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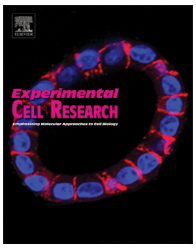

Research Article

Calpain-mediated proteolysis of polycystin-1 C-terminus induces JAK2 and ERK signal alterations

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD), a hereditary renal disease caused by mutations in *PKD1* (85%) or *PKD2* (15%), is characterized by the development of gradually enlarging multiple renal cysts and progressive renal failure. Polycystin-1 (PC1), *PKD1* gene product, is an integral membrane glycoprotein which regulates a number of different biological processes including cell proliferation, apoptosis, cell polarity, and tubulogenesis. PC1 is a target of various proteolytic cleavages and proteosomal degradations, but its role in intracellular signaling pathways remains poorly understood. Herein, we demonstrated that PC1 is a novel substrate for μ- and m-calpains, which are calcium-dependent cysteine proteases. Overexpression of PC1 altered both Janus-activated kinase 2 (JAK2) and extracellular signal-regulated kinase (ERK) signals, which were independently regulated by calpain-mediated PC1 degradation. They suggest that the PC1 function on JAK2 and ERK signaling pathways might be regulated by calpains in response to the changes in intracellular calcium concentration.

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; PC1, polycystin-1; PC2, polycystin-2; CT, C-terminal; JAK, Janus kinase; STAT, signal transducer and activator of transcription; TRP, transient receptor potential; HA, haemagglutinin; PKD, polycystic kidney disease; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary renal disease with a prevalence of one in every 400–1000 live births [1]. ADPKD is caused by mutations in either *PKD1* (16p13.3) or *PKD2* (4q21-23). *PKD1* encodes polycystin-1 (PC1), which is an integral membrane glycoprotein of 4302 amino acids [2]. PC1 has 11 transmembrane domains with a large extracellular segment. This segment has a novel combination of protein–protein interacting domains including a cluster of 15 PKD repeats that may mediate homophilic interactions [3]. *PKD2* encodes polycystin-2 (PC2), an integral membrane protein of 968 amino acids, which is a member of the TRP-like superfamily and supposed to act as a nonselective cation channel with a preference of calcium [4]. The short cytoplasmic C-terminus of PC1 has been shown to interact with PC2 [4], tuberin [5], STAT6 [6], and activates JAK2-STAT3 [7]. The PC1-PC2 complex can regulate a number of different biological processes including cell proliferation, apoptosis, cell polarity, and tubulogenesis [8,9].

The hallmark of ADPKD is the progressive enlargement of innumerable fluid-filled cysts derived from tubular epithelia in kidneys. Several lines of evidence suggest that the dysregulation of epithelial cell growth is a key step in this process. However, the pathogenetic mechanism of cyst formation and growth has not been well understood. It may involve disrupted intracellular calcium homeostasis, increased cAMP levels, and enhanced Ras/MAPK signaling [10].

Calpain is a member of the calcium-dependent cysteine protease family that modulates various cellular actions. There are at least 14 genes encoding the members of calpain superfamily. Among them, μ- and m-calpain, which require a micromolar and a millimolar concentration of calcium for activation respectively, have been mainly studied [11,12]. It has been proposed that the polypeptide sequence enriched in proline(P), glutamic acid(E), serine(S), and threonine(T), known as "PEST" domain, is a target for rapid degradation by calpain [13]. Calpain can recognize these sequences and degrade PEST domain-containing proteins.

PC-1 has been recognized as a target of proteolytic cleavage and proteosomal degradation. We previously demonstrated that Siah-1 interacts with PC1, which affects its stability via the ubiquitin-dependent proteasome pathway [14]. Further, the potential PEST domain in the C-terminus of PC1 has been suggested as a mediator motif of rapid degradation of PC1 [6,15]. However, the role and degradation mechanism of PEST domain in PC1 has not been elucidated in depth. In this study, we report for the first time that PEST domain in PC1 C-terminus is calpain-sensitive. The role of calpain-mediated degradation of PC1 on regulation of JAK2 and ERK signals is also addressed.

Materials and methods

Plasmids

The cytoplasmic tail of PC1 (PC1-CT), encoding 4147–4302 amino acid sequences of PC1, was generated by digestion of pcDNA 3.0/HA (Invitrogen, Carlsbad, CA) with *Eco*RI and *Xho*I as previously described [14]. PC1-CT and PC1-CT ΔPEST constructs were subcloned into pGEX-4T-1 vector (Promega, Madison, WI). PC1-CT ΔPEST was generated by deletion of PEST domain (4168–4183 aa) from PC1-CT. pEGFP-N3 (Clontech, Palo Alto, CA) expressing GFP protein was used as transfection and loading control. FLAG-PKD1 [16], which encodes full length PC1 tagging FLAG epitope at C-terminus, was kindly provided from Gregory G. Germino (NIDDK, NIH, Bethesda, MD).

In vitro degradation reaction

HEK293 cell lines were maintained in DMEM (Gibco, New York, NY) supplemented with 10% FBS and 1% antibiotic-antimycotic. Then, HEK293 cells were transfected using lipofectamine 2000 (Invitrogen) with PC1-CT, FLAG-PKD1 constructs. In order to activate calpains, the transfected cells were treated with $CaCl_2$ and A23187, a calcium ionophore, in serum-free conditions at 95% confluence. For the inhibition of calpains, calpeptin and calpain inhibitor IV (Calbiochem, La Jolla, CA) were co-treated with $CaCl_2$ and A23187. Chemical reagents were purchased from Sigma (St Louis, MO).

GST-pull down assay

GST-fusion proteins, pGEX plasmids containing GST-PC1-CT, GST-PC1-CT-ΔPEST, and GST alone, were overexpressed in *Escherichia coli* DH5α and were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following sonication for cell lysates, GST fusion proteins were batch-purified from extracts by binding to Glutathione Sepharose 4B beads (Sigma) according to the manufacturer's instruction.

FLAG tagged full length PC1 purification and reaction with purified calpains

FLAG-PKD1 was transfected into HEK293 cells, and then full length PC1 tagging FLAG epitope at C-terminus was immunoprecipitated by FLAG-M2 agarose conjugating FLAG antibody (Sigma). To elute full length PC1, it was incubated in 0.1 M glycine–HCl (pH 3.5) for 10 min.

Proteins and $CaCl_2$ (750 μM for μ-calpain or 5 mM for m-calpain) were incubated in a reaction mixture (30 mM Tris–HCl (pH 7.5) and 1.5 mM dithiothreitol) at 30 °C for 20 min. After incubation, reactions were terminated by adding SDS sample buffer.

Mouse embryo fibroblast (MEF) isolation

Primary MEF cells were isolated from either E11.5 $Pkd1^{-/-}$ or $Pkd1^{+/+}$ mouse embryos. Harvested embryos were dissected and digested with trypsin-EDTA for 20 min at 37 °C. Primary MEF cells were cultured in DMEM (Gibco) with 10% FBS in a humidified incubator with 5% CO_2 and 95% air, and the cells were used for Western blot analysis at passage 1–2.

Western blot analysis

Anti-ERK, phospho-ERK, JAK2, phospho-JAK2, GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG and β-actin antibodies from Sigma, and anti-μ-calpain antibody from Cell Signaling Technology (Beverly, MA). Proteins purified from transfected cells were separated by SDS-PAGE and transferred to nitrocellulose filters. The membrane-bound antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham Bioscience, Buckinghamshire, UK).

Results

The cytoplasmic tail of PC1 contains a calpain-sensitive PEST sequence

Since potential PEST domain at PC1 C-terminus has been reported without any functional study [15], we focused on the role of PEST domain at PC1. To confirm that PC1 contains a PEST domain, the web-based algorithm called PEST-FIND was used [17]. PEST-FIND produces a score ranging from −50 to +50. By definition, a score above zero denotes a possible PEST region, but a value greater than +5 sparks real interest. The annotated region in Fig. 1A was found to be 16 amino acids between positions 4168–4183 of PC1 showing +5.76 PEST-FIND score.

Rogers et al. [17] have suggested that PEST domain may be required for rapid degradation by calpains, which are calcium dependent proteases. To determine whether the PEST domain in PC1-CT is susceptible for calpain-mediated degradation, we induced calpain activation by treating PC1-CT transfected HEK293 cells with $CaCl_2$ and a calcium ionophore A23187. We assumed that μ-calpain would be activated at the lower concentration of intracellular calcium level (4 μM of A23187 for 2 h, Fig. 1B) whereas m-calpain will be only activated at the higher concentration of intracellular calcium level (10 μM of A23187 for 4 h, Fig. 1C). Then we treated cells with calpain inhibitors, calpeptin and calpain inhibitor IV. Calpeptin is a cell permeable calpain inhibitor which inhibits both μ- and m-calpain, whereas calpain inhibitor IV is a specific inhibitor only for m-calpain. As shown in Fig. 1B-lane 2, the degradation of PC1-CT was observed by treatment with $CaCl_2$ and A23187, and it was recovered by treatment with calpeptin but not by calpain inhibitor IV (Fig. 1B-lane 3 and lane 4, respectively). This finding tells us that degradation of PC1-CT at the condition indicated in Fig. 1B was induced mainly by μ-calpain. On the other hand, when we treated PC1-CT transfected cells with higher concentration of A23187 (10 μM) for longer time (4 h), PC1-CT band was recovered both by treatment with calpeptin and calpain inhibitor IV. Indeed, this indicates us that PC1-CT was degraded mainly by m-calpain at the condition indicated in Fig. 1C. As shown in Fig. 1D, we could not observe the degradation of PC1-CT without PEST domain even at the conditions that activate either μ- or m-calpain. Taken together, these results suggest that PC1-CT is degraded by both μ- and m-calpain and that the PEST domain is required for the calpain-mediated PC1-CT degradation.

PC1 is degraded by μ- and m-calpain through PEST domain *in vitro*

To determine whether the degradation of PC1-CT is caused by calpains *in vitro*, GST-PC1-CT was constructed (Fig. 2A) and reacted with μ- and m-calpain at the different calcium concentrations (Fig. 2B

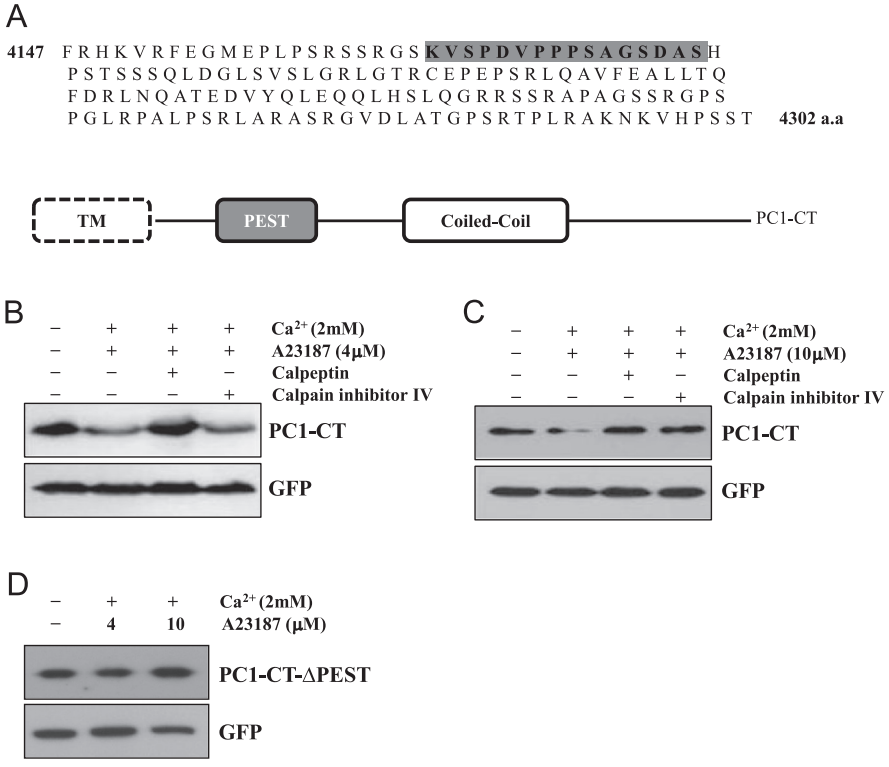

Fig. 1 – The cytoplasmic tail of PC1 (PC1-CT) was degraded by calpains. (A) Location of PEST domain within PC1-CT. A potential PEST domain is depicted as a shaded box. (B and C) Western blot analyses of the degradation of PC1-CT by calpains. HEK293 cells were transfected with PC1-CT plasmid and GFP empty vector as a control. After 48 h, cells were treated with indicated concentrations of Ca^{2+} and A23187, a calcium ionophore, for 2 h (B) and 4 h (C). B and C show the degradation of PC1-CT. The degradation of PC1-CT was inhibited by treatment of calpeptin, an inhibitor of μ- and m-calpain, and calpain inhibitor IV, a specific inhibitor of m-calpain (C). (D) PEST domain-mediated degradation by calpains. Degradation of PC1-CT by calpains required PEST domain. GFP was used as the transfection and loading control.

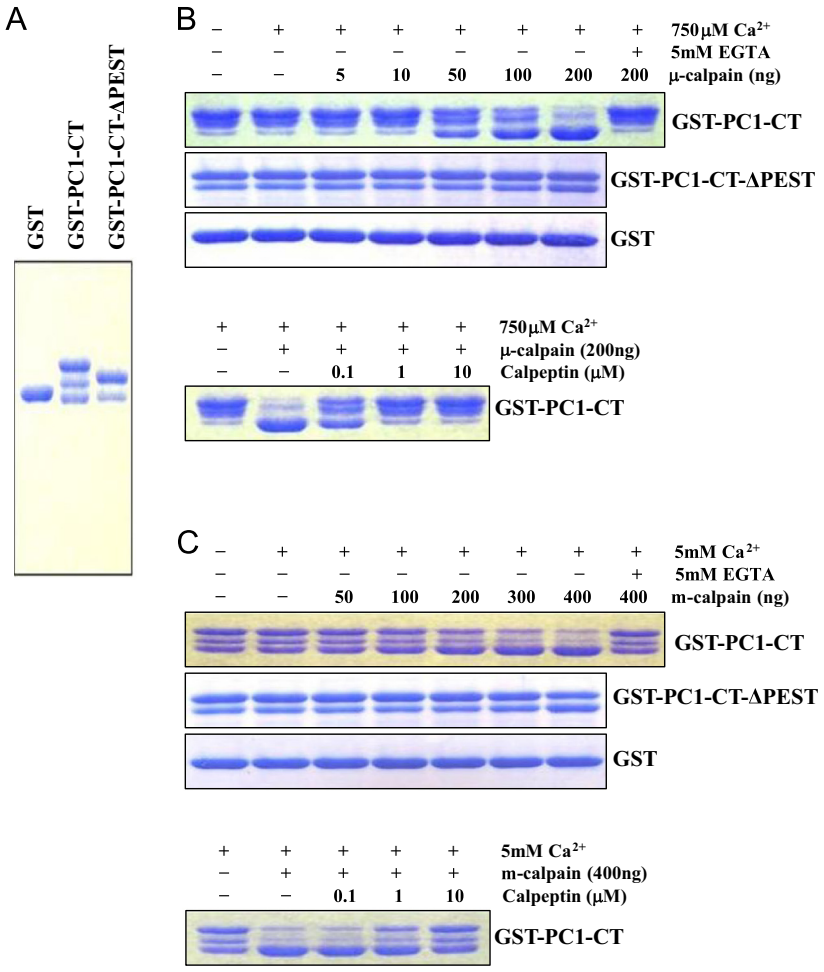

Fig. 2 – *In vitro* degradation of GST-PC1-CT by μ-calpain and m-calpain. (A) Coomassie Blue staining of purified GST fusion proteins, GST alone, GST-PC1-CT, and GST-PC1-CT-ΔPEST. (B) Degradation of GST-PC1-CT by μ-calpain. Degradation of GST-PC1-CT was inhibited by treatment of 5 mM EGTA, calcium chelator (*upper panel*). Degradation of GST-PC1-CT was blocked by calpeptin treatment (*lower panel*). (C) Degradation of GST-PC1-CT by m-calpain. Degradation of GST-PC1-CT was inhibited by treatment of 5 mM EGTA, calcium chelator (*upper panel*). Degradation of GST-PC1-CT was blocked by calpeptin treatment. The lowest band of GST-PC1-CT (B) and (C) represents GST protein, and this band became larger corresponding to degradation of PC1-CT because calpain can only degrade PC1-CT of GST-PC1-CT followed by accumulation of GST band. GST was used as a negative control (*lower panel*). These data were analyzed by SDS-PAGE and Coomassie blue staining.

and C). As shown in Fig. 2B (*upper panel*), GST-PC1-CT was degraded in a dose-dependent manner by μ-calpain. Furthermore, the degradation of GST-PC1-CT was calcium-dependent, because the addition of the calcium chelator EGTA to the reaction mixture completely inhibited the degradation of GST-PC1-CT even at the highest calpain concentration. Calpeptin also inhibited the degradation of GST-PC1-CT in a dose-dependent manner (Fig. 2B, *lower panel*). Similar to μ-calpain, m-calpain also degraded GST-PC1-CT in a dose- and calcium-dependent manner (Fig. 2C). These data indicate that PC1-CT is degraded by both μ- and m-calpain *in vitro*.

To further examine whether the PEST domain determines the degradation of PC1-CT by calpains *in vitro*, GST-PC1-CT-ΔPEST was constructed (Fig. 2A). The purified GST protein was used as a negative control. GST-PC1-CT-ΔPEST was incubated with graded concentrations of μ- and m-calpain. As shown in Fig. 2B and C, GST-PC1-CT-ΔPEST was not degraded in contrast to GST-PC1-CT. It indicates that the PEST domain is required for the degradation of PC1-CT.

To confirm that full-length PC1 is also degraded by calpains, we purified full-length PC1 and subsequently treated it with μ- or m-calpain. Full-length PC1 (~460 kDa) was immunoprecipitated with FLAG antibody conjugating agarose (FLAG-M2 agarose) and then detected by FLAG antibody (Fig. 3A). Purified full-length PC1 was reacted with μ- or m-calpain for 20 min, and then analyzed by Western blot. Similar to GST-PC1-CT, the full-length PC1 was also degraded by both μ- and m-calpain (Fig. 3B and C).

Calpain-mediated degradation of PC1 independently affects JAK2 and ERK signaling pathways

Since PC1 physically interacts with JAK2 and transmits its growth inhibitory signals to the nuclei via direct activation of the JAK-STAT pathway [18], we postulated that calpain-mediated proteolysis of PC1-CT may reduce JAK2 phosphorylation. In addition, since PC1 regulates ERK phosphorylation to control cell size [19],

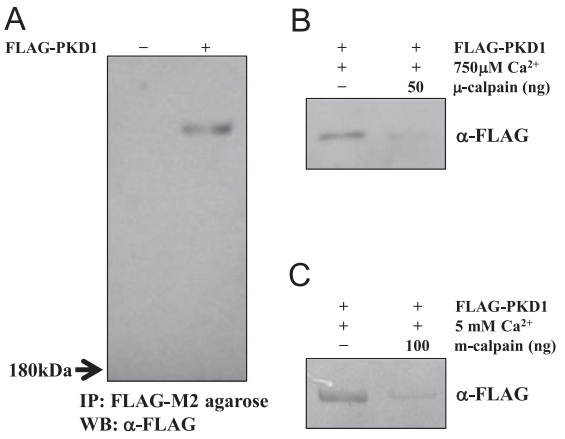

Fig. 3 – *In vitro* degradation of full-length PC1 by calpains. (A) Purification of FLAG-tagged full-length PC1 using FLAG-M2 agarose bead. Purified full-length PC1 was detected by Western blot analysis using anti-FLAG antibody. The CTF, which is generated by the cleavage at GPS, could not be seen in this experiment since the band representing CTF (150 kDa) was already run out from the 4% gel. (B) Full-length PC1 was degraded by μ-calpain (50 ng). (C) Full-length PC1 was also degraded by m-calpain (100 ng).

we analyzed the change of phosphorylation of JAK2 and ERK by calpain-mediated PC1 degradation. Firstly, recombinant PC1 was overexpressed in HEK293 cells, and phosphorylation of JAK2 and ERK was detected by Western blot. As expected, JAK2 phosphorylation was increased, while ERK phosphorylation was decreased in PC1 overexpressed cells (Fig. 4A). The regulation of JAK2 and ERK by PC1 was further confirmed using MEF cells isolated from *Pkd1*$^{-/-}$ mice, and we observed down-regulation of phosphorylated JAK2 (pJAK2) and up-regulation of phosphorylated ERK (pERK) in *Pkd1*$^{-/-}$ MEF cells (Fig. 4C). Interestingly, the calpain-mediated degradation of PC1 reversed the regulatory effects of PC1 on JAK2 and ERK (Fig. 4B and C). To activate endogenous μ-calpain, we added A23187 to FLAG-PKD1 plasmid transfected HEK293 cells and detected autolysed μ-calpain, which indicates μ-calpain activation since autoproteolysis of μ-calpain occurs at the amino-terminal region and generates cleaved activated μ-calpain in the presence of calcium. As shown in Fig. 4B, pERK was increased whereas pJAK2 was decreased at the condition degrading both full-length PC1 (450 kDa) and the C-terminal fragment (CTF, 150 kDa), which is generated by the cleavage at G-protein coupled proteolytic site (GPS) [20]. Consistent results were observed with *Pkd1*$^{+/+}$ MEF cells, and those effects were recovered by treatment with calpeptin suggesting that calpains play crucial roles in regulation of JAK2 and ERK by PC1 (Fig. 4C). Since JAK2 was suggested to negatively regulate ERK [21], we examined whether JAK2 and ERK signal changes were correlated each other in the presence of PC1. To address this question, we induced calpain-mediated degradation of PC1 using A23187 and then treated JAK2 inhibitor II (Fig. 4D). Though activated μ-calpain degraded PC1 (data not shown), JAK2 inhibition had no effect on ERK phosphorylation (Fig. 4D). The data demonstrate that JAK-STAT and ERK pathways may be independently controlled by PC1 in response to the changes in intracellular calcium level.

Discussion

PC1 is a target for a series of proteolytic cleavage events that release several fragments with different size. Cleaved fragments can enter the nucleus where they bind various transcription factors to affect downstream signaling pathways [9]. On the other hand, we previously demonstrated that PC1 is also a target for proteosomal degradation mediated by Siah-1, which interacts with C-terminal domain of PC1 [14]. In addition, a rapid degradation of PC1 has been speculated through the presence of potential PEST domain contained in the PC1 C-terminus [6,15]. However, the exact mechanism and role of PEST domain on PC1 degradation have not been studied in depth.

In this study, we showed a ubiquitin-independent degradation of PC1. We found that PC1 is a novel substrate for μ- and m-calpains, which are nonlysosomal, calcium-activated cysteine proteases. It was clearly shown that activated calpains directly recognize PEST domain at cytoplasmic tail of PC1. Calcium is not only an important mediator to activate calpain but also a universal secondary messenger in many signal transduction pathways [22]. Indeed, disturbance in calcium homeostasis caused by the mutations either in *PKD1* or *PKD2* induces dysregulation of various signaling pathways and abnormal cell functions in PKD [22–24]. Therefore, we speculated that calpain-mediated PC1 degradation may be implicated in the control of signaling pathways rather than the removal of useless proteins.

PC1 is constitutively bound to JAK2, but JAK2 becomes activated only when PC1 is co-expressed with PC2 [18]. Overexpression of PC1 transmits its growth inhibitory signals from the cell surface to the nucleus via direct activation of the JAK-STAT pathway. This, in turn, increased expression of $p21^{waf1}$, inducing cell cycle arrest in G0/G1. We postulated that calpain-mediated proteolysis of PC1 would affect the coiled-coil domain, which may result in failure to interact with both JAK2 and PC2 and finally inhibit JAK2 phosphorylation. When PKD1 cDNA was transfected in HEK293 cells, PC1 overexpression induced JAK2 phosphorylation whereas treatment of $CaCl_2$ and A23187 in PC1 overexpressing HEK293 cells activated μ-calpain and reduced pJAK2. Similar effects were observed with the experiments using *Pkd1*$^{+/+}$ and *Pkd1*$^{-/-}$ MEF cells, and either degradation of PC1 by calpains or loss of PC1(*Pkd1*$^{-/-}$ MEF cells) decreased pJAK2. This observation supports the previous finding that C-terminus of PC1 could interact with JAK2. PC1 down-regulates the mTOR pathway through direct regulation of the ERK-specific phosphorylation sites on tuberin (serine 664) to reduce cell sizes [19]. A recent report has also demonstrated that the inactivation of the *PKD1* gene in the kidney of *PKD1* conditional knockout mice results in massive renal cystogenesis accompanied by increased activation of the ERK pathway [25]. Our results also revealed that overexpression of PC1 inhibited ERK phosphorylation independent of JAK2 signaling because JAK2 inhibition had no effect on ERK phosphorylation.

In conclusion, this study demonstrated that PEST domain of PC1 is the target of a ubiquitin-independent degradation by calpains. The function of PC1 on JAK2 and ERK signaling pathways might be independently regulated by calpains in response to changes in intracellular calcium level.

Declaration of interest

Authors have nothing to declare.

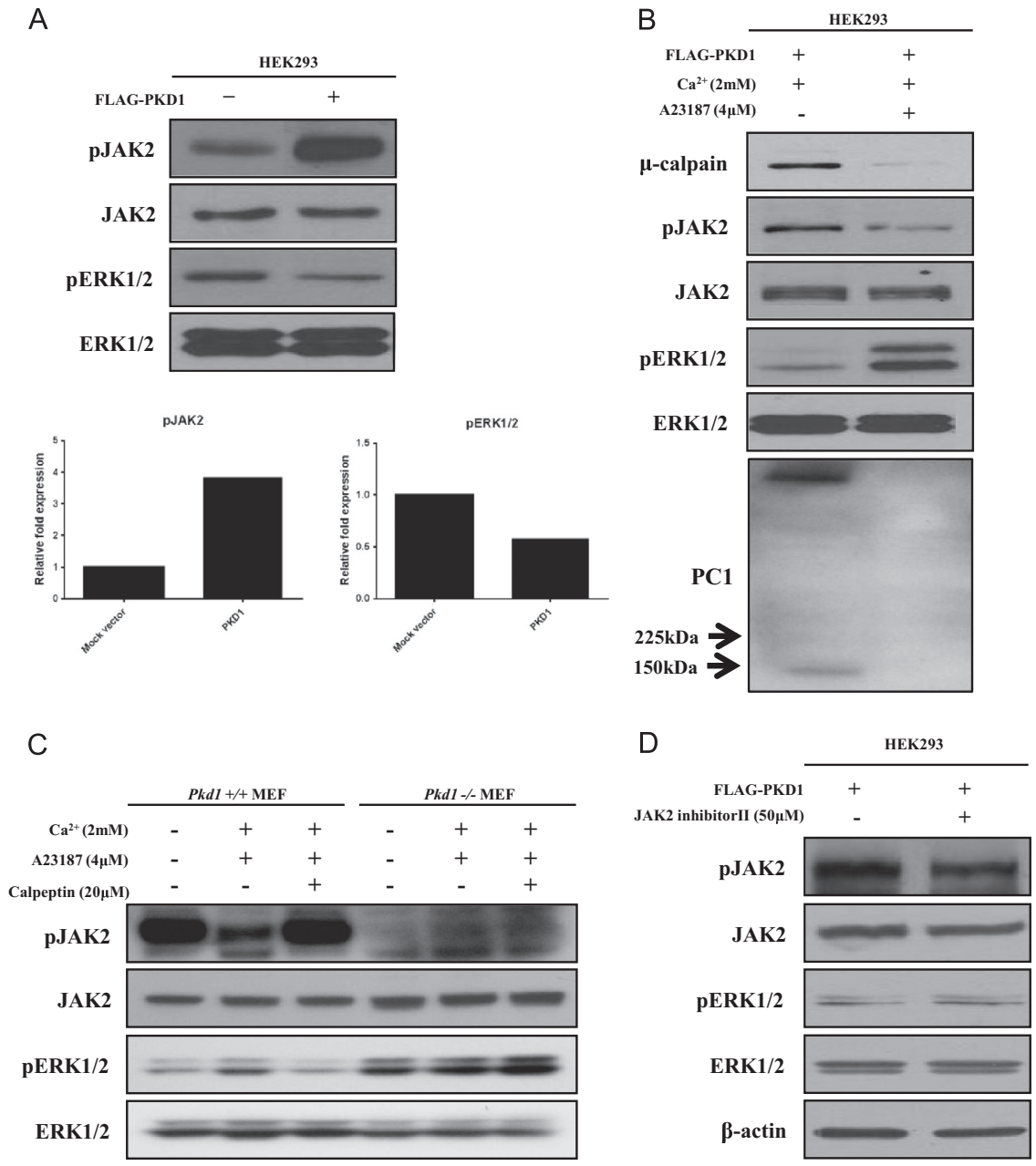

Fig. 4 – The effect of calpain-dependent degradation of PC1 on JAK2 and ERK phosphorylation. (A) JAK2 and ERK regulation by PC1. Total cell lysates of HEK293 cells transfected with FLAG-PKD1 plasmid were resolved in SDS-PAGE and Western blotted to detect pJAK2 and pERK. PC1 overexpression induced JAK2 phosphorylation but reduced ERK phosphorylation. (B) The effects of calpain-mediated degradation of PC1 on JAK2 and ERK signals. HEK293 cells transfected with FLAG-PKD1 plasmid were treated with A23187 and $CaCl_2$ to activate μ-calpain. When μ-calpain was activated, JAK2 phosphorylation was decreased, whereas ERK phosphorylation was increased. (C) JAK2 and ERK regulation in *Pkd1*$^{+/+}$ and *Pkd1*$^{-/-}$ MEF cells. *Pkd1*$^{-/-}$ MEF cells showed down-regulated pJAK2 and up-regulated pERK. Calpain activation in *Pkd1*$^{+/+}$ MEF cells induced a decrease in pJAK2 and an increase in pERK. (D) Correlation between JAK2 and ERK signals in PC1 overexpressed cells. To examine the correlation between JAK2 and ERK signaling, 50 μM of JAK2 inhibitor II was treated for 24 h after PC1 overexpression. JAK2 phosphorylation was reduced but JAK2 inhibition did not affect ERK phosphorylation.

Acknowledgments

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