 cells were incubated in the absence (0) or in the presence of increasing concentrations of IPA-3 or 30 μM PIRS for 1 h and then stimulated without (-) or with 10 nM ANGII for 15 min. Then, the cultures were lysed with 2×SDS–PAGE sample buffer and the extracts analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) and PKD1 phosphorylated at Ser⁹¹⁶ (pSer⁹¹⁶). Lysates were also analyzed for total PKD1 (PKD). **M**, Quantification of 4 independent experiments similar to those shown in L but including additional concentrations of IPA-3. **N**,

Confluent cultures of 3T3.PKD1 cells were incubated in the absence (0) or in the presence of increasing concentrations of IPA-3 or 20 μ M PIRS for 1 h and then stimulated without (-) or with 10 nM bombesin for 15 min. Then, the cultures were lysed with 2 \times SDS-PAGE sample buffer and analyzed by immunoblotting with antibodies that detect PKD1 phosphorylated at Ser²⁰³ (pSer²⁰³) or at Ser⁹¹⁶ (pSer⁹¹⁶). Lysates were also analyzed for total PKD1 (PKD). **O**, Quantification of 4 independent experiments similar to those shown in N but including additional concentrations of IPA-3.

Figure 4. Identification of PAK1 and PAK2 as the upstream protein kinases that phosphorylate PKD1 at Ser²⁰³. **A**, Cultures of IEC-18 cells were transfected with non-targeting siRNA (N. Targ) or with a mixture of siRNAs targeting PAK1 and PAK2 (siPAK1+2) for 5 days. Then, the cultures were stimulated with 10 nM ANGII for 10 min. **C**, Cultures of 3T3.PKD1 cells were transfected with non-targeting siRNA (N. Targ) or with a mixture of siRNAs targeting PAK1 and PAK2 (siPAK1+2) for 5 days. Then, the cultures were stimulated with 10 nM bombesin for 10 min. Following stimulation, the cultures in **A** and **C** were lysed with 2 \times SDS-PAGE sample buffer and the extracts analyzed by immunoblotting with antibodies that detect phosphorylated PKD1 at Ser²⁰³ (pSer²⁰³) or Ser⁹¹⁶ (pSer⁹¹⁶) and PAK1/2 at Ser^{144/141} (PAK1pS^{144/141} (**A**), PAKpS¹⁴¹ (**B**)). Lysates were also analyzed for total PKD1 (PKD). **B**, Quantification of 5 independent experiments similar to those shown in **A** are included. **D**, Quantification of 3 independent experiments similar to those shown in **B** are included. **E**, COS-7 cells were co-transfected with either GFP-PKD and bombesin receptor, GFP-PKD, bombesin receptor and pCMV6M-PAK1, GFP-PKD, bombesin receptor and pCMV6M-PAK1^{T423E} or GFP-PKD, bombesin receptor and GST tagged pEBG-PAK1 83-149 (AID). After 4 days, the cultures were incubated in the absence (-) or presence of 10 nM Bombesin (Bom) for 10 min. Lysates were analyzed for phosphorylated PKD1 at Ser²⁰³ (pSer²⁰³), PAK1, GST, GFP and GAPDH. **F**, Quantification of 6 independent experiments similar to those shown in **E**, all conditions also included GFP-PKD1 and bombesin receptor.

Figure 5. Inducible EGFP-PKD1 remains associated to the plasma membrane in the presence of inhibitors of the group I PAK or in cells transfected with siRNAs targeting PAK1 and PAK2. Confluent cultures of IEC-18 cells that express PKD1-EGFP in an inducible manner were treated with 1 μ g/ml doxycycline for 24 h to promote the expression of PKD1-EGFP. Then, the cells were treated in the absence (**A and B**) or presence of 3.5 μ M PF-3758309 (**C and D**), 5 μ M FRAX597 (**E and F**) or 30 μ M IPA-3 (**G and H**) for 1 h and subsequently stimulated with 10 nM ANG II for various times, as indicated. **I, J and K** Cultures of IEC-18 cells that express inducible PKD1-EGFP were transfected with non-targeting siRNA (N. Targ) or with a mixture of siRNAs targeting PAK1 and PAK2 (siPAK1+2) for 5 days. Then, the cultures were stimulated with 10 nM ANGII for the indicated times. All other cultures were fixed with 4% paraformaldehyde. Quantitative analysis of the relative change in plasma membrane and cytosol fluorescence intensity (expressed as ratio of membrane/cytosol) are shown in panels **B, D, F, H, J** and **K**. In panel **L**, cultures of IEC-18 cells that express inducible PKD1-EGFP were transfected with non-targeting siRNA (N. Targ) or with a mixture of siRNAs targeting PAK1 and PAK2 (siPAK1+2) for 5 days. Then, the cultures were stimulated without (-) or with 10 nM ANGII for 10 min. The extracts were analyzed by immunoblotting with antibodies that detect PAK1, PAK2 and GAPDH.

Figure 6. Endogenous PKD1 remains associated to the plasma membrane in the presence of inhibitors of the group I PAK or in cells transfected with siRNAs targeting PAK1 and PAK2.

Confluent cultures of IEC-18 cells were treated in the absence (A) or presence of 3.5 μ M PF-3758309 (B), 5 μ M FRAX597 (C) or 30 μ M IPA-3 (D) for 1 h and subsequently stimulated with 10 nM ANG II for various times, as indicated. E and F, Cultures of IEC-18 cells were transfected with non-targeting siRNA (N. Targ) or with a mixture of siRNAs targeting PAK1 and PAK2 (siPAK1+2) for 5 days. Then, the cultures were stimulated with 10 nM ANGII for the indicated times. The cultures were then washed and fixed with 4% paraformaldehyde and stained with an antibody that detects the PKD1 and Hoechst 33342 stain to visualize the nuclei. The bars represent the analysis of the relative change in plasma membrane fluorescence intensity (expressed as ratio of membrane/cytosol).

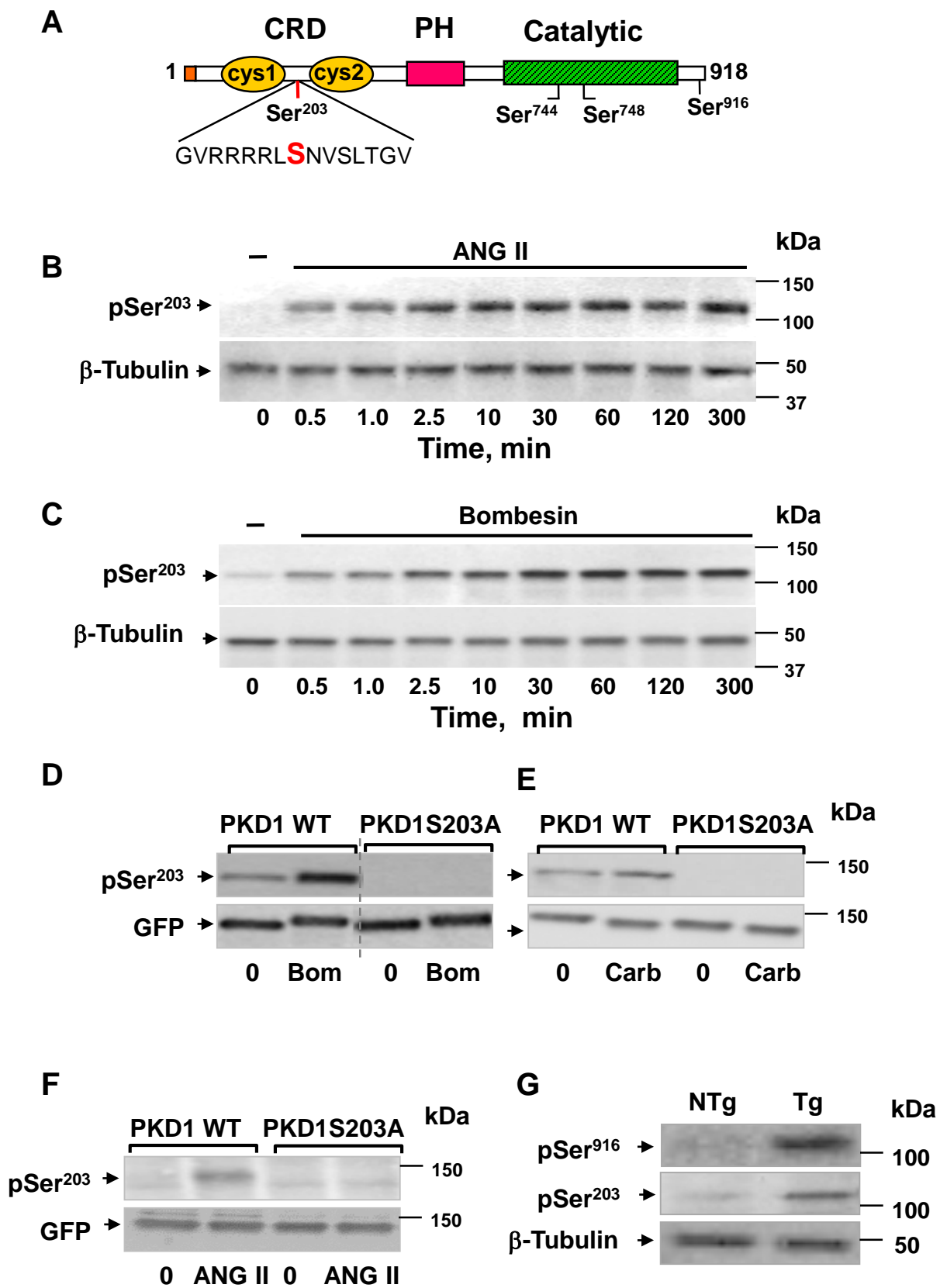
Figure 7. Identification of a novel PAK/PKD/HDAC signaling pathway.

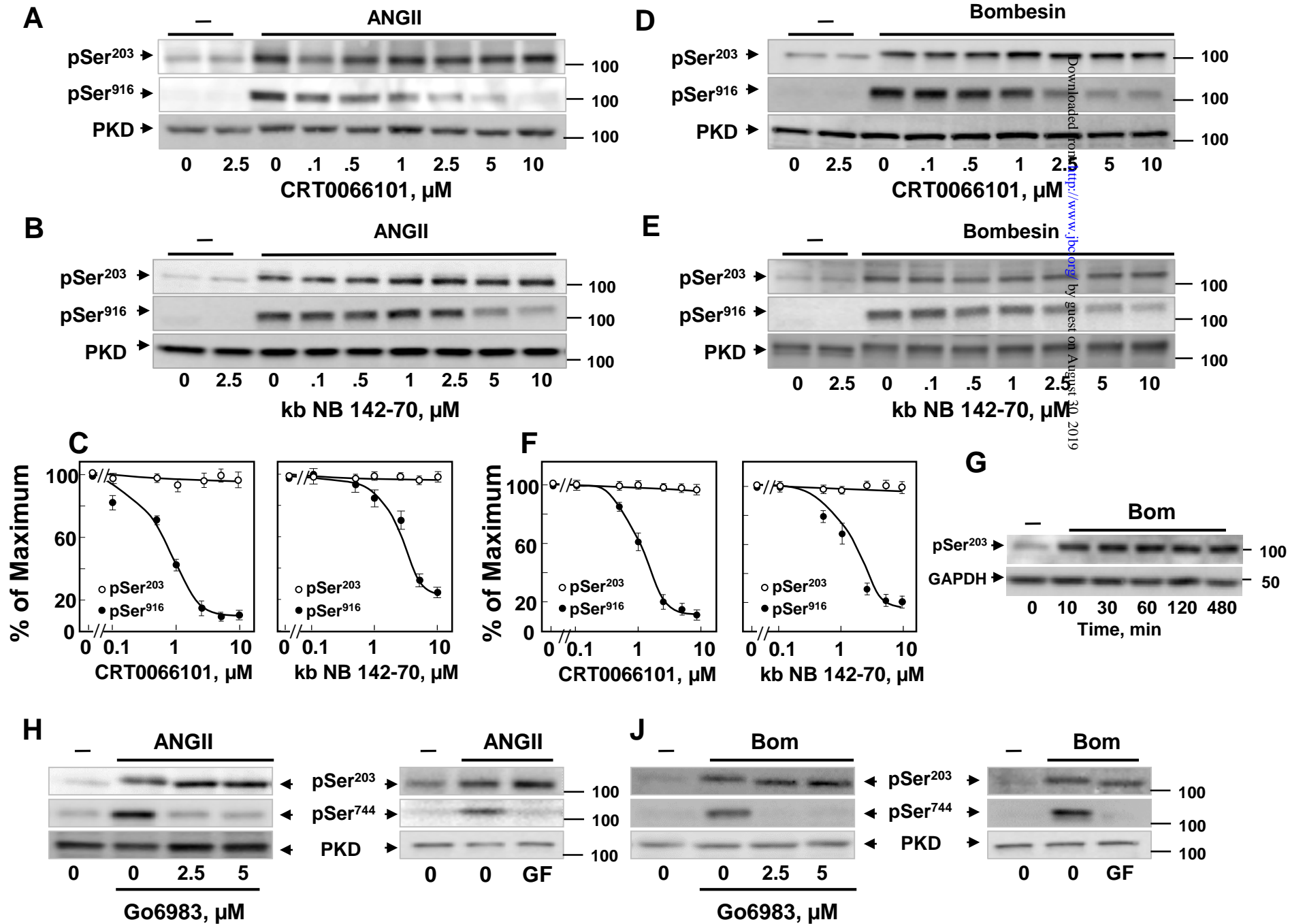
A, IEC-18 cells were transiently transfected with constructs encoding GFP-PKD1 or GFP-PKD1S203A. After 48 h, the cultures were incubated in the absence (-) or presence of 10 nM ANG II for various times, as indicated. B, Analysis of 70 cells at each time point. Results are expressed as the % of cells with GFP-PKD1 localized on the cell membrane. C, IEC-18 cells were transiently transfected with a plasmid encoding FLAG tagged-HDAC5. The cultures were incubated in the absence (-) or presence of 3.5 μ M CRT0066101, 3.5 μ M PF-3758309, 3.5 μ M FRAX597 or 30 μ M IPA-3 for 1 h prior to stimulation with 10 nM ANG II for 1 h. The cultures were then washed and fixed with 4% paraformaldehyde and stained with an antibody that detects the FLAG tag and Hoechst 33342 stain to visualize the nuclei. D, Analysis of 100 cells for each treatment. Results are expressed as the % of cells with FLAG-HDAC5 localized in the nucleus. In each case, the closed bars represent cultures stimulated with 10 nM ANG II. E, Confluent cultures of IEC-18 cells were incubated in the absence (0) or in the presence of 3.5 μ M CRT0066101 (CRT), 3.5 μ M PF-3758309 (PF), 3.5 μ M FRAX597 (FRAX) or 30 μ M IPA-3 (IPA) for 1 h and then stimulated without (-) or with 10 nM ANG II for 15 min. F and G, IEC-18 cells were incubated in the absence (0) or in the presence of increasing concentrations of PF-3758309 (F) or FRAX597 (G) for 1 h and then stimulated without (-) or with 10 nM ANG II for 15 min. H, Cultures of IEC-18 cells were transfected with non-targeting siRNA (N. Targ) or with a mixture of siRNAs targeting PAK1 and PAK2 (siPAK1+2) for 5 days. Then, the cultures were stimulated with 10 nM ANG II for 10 min. In E, F, G and H, all incubations were terminated by addition of 2 \times SDS-PAGE sample buffer and cell lysates resolved by SDS-PAGE. HDAC5 phosphorylation was determined by Western blot analysis using the antibody that detects its phosphorylated state on Ser⁴⁹⁸ and GAPDH as a loading control. J, GPCR signaling induces DAG accumulation at the plasma membrane, which mediates the translocation of inactive PKD1 from the cytosol to that cellular compartment. DAG also recruits and activates novel PKCs which mediate the transphosphorylation of PKD1 on Ser⁷⁴⁴ (in mouse PKD1). DAG and PKC-mediated transphosphorylation of PKD1 act synergistically to promote PKD1 catalytic activation and autophosphorylation on Ser⁷⁴⁸. PAK1 and PAK2 also activated in response to GPCR signaling phosphorylate PKD1 at Ser²⁰³ thereby facilitating its dissociation from the membrane to the cytosol (PKD_{cyt}) and to the nucleus (PKD_{nucleus}) where PKD1 phosphorylates class IIa HDAC, including HDAC5 and HDAC7. In this manner, the PAKs regulate class IIa HDAC phosphorylation and localization through phosphorylation of PKD1 on Ser²⁰³. Thus, our results identify a novel PAK/PKD/HDAC5 pathway in signal transduction. More details in the text.

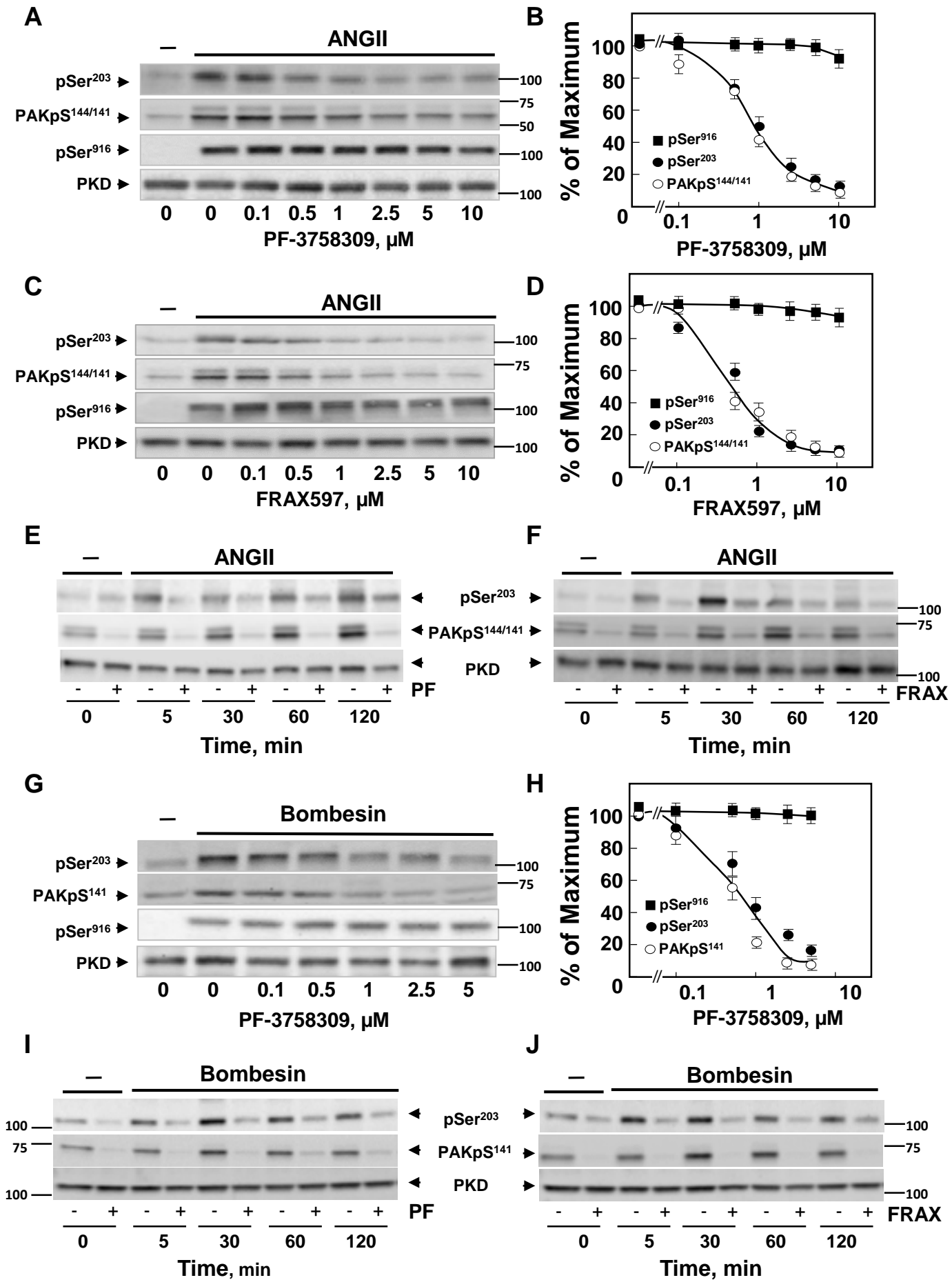
Table 1. Multiple protein kinase inhibitors did not prevent PKD1 phosphorylation at Ser203 in IEC-18 cells

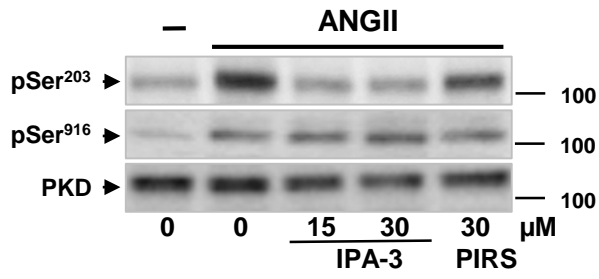
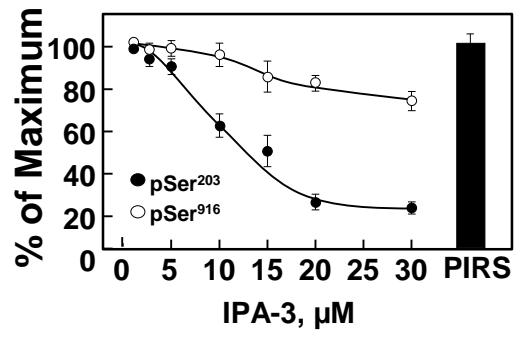
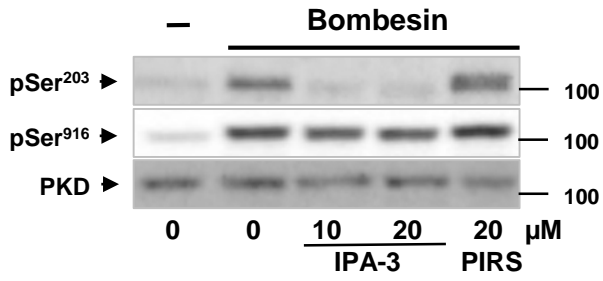
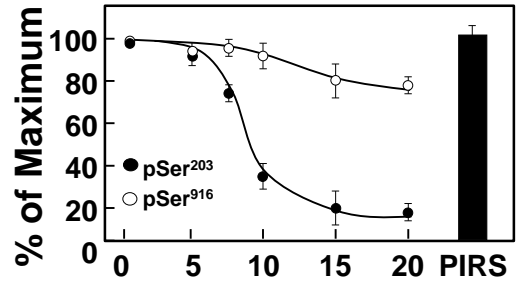
Inhibitor	Primary target	Concentration (μ M)
kb NB 142-70	PKD	1-10
CRT 0066101	PKD	1-5
Go6983	cPKC, nPKC, aPKC	1-10
GF 109203X	cPKC, nPKC	3.5
KT 5720	PKA	1
KU 0063794	mTOR	1-5
GSK 690693	Akt, PKC, PKA	1-5
KU 55933	ATM	5-25
HA 1077	ROCK-II, PKA, PKG, PKC α	10
Y-27632	ROCK	10
Compound C	AMPK	5-25
Aurora Kinase Inhibitor III	Aurora A	10
Aurora Kinase Inhibitor II	Aurora A and B	10
GSK-3 Inhibitor XVI	GSK-3	5
LY 294002	PI3K, mTOR	10
PP2	Src	10
AG1478	EGFR	1
U0126	MEK1/2	5
PD98059	MEK1/2	5
PIM-1 Inhibitor 2	Pim-1 kinase	1-10
TG003	Clk family	1-10
Enzastaurin (LY317615)	PKC β	1-5
GO6976	cPKC, PKD	10
KN92	CaMKII	10
KN62	CaMKII	10
FAK Inhibitor 14	FAK	1-5
UCN-01	Chk1	10

Confluent cultures of IEC-18 cells were incubated with each of the inhibitors listed above for 1 h. Then, the cells were stimulated with ANG II for 60 min, lysed and the phosphorylation of PKD1 at Ser²⁰³ was determined by immunoblotting using the antibody that detects the phosphorylated state of this residue. The inhibitors listed in this Table did not prevent Ser²⁰³ phosphorylation at the concentrations tested.







L**M****N****O****Fig. 3 Part B**

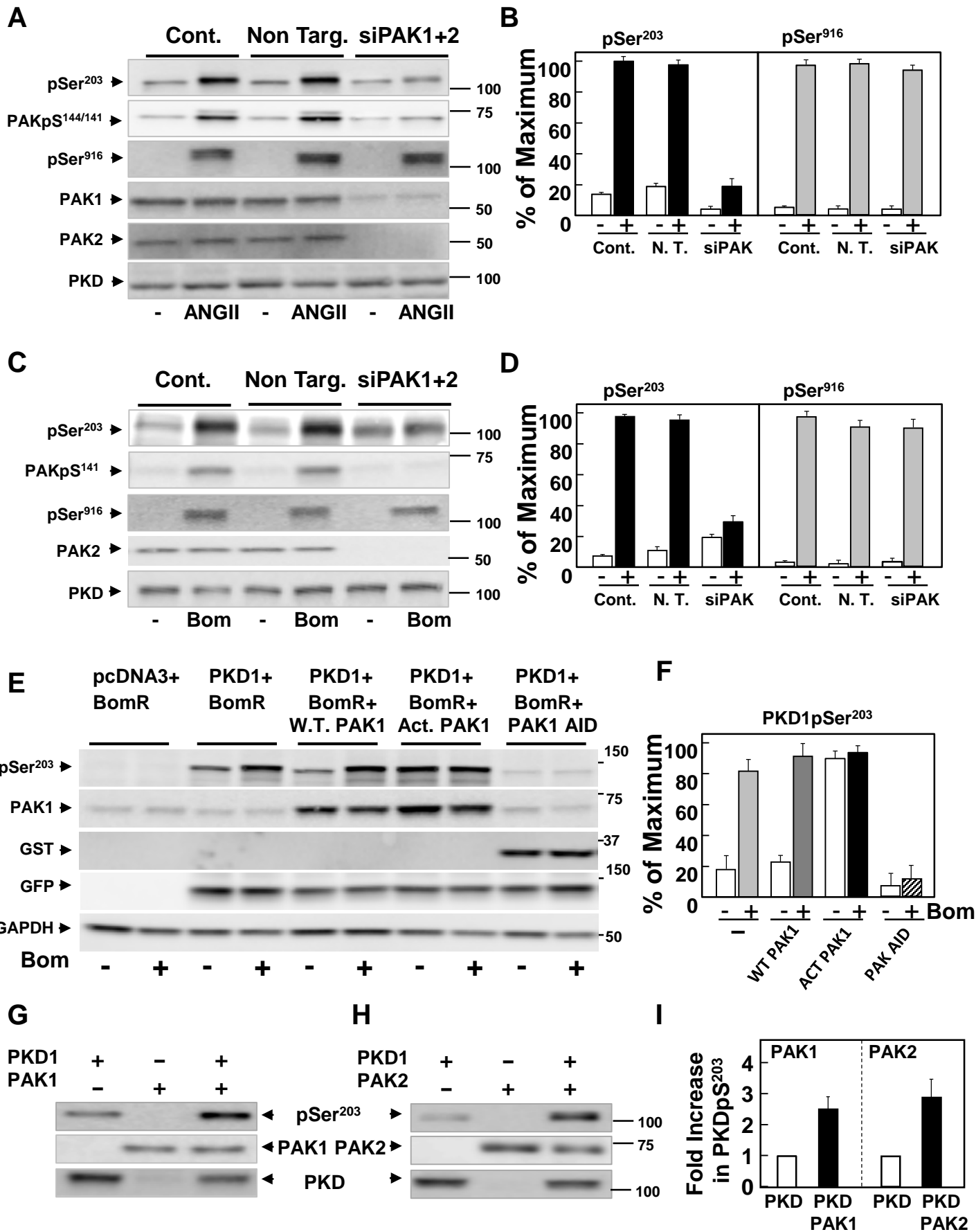


Fig. 4

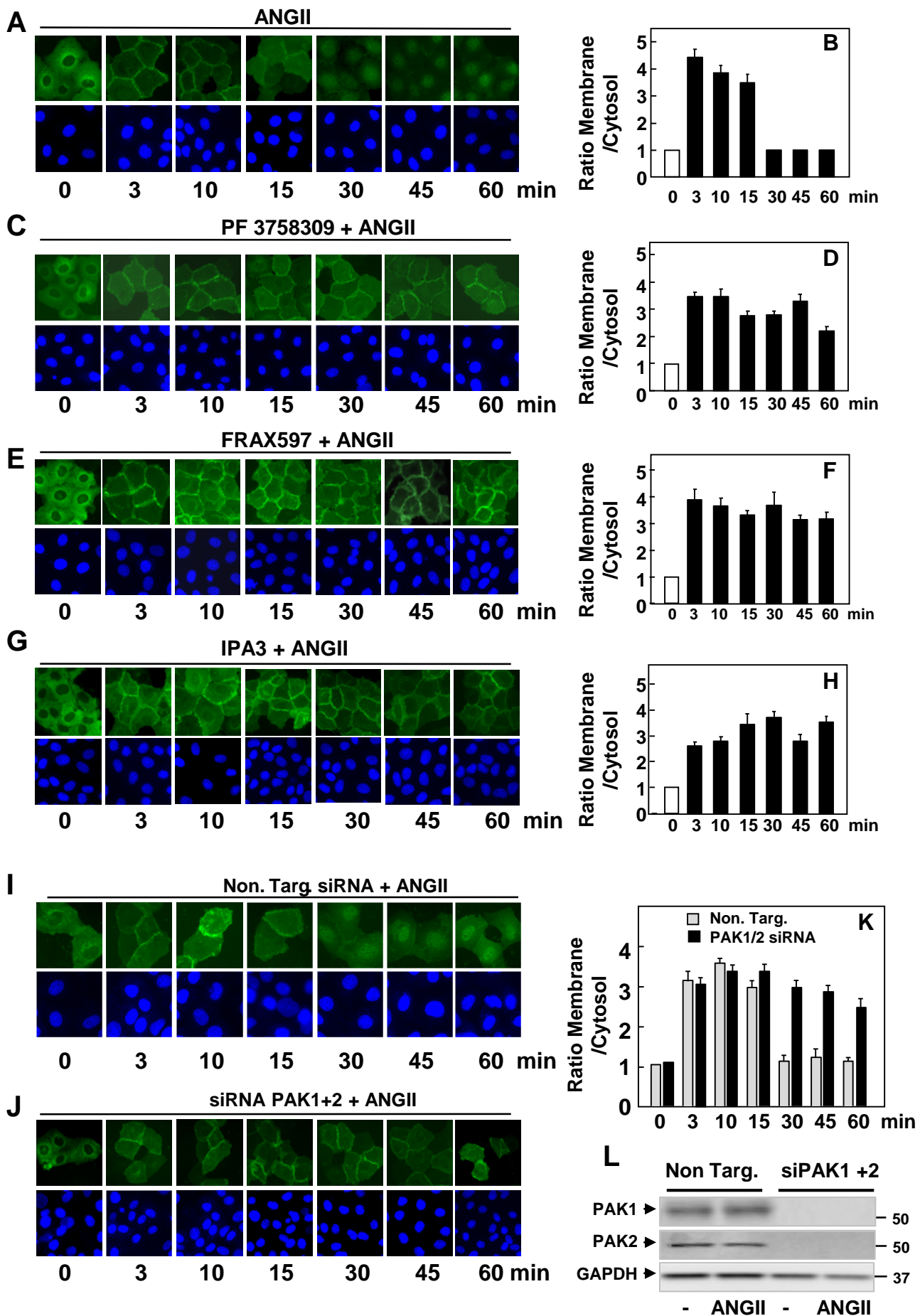
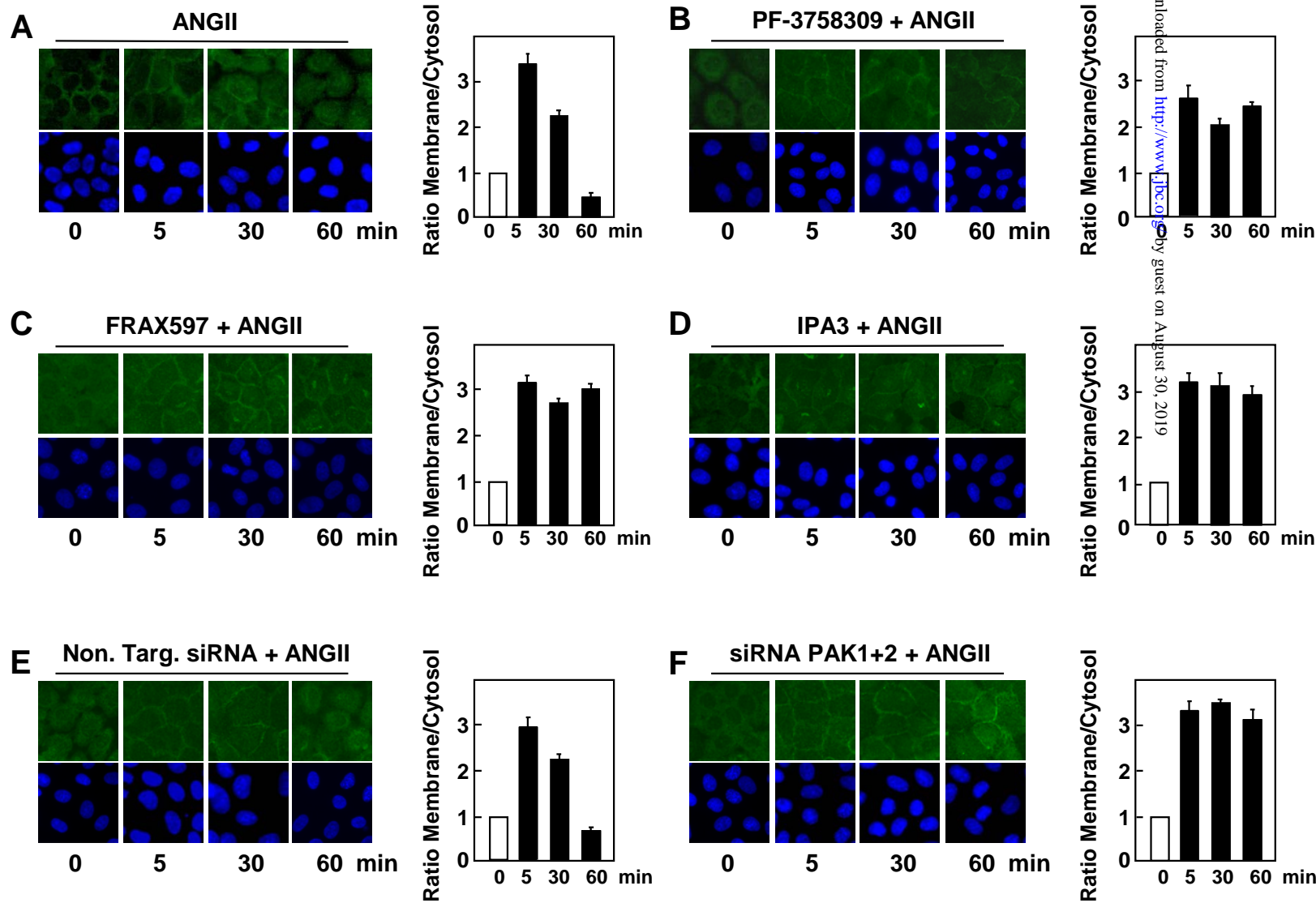


Fig. 5



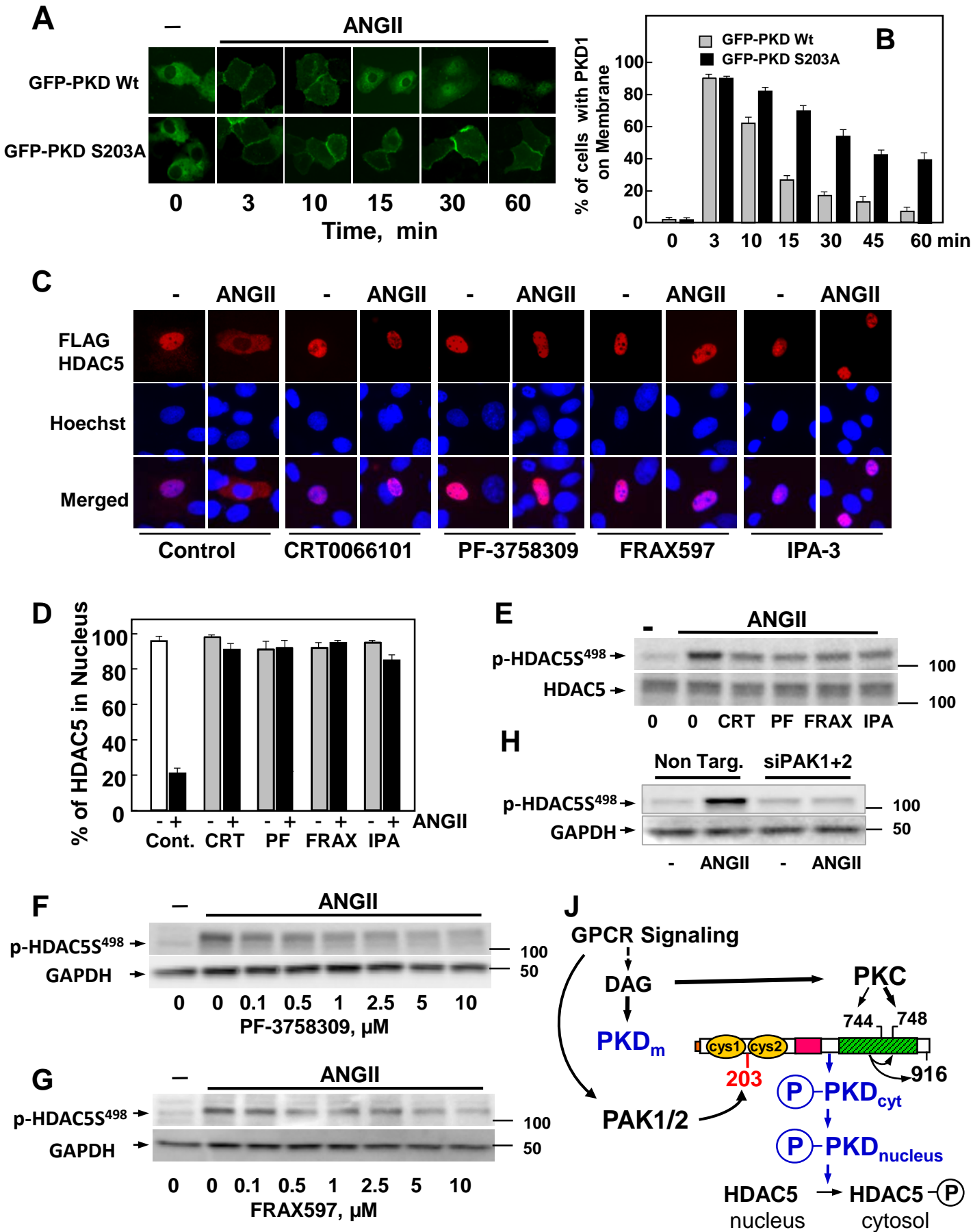


Fig. 7

Protein kinase D1 (PKD1) phosphorylation on Ser²⁰³ by type I p21-activated kinase (PAK) regulates PKD1 localization

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