Calpain mediated proteolysis of polycystin biology essay

Science, Biology

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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 their role in intracellular signaling pathways remain 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. Keywords: polycystic kidney disease; polycystin-1; calpain; PEST; calcium homeostasis

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary renal disease with a prevalence of one in every 400-1000 live births . 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 . 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 . 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 . The short cytoplasmic C-terminus of PC1 has been shown to interact with PC2 , tuberin , STAT6 , and activates JAK2-STAT3 . The PC1-PC2 complex can regulate a number of different biological processes including cell proliferation, apoptosis, cell polarity, and tubulogenesis . 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 . 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 . 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 . 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 . Further, the potential PEST domain in the C-terminus of PC1 has been suggested as a mediator motif of rapid degradation of PC1 . 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 PC1C-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 EcoRI and XhoI as previously described . 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 , 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, Carlsbad, CA) with PC1-CT, FLAG-PKD1 constructs. In order to activate calpains, the

transfected cells were treated with CaCl2 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 CaCl2 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. 2mM isopropyl-🚲-D-thiogalactopyranoside (IPTG). Following sonication for cell lysates, GST fusion proteins were batch-purified from extracts by binding to Glutathione Sepharose 4B beads (Sigma, St Louis, MO) 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, St Louis, MO). To elute full length PC1, it was incubated in 0. 1 M glycine-HCl (pH 3. 5) for 10 minutes. Proteins and CaCl2 (750 ❣M for ❣-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 minutes. After incubation, reactions were terminated by adding SDS sample buffer.

Western blot analysis

Anti-ERK, phospho-ERK, JAK2, phospho-JAK2, GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG and -actin antibodies from Sigma (St Louis, MO), and anti--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 , 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 . 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 Figure 1A was found to be 16 amino acids between positions 4168-4183 of PC1 showing +5. 76 PEST-FIND score. To identify whether PC1 is regulated by calpains, we transfected PC1-CT into HEK293 cells and then treated with CaCl2 and a calcium ionophore A23187 at indicated concentrations to activate different subtype of calpains, - and m-calpain (4μM and 10 μM of A23187, respectively). Western blot analysis showed that PC1-CT was degraded by

activated calpains after treatment with Ca2+ and A23187 (Fig. 1B-lane 2 and 1C-lane 2). To identify which subtypes of activated calpains degraded PC1-CT, the transfected cells were treated with two different kinds of calpain inhibitors: calpeptin, which inhibits both ı̣- and m-calpain and calpain inhibitor IV, which inhibits only m-calpain. As shown in lane 3 and 4 of Fig. 1B, the degradation of PC1-CT was inhibited by calpeptin but not by calpain inhibitor IV. This revealed that the degradation of PC1-CT in this condition was induced by ı̣-calpain. However, in lane 3 and 4 of Fig. 1C, the degradation of PC1-CT was inhibited by both calpeptin and calpain inhibitor IV. It demonstrated that this degradation was induced by m-calpain. Taken together, these results suggest that PC1-CT is degraded by both ı̣- and m-calpain.

PC1 is degraded by ı̣- 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 reacted with ı̣- and m-calpain at the different calcium concentrations (Fig. 2A). As shown in Fig. 2B (upper panel), GST-PC1-CT was degraded in a dose-dependent manner by ı̣-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 ı̣-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 ı̣- and m-calpain in vitro. To

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 2C, 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 further 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 minutes, 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 3C).

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, we postulated that calpain-mediated proteolysis of PC1 C-terminus may promote JAK2 phosphorylation. In addition, since PC1 regulates ERK phosphorylation to control cell size , we analyzed the change of phosphorylation of JAK2 and ERK by calpain-mediated PC1 degradation. 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). Since JAK2 was suggested to negatively regulate ERK , 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. 4B and 4C). To activate endogenous ɨ-calpain, we added A23187 to FLAG-PKD1 plasmid transfected cells and detected autolysed ɨ-calpain, which indicates ɨ-calpain activation since autoproteolysis of ɨ-calpain occurs at the amino-terminal region and generates cleaved activated ɨ-calpain in the presence of calcium. Though activated ɨ-calpain degraded PC1 (data not shown), JAK2 inhibition had no effect on ERK phosphorylation (Fig. 7). 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 . 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 . In addition, a rapid degradation of PC1 has been speculated through the presence of potential PEST domain contained in the PC1 C-terminus . 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 ɨ- and m-calpains. It was clearly shown that activated calpains directly recognize PEST domain at cytoplasmic tail of PC1.

Calpains are nonlysosomal, calcium-activated cysteine proteases. Of the several members of calpain superfamily, µ-calpain and m-calpain are the two most well studied calpains because of their ubiquitous expression . Calcium is not only an important mediator to activate calpain but also a universal secondary messenger in many signal transduction pathways . 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 . 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 . 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 p21waf1, 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 CaCl2 and A23187 in PC1 overexpressing HEK293 cells activated ı̣-calpain and reduced phosphorylated JAK2. 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 . 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 . Our results also revealed that overexpression of PC1 inhibited ERK phosphorylation independent of JAK2 signaling because JAK2 inhibition had no effect on ERK phosphorylation. Taken together, 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.

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Declaration of interest

Authors have nothing to declare.