University of Alberta

Function and Regulation of PKD2 and PKD2L1

by

Jungwoo Yang

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited genetic disorder, inducing cysts in kidney, liver and pancreas. It is caused by pathogenic mutations in the *PKD1* or *PKD2* gene encoding PKD1 or PKD2 respectively. Cellular abnormalities in ADPKD include cell over-proliferation and apoptosis. PKD2 is a Ca^{2+}-permeable non-selective cation channel and functions as an anti-apoptotic and anti-cell proliferation protein. Endoplasmic reticulum (ER) stress induces the phosphorylation of eukaryotic initiation factor 2α (eIF2α) by pancreatic ER-resident eIF2α kinase (PERK), which inhibits global protein synthesis and up-regulates the translation of selected target proteins.

In Chapter 2, using cultured mammalian cell lines, we found that PKD2 represses cell proliferation through promoting the eIF2α phosphorylation by PERK. PKD2 knock-down repressed ER stress-induced eIF2α phosphorylation, indicating that PKD2 facilitates the eIF2α phosphorylation by PERK. We also found that PKD2 interacts with PERK and eIF2α. Together, we demonstrate that PKD2 down-regulates cell proliferation by promoting PERK-mediated eIF2α phosphorylation, presumably through a physical interaction.

In Chapter 3, using cultured mammalian cell lines, we showed that PKD2 protein expression is up-regulated by cellular stresses which all increase P-eIF2α without increasing PKD2 mRNA. Increasing P-eIF2α by salubrinal or by over-expression of eIF2α up-regulates the PKD2 level. Over-expression of Gadd34 or eIF2α knock-down suppressed ER stress-induced PKD2 up-regulation. We also

found that ER stress increases binding of PKD2 mRNA molecules with ribosomes, and the regulation of PKD2 by P-eIF2α is mediated by the upstream open reading frame (uORF) in the PKD2 5'-untranslated region. Therefore, this study demonstrates that P-eIF2α translationally up-regulates PKD2 protein expression through the uORF of PKD2.

PKD2L1 is a Ca^{2+}-modulated non-selective cation channel. In Chapter 4, we found, using *Xenopus* oocyte, that the receptor for activated C kinase 1 (RACK1) binds with the PKD2L1 N-terminus and inhibits its channel activity. This was supported by our finding that over-expressed N-terminal fragment Met^{1}–Pro^{45} acts as a blocking peptide and abolishes the inhibitory effect of RACK1. Therefore, we demonstrate that RACK1 binds with Met^{1}–Pro^{45} of PKD2L1 to inhibit its channel activity.

Therefore, our studies constitute valuable contributions to understanding the function and regulation of PKD2 and PKD2L1.

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LIST OF ABBREVIATIONS

aa	**amino acid**
ADPKD	**autosomal dominant polycystic kidney disease**
ATF	**activating transcription factor**
ATF4/6	**activating transcription factor 4/6**
ATP	**adenosine triphosphate**
bZIP	**basic Leu-Zipper**
CHOP/Gadd153	**C/EBP Homology Protein/Growth Arrest and DNA**
cAMP	**cyclic adenosine monophosphate**
co-IP	**co-Immunoprecipitation**
DMEM	**dulbecco's modified eagle medium**
DMSO	**dimethyl sulphoxide**
DNA	**deoxyribonucleic acid**
dsRNA	**double stranded RNA**
ECL	**enhanced chemiluminescence**
EDTA	**ethylenediaminetetraacetic acid**
eIF2α	**eukaryotic initiation factor 2α**
eIFs	**eukaryotic initiation factors**
Endo H	**endoglycosidase H**
ER	**endoplasmic reticulum**
ERAD	**endoplasmic reticulum associated degradation**

ERSE	**endoplasmic reticulum stress element**
ESRD	**end stage renal disease**
FBS	**fetal bovine serum**
Gadd34	**growth arrest and DNA damage-inducible gene 34**
GCN2	**general control non-depressible**
GDP	**guanosine diphosphate**
GFP	**green fluorescent protein**
GRP78	**glycoprotein 78 kDa**
GTP	**guanosine triphosphate**
HEK293	**human embryonic kidney 293**
HeLa	**henrietta Lack's cells**
HEPES	**4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**
Herp	**homocysteine-inducible endoplasmic reticulum resident protein**
HRD1	**hydroxymethylglutaryl-CoA reductase degradation 1**
HRI	**heme-regulated inhibitor**
HRP	**horseradish peroxidase**
HSP-60	**heat shock protein 60 kDa**
IMCD	**inner medullary collecting duct**
IP3R	**inositol triphosphate receptor**
IRE1α	**inositol requirement 1α**
kDa	**kiloDaltons**
MDCK	**madine darby canine kidney**

MEF	**mouse embryonic fibroblast**
Met-tRNAi	**initiator methionine transfer RNA**
mRNA	**messenger RNA**
mTOR	**mammalian target of rapamycin**
NMDAR	**N-methyl-D-aspartate receptor**
ORF	**open reading frame**
PAGE	**polyacrylamide gel electrophoresis**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**
PDI	**protein disulphide Isomerase**
PERK	**pancreatic ER-resident eIF2*α* kinase**
PKC/A	**protein Kinase C/A**
PKD	**polycystic kidney disease**
PKD1	**polycystic kidney disease 1**
PKD1L3	**polycystic kidney disease 1 like 3**
PKD2	**polycystic kidney disease 2**
PKD2L1	**polycystic kidney disease 2 like 1**
PKR	**the double-stranded RNA-dependent kinase**
PM	**plasma membrane**
RACK1	**receptor for activated C-kinase 1**
RNA	**ribonucleic acid**
RT	**room temperature**
RT-PCR	**reverse transcription polymerase chain reaction**

RyR	**ryanodine Receptor**
SDS	**sodium dodecyl sulphate**
SERCA	**sarcoplasmic/endoplasmic reticulum calcium ATPase**
TM	**Trans membrane domain**
TRP channel	**transient receptor potential channel**
TRPM6	**transient receptor potential melastatin 6**
TRPP	**transient receptor potential polycystin**
uORF	**upstream open reading frame**
UPR	**unfolded protein response**
UTR	**untranslated region**
WB	**western blot**
WT	**wild type**
XBP1	**X-Box binding protein 1**

CHAPTER 1

INTRODUCTION

1.1. Transient Receptor Potential Channels

The transient receptor potential (TRP) channel proteins are divided into seven subfamilies that include TRPC (canonical) family, TRPV (vanilloid) family, TRPM (melastatin) family, TRPN (NOMP-C, from no mechanoreceptor potential-C) family, TRPA (ankyrin-like with transmembrane domains 1) family and TRPP (polycystin) family in which 56 related six-transmembrane domain cation channels are classified in seven subfamilies (6). The classification of TRPP subfamilies relies more on topology than homology. The TRPP subfamily is structurally divided into two groups. (1) The polycystic kidney disease 1 (PKD1 known as TRPP1)-like proteins including PKD1 (TRPP1), PKDREJ, PKD1L1, PKD1L2, and PKD1L3, and (2) the polycystic kidney disease 2 (PKD2 known as TRPP2)-like proteins including PKD2 (TRPP2 and PC2), PKD2L1 (TRPP3), and PKD2L2 (TRPP5) (7,8). PKD1-like proteins have 11 transmembrane domains, a more than 2500 amino acid extracellular N- terminal domain and an intracellular C-terminal domain (9). The N-terminal domain includes several protein-protein interaction domains such as the Receptor for Egg-Jelly (REJ) domain, a G protein coupled receptor proteolytic site, and many motifs for cell-cell/cell-matrix interactions (10,11). The function of these PKD1-like proteins is still unclear. PKD2-like proteins have 6 putative transmembrane domains which forms channels, and both N- and C- terminals are localized in intracellular region (11,12). PKD1 and PKD2 form a complex which functions as a mechanosensor. Gene mutations of these genes are strongly related to autosomal dominant

polycystic kidney disease (ADPKD) (13). Moreover, both PKD1L3 and PKD2L1 are necessary to form a functional channel which is associated with the sour taste in the tongue (14,15).

1.2. Polycystic Kidney Disease

Polycystic kidney disease (PKD) is a group of inherited disorders that result in the development of fluid-filled cysts in the kidney and/or tubular dilatation (16). Although there are a number of inherited cystic kidney disorders, PKD is most often caused by two types of inherited cystic kidney disorders. Autosomal Dominant Polycystic Kidney Disease (ADPKD), which is often diagnosed in adults (17) and Autosomal Recessive Polycystic Kidney Disease (ARPKD), which mainly affects infants (18). ADPKD is caused by mutations in *PKD1* or *PKD2* genes. *PKD1* and *PKD2* encode PKD1, a large receptor-like protein, and PKD2, a Ca^{2+} permeable cation channel respectively. ARPKD is caused by mutations in the *PKHD1* gene encoding fibrocystin, which is a large receptor-like protein (19). Furthermore, there are numerous rare types of PKD which include nephronophthisis (NPHP) (20), Joubert syndrome and related disorder (JSRD) (21), Meckel syndrome (MKS) (22), orofacial digital syndrome (OFDS) (23).

1.3. Autosomal Dominant Polycystic Kidney Disease

1.3.1. Genes in ADPKD and genotype-phenotype correlations

ADPKD is genetically heterogenous and is mainly caused by mutations in two genes: PKD1 (16p13.3) and PKD2 (4p21) that encode PKD1 (or PKD1; around 460 kDa) and PKD2 (or polycystin-2, around 110 kDa). Whether a third gene is associated with ADPKD is not clear. Pathogenic mutations in the PKD1 and PKD2 genes account for ~85% and ~15% of the ADPKD cases, respectively (13,24-26). The PKD1 gene (46 exons; genomic extent, 50 kb) encodes a large transcript with an open reading frame (ORF) of 12,909 bp (25,26). The PKD2 gene (15 exons; 68 kb) encodes an ORF of 2904 bp (13). Homozygous and compound heterozygous gene mutations in PKD1 or PKD2 are lethal *in utero* (27). Individuals with heterozygous mutations in PKD1 or PKD2 can survive to adulthood (28,29). In general, ADPKD caused by PKD1 mutations is more severe than that caused by PKD2 mutations; the average age at the onset of end stage renal disease (ESRD) in the PKD1 type is 54.3 years, on the other hand, the PKD2 type corresponds to 74.0 years (30). Furthermore, PKD2 females have a later age of ESRD onset at 76.0 years, whereas in males the age of onset is 68.1 years. In disease severity of PKD1, there is almost no difference between males and females (31,32).

ADPKD has vast interfamilial and intrafamilial phenotypic variability. However, genic or allelic effects do not strongly influence ADPKD phenotypic variability. In reported 461 patients affected by PKD2 from 71 families, there was no clear correlation of the severity of kidney disease between mutation type and mutation position (32). In PKD1, no correlation was likewise shown between mutation type and disease severity (31). In monozygotic twins and siblings there

is a large variance in time to ESRD. One pair of dizygotic twins was seen with significantly divergent phenotypes, supporting the hypothesis that genetic modifying effects influence phenotype (33,34). In several reports, nongenetic factors like environmental factors were shown to have certain effects on the severity in ADPKD phenotypes. For examples, exposure of estrogen or pregnancy in women has more exacerbated polycystic liver disease (PLD; an extrarenal disease in ADPKD). Besides, in the PKD2 type, males have more progressive and faster cystic growth than women, supporting the potential influence of male hormones. This may mean that hormonal factors play pivotal roles in determining ADPKD phenotypes (32,35,36). Caffeine also has negative effects on PKD, presumably by increasing the production of cAMP in cyst-derived cells, which induces cell proliferation and fluid secretion (37).

Therefore, genetic modifier (environmental background) and environmental factors are particularly important factors to decide phenotypic variability.

1.3.2. The structures of PKD1 and PKD2

PKD1 is a large integral membrane protein (4303 amino acids) with 11 transmembrane domains, a very large extracellular region putatively involved in protein-protein and protein-carbohydrate interactions and a short cytoplasmic tail (25,38,39) (Fig. 1-1). The overall structure of PKD1 suggests a role in cell-cell and/or cell-matrix interactions, and receptor functions (40-42). PKD2 is a 968

amino-acid membrane protein and acts as a nonselective cation channel permeable to Ca^{2+} (43,44). PKD2 is predicted to have cytoplasmic N- and C-termini and putative six transmembrane domains (45) (Fig. 1-1). PKD2 shows homology with last six-transmembrane domain regions of PKD1, and it belongs to polycystin

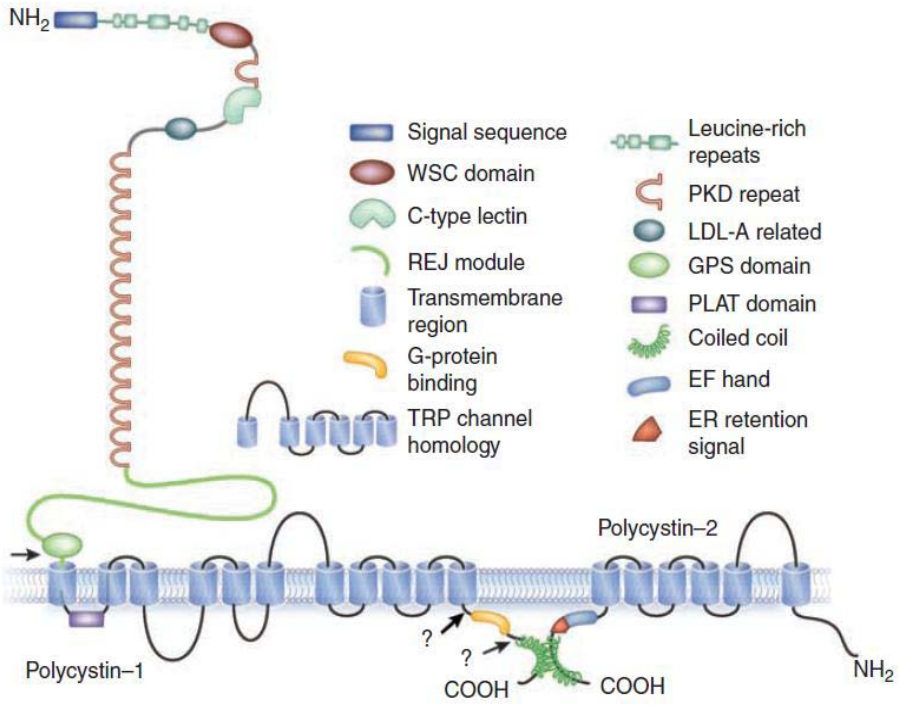

Fig. 1-1. Diagram of the PKD1 and PKD2 proteins. PKD1 (left) and PKD2 (right), and their interaction through coiled-coil domains in the C-terminal tails. The domains and regions of homology are shown. The arrows are indicated the cleavage site in the GPS region and potential cleavage sites in the C-terminal tail of PKD1 (1)

subfamily of TRPs (46). The polycystins localizes to various tissues and subcellular compartments. PKD1 is found in the plasma membrane (PM) at focal adhesions, desmosomes, and adherent junctions. PKD2 is found in the ER and PM (25,41,47-50). PKD1 and PKD2 are also located in the primary cilium, which is a single hair-like organelle projecting from the surface of cells (51-54). These polycystins are arranged in a complex and act as a flow sensor on the cilium (Nauli et al., 2003). Mechanical or flow-induced bending of cilia is associated

with a Ca^{2+} influx into the cell, which occurs through the PKD2 channel (55). Indeed, although $PKD1^{-/-}$ cells have normal cilia shape they do not exhibit the normal flow-induced Ca^{2+} response suggesting that PKD1 regulates PKD2 (53,56).

1.3.3. Functions of PKD2 in different subcellular compartments

Functions of PKD2 in the ER

$PKD2^{+/-}$ vascular smooth muscle cells has been shown to alter intracellular Ca^{2+} homeostasis, which may be associated with PKD2 channel function in the ER (57). PKD2 reconstituted in lipid bilayers was shown to be directly activated by Ca^{2+} (44). It was subsequently shown that PKD2 interacts with the IP_3 receptor using a Ca^{2+} imaging system in *Xenopus* oocytes (50). This interaction significantly prolongs the half-decay time of IP_3 induced Ca^{2+} transients. Further, a pathogenic mutant with defective channel activity (D511V) and a mutant with C-terminal truncation (R742X) were not shown to alter the half-decay time. Thus, ADPKD might be induced by misregulation of intracellular Ca^{2+} concentration through pathogenic mutations of PKD2.

Cai et al reported that native and transfected PKD2 was likely expressed in the ER and that no significant amount of PKD2 is detected in the plasma membrane (48). It was also found that a cluster of acidic residues is essential for PKD2 retention in the ER through a deletion study. For example, a pathogenic mutant of R742X where part of the C-terminus including the ER retention domain is deleted is transported to the plasma membrane and show channel activity (5).

Possibly, accumulation of PKD2 in the ER may be a pathogenic factor in ADPKD, or the targeting of PKD2 to the plasma membrane may not be sufficient for its functions.

Afterward, it was reported that PKD2 functions as an intracellular Ca^{2+} channel in response to elevated intracellular, but not extracellular Ca^{2+} concentration. This suggests that an initial increase in the intracellular Ca^{2+} concentration activates intracellular PKD2, thereby increasing intracellular Ca^{2+} concentration (44). For example, overexpression of PKD2 increases the duration of the amplitude of Ca^{2+} release, but a mutant with defective channel activity (D511V) only showed transient amplitude of Ca^{2+} release, suggesting that PKD2 functions as a Ca^{2+}-induced Ca^{2+} release channel.

Functions of PKD2 in the plasma membrane

Functional expression of PKD2 was first reported by Hanaoka et al in the plasma membrane (58). This report demonstrated that PKD2 alone is not capable of forming a functional channel. However, interaction with PKD1 allowed PKD2 to have ion channel activity. This interaction between PKD1 and PKD2 take places *in vivo* and *in vitro*, suggesting that these two proteins may be a complex like a receptor-ion channel complex that senses extracellular signals and transduces them into intracellular Ca^{2+} signals. Interestingly, naturally occurring mutations in PKD1 or PKD2, which disrupt their interaction and lose PKD2 channel activity (58).

1.3.4. Clinical manifestation in ADPKD

ADPKD is one of the most common monogenic disorders, with incidence of 1:400-1000. It is typically diagnosed in adults, though there are rare infantile cases. ADPKD occurs in all races. The disease is characterized by progressive cyst formation, bilateral cyst development and enlargement of focal cysts in both kidneys that typically result in end-stage renal disease (ESRD) by the fifth decade. Since ADPKD is a systemic disease (Pleiotropic), it has to a great extent extrarenal phenotypic variability with cysts formation also taking place in the liver (approximately 75% of patients develop liver cysts), pancreas, seminal vesicle, and arachnoid membrane. Other non-cystic phenotypes in ADPKD include intracranial aneurysms (around 5 times higher than the general population), dolichoectasias, aortic root dilatation, mitral valve prolapse, and abdominal wall hernias (59). The severity of the renal disease in ADPKD is greatly variable. Though massively enlarged cystic kidneys infrequently occur in utero, typically ESRD occurs in the sixth decade. The considerably high variability of ADPKD's phenotypes indicates that other factors including gene modifiers, environmental conditions and hormonal influence play crucial roles in ADPKD (60). Estimates of ESRD caused by ADPKD in annual incidence rates in men and women are 8.7 and 6.9 per million (1998-2001, USA), 7.8 and 6.0 per million (1998-1999, Europe) (61), and 5.6 and 4.0 per million (1999-2000, Japan) (62), respectively. Thus, men are involved in more progressive disease in ADPKD than women.

Cyst development and growth

Various manifestations are connected with the development and enlargement of renal cysts. Total volumes of kidney and cysts are significantly increased in ADPKD. Interestingly, the proportion of volume change between total kidney and cysts and right and left kidney volumes, were appreciably correlated by investigation of the Consortium of Radiologic Imaging Studies to understand the progression of PKD. Furthermore, the increase in the total kidney volumes is strongly associated with the decline of glomerular filtration rate (GFR) in patients with higher than 1500 mL of baseline total kidney volume. In the absolute change, PKD1 kidneys have much higher volume (74.9 mL/year) and more cysts than PKD2 kidneys (32.2 mL/year). However, the rates of cyst growth were not significantly different (PKD1: 5.68%/year VS PKD2: 4.82% /year). This means that both the cyst initiation and the enlargement of cysts are regulated by PKD1 and PKD2 genes (36,63,64).

Abnormalities of early renal function

In the early stages, the capacity of concentrating urine is usually impaired for 60% of children (65); Also, the concentration of vasopressin is increased in plasma. Interestingly, previous reports suggested that translocation of aquaporin-2, affected by vasopressin to the apical membrane is impaired in a mouse model but not in humans (66). This defect of urinary concentrating capacity frequently corresponds to a disorder of renal medullary architecture, which strongly

correlates with the severity of cystic disease (66). Recent studies suggest that the impaired urinary concentrating capacity and elevated vasopressin concentration in plasma can affect cystogenesis, glomerular hyperfiltration, development of hypertension, and chronic kidney disease progression (65-74).

Hypertension

If blood pressure is higher than 140/90 mmHg, we call it hypertension. Around 50% in ADPKD patients aged 20-34 have normal renal function but almost 100% patient with ESRD have hypertension (75). Usually, hypertension develops with a reduction in renal blood flow, increased filtration fraction, abnormal control of Na^+ in the kidneys and widespread modification of the renal vasculature (76,77). Some studies showed that stretching and compression of the vasculature by cyst initiation and growth can cause renal ischemia and stimulation of the rennin-angiotensin system, which elicits several cellular stresses and regulates blood pressure and fluid balance respectively (78). For example, administration of an angiotensin-converting enzyme (ACE) inhibitor increased renal vascular resistance, increased filtration fraction, reversed the reduced renal blood flow (75,76,79), stimulated ectopic synthesis of renin in the endothelium of dilated tubules and cysts (80,81), and caused ACE-independent generation of angiotensin II by chymase-like enzyme (82). The level of expression of PKD1 and PKD2 in vascular smooth muscle (83-85) and endothelium accompanies enhanced vascular smooth muscle contractility (86) and impaired endothelial-dependent

vasorelaxation (87). It was suggested that functional defects of PKD1 and PKD2 play a partial role in hypertension development and renal vascular remodeling (87-89).

1.3.5. Extrarenal manifestations in ADPKD

Cyst formation in multiple organs

In addition to hepatic cysts in ADPKD, several other organs such as the seminal vesicles, pancreas and arachnoid membrane also have cyst formation (90-95). Seminal vesicle cysts are induced in around 40% of male patients, though they rarely cause infertility (96). However, ADPKD does result in defective sperm motility, which leads to male infertility (97). Most of the pancreatic cysts (5% in patients) are asymptomatic with recurrent pancreatitis and intraductal papillary mucinous tumor (97). Arachnoid membrane cysts (8% in patients) are also generally asymptomatic with very rare cases of cerebrospinal fluid leak (98), and subdural haematomas (92,99).

Polycystic liver disease (PLD)

Polycystic liver disease (PLD) is one of the most common extrarenal manifestations in ADPKD. Mutations in either the PKD1 or PKD2 gene are strongly associated with PLD (100-102). The PLD also occurs as distinct from ADPKD with mutations in the PKCSH and SEC61 genes that lead to autosomal

polycystic liver disease (ADPLD). ADPLD accounts for a third of the PLD cases (103-105). Liver cysts begin by excessive proliferation and dilatation of biliary ductules and peribiliary glands. Interestingly, estrogen elicits hepatic-cyst-derived cell proliferation through binding with estrogen receptors which are expressed in the epithelium lining hepatic cysts (106). Secreted growth factors and cytokines in the cyst fluid also induce growth of liver cysts (107,108). Therefore, women have a higher volume and more prevalent hepatic cysts than men. For example, women who have experienced multiple pregnancies or estrogen replacement therapy have more severe hepatic disease (109,110).

Vascular abnormalities

Intracranial aneurysms and dolichoectasias, thoracic aortic, cervicocephalic artery dissections, and coronary artery aneurysms are all associated with the vascular manifestations in ADPKD. These manifestations are directly affected by the changes of vasculature which are related to mutation of the PKD1 or PKD2 gene. Both PKD1 and PKD2 are localized in vascular smooth muscle cells (83-85). For example, vascular smooth muscle cells with $PKD2^{+/-}$ have a high rate of cell proliferation and apoptosis, and an increased susceptibility to vascular injuries (57,111).

Intracranial aneurysms occur in ~16% of ADPKD patients with a positive family history of intracranial aneurysms and ~6% of patient with no negative

family history (112). It is almost always asymptomatic, but rupture by intracranial aneurysms brings high risk (35~55%) of morbidity and mortality (113).

1.3.6. Cellular abnormalities in ADPKD

Previous reports demonstrated that kidneys from PKD patients show high levels of apoptosis and cell proliferation (114-116). The c-myc and bcl-2 genes are significantly increased in the renal cysts in ADPKD. These genes are strongly associated with improper regulation of cell proliferation and apoptosis (115).

Cell over-proliferation in ADPKD

A primary morphological feature of all of human PKD is tubular hyperplasia. In addition, the inappropriate cell proliferation of tubular epithelial cells is strongly associated with renal cystic cell growth and development (117). These cell proliferation indicators are highly elevated in non-cystic tubule segments in ADPKD, ARPKD and renal cystic kidneys as well (114). The elevated indicators are found in collecting duct in non-cystic regions of ARPKD kidneys, and the distal tubule and non-cystic regions in ADPKD. These indicators are believed to initiate in both proximal and distal tubules in renal cystic disease (114). This suggests that improper elevated proliferation has susceptibility to cystic formation. Moreover, a number of proto-oncogene molecules are increased in human and mouse models with polycystic kidneys, including the c-myc, c-jun,

c-fos, c-ha-ras and c-ki-ras (118-120), which suggests that inappropriate expression of proto-oncogenes may cause hyperplasia and cystic formation (121).

In mouse model study of over-expression of transgenic oncogenes such as *H-ras* and *c-myc*, formation of cystic kidneys and tubular cysts have been shown to occur (121-123). In cultured epithelial cells from ADPKD cysts showed elevated proliferation with overexpressed c-myc (124,125). Fascinatingly, a study reported that haploinsufficiency of PKD2 can induce elevated tubular proliferation (126). The basal and epidermal growth factor (EGF)-induced rate of cell proliferation in PKD2 knock-out cells are higher compared to cells expressing PKD2. Furthermore, when comparing cell proliferation rate in cells expressing PKD2 wild-type and a channel dead mutant of PKD2 (D511V), the cells expressing D511V proliferated faster than the cells expressing wild-type. Presumably, PKD2 is strongly implicated in negatively controlling cell proliferation and its channel activity plays a crucial role in the process (127). In addition, PKD2 influences the anti-proliferative function of PKD1 by facilitating up-regulation of p21 which is cell cycle arrest protein (128).

Elevated apoptosis in ADPKD

Interference in the balance between the rate of cell proliferation and apoptosis has been shown to cause cyst formation (129,130). Polycystic kidney disease also is strongly related to elevated apoptosis, which is a crucial cause of cyst formation (131). For example, tubular epithelial cells with ADPKD have

been reported to elicit increased apoptosis in human and most animal model of PKD (116,132), however, cystogenesis induced by elevated apoptosis is inhibited by over-expression of the protein Bcl-2, which is an anti-apoptotic factor (133). A previous study also reported that the inhibition of a caspase that leads to apoptosis attenuates cyst formation and kidney failure in PKD animal models as well as cell proliferation in renal epithelia (132). Although the correlation between PKD and apoptosis is well studied, it is still unclear how PKD2 is associated with the molecular mechanism of apoptosis. Recently, many reports studied with how Ca^{2+} regulates apoptosis (134,135). Usually, increased cytosolic Ca^{2+} is observed during cell death, which is promoted by Ca^{2+} release from the ER, hyper-activation of cation channel and, damage of the extracellular membrane (134,136). For instance, thapsigargin and ceramide induce Ca^{2+} release from the ER, and this causes an increase in mitochondrial Ca^{2+} concentration, which leads to apoptosis (137). Very interestingly, PKD2, when acting as a Ca^{2+} -permeable non-selective cation channel (138), in the ER facilitates cell protection from apoptosis by decreasing the concentration of releasable Ca^{2+} in the ER (139). Moreover, PKD1 also has a protective effect on cells from apoptosis through controlling the phosphatidylinositol 3-kinase (PI3-kinase)/Akt signal pathway (130,140). However, the molecular mechanism by which PKD1 and -2 inhibit apoptosis is still unknown.

1.4. Translational initiation

Translation consists of three steps: initiation, elongation and termination (141). The translational initiation factor associated with introducing methionyl-tRNA (Met-tRNAi) to the 40S ribosomal subunit is eukaryotic initiation factor 2 (eIF2), which consists of α, β, γ subunits and mediates the binding between 40S ribosome and the initiator methionyl-tRNA (Met-tRNAi). Before binding with the 40S ribosome, eIF2 needs to bind with Guanosine-5'-triphosphate (GTP), so called GTP-eIF2 (active state to initiate translation) and this GTP-eIF2 only can bind with Met-tRNAi. The eIF2·GTP·Met-tRNAi which is called a ternary complex subsequently can bind with the 40S ribosome, which leads to the formation of the 43S preinitiation complex (4). Another important factor, eukaryotic initiation factor-4F (eIF4F) is a cap-binding protein complex that interacts with the mRNA 5'end cap structure (m^7GpppN: methylated guanosine at 7 positions with nucleotide). The eIF4F complex is comprised of three subunits (eIF4E, eIF4G and eIF4A): eIF4E is a cap structure binding protein, eIF4A that is a RNA helicase unwinding secondary structure of the mRNA 5' untranslated region (UTR), and eIF4G is a scaffolding binding protein interacting with eIF3 that is involved in the 43S preinitiation complex (a 40S subunit, the eIF2–GTP–Met-tRNAMeti ternary complex, eIF3, eIF1, eIF1A and probably eIF5). The eIF4F complex acts to connect the mRNA to 43S preinitiation complex as well as binding with Poly(A)-binding protein (PABP), which binds with poly(A) tail in 3'UTR to make a circular form of mRNA (141). The anti-codon of Met-tRNA in 43S preinitiation complex recognizes the initiation codon (AUG) of mRNA to initiate translation. The 43S preinitiation complex with eIF4F complex can start

scanning for 5’ to 3’UTR direction until it reaches the initiation codon (AUG) (142). After recognition of the initiation codon by Met-tRNA in 43S preinitiation

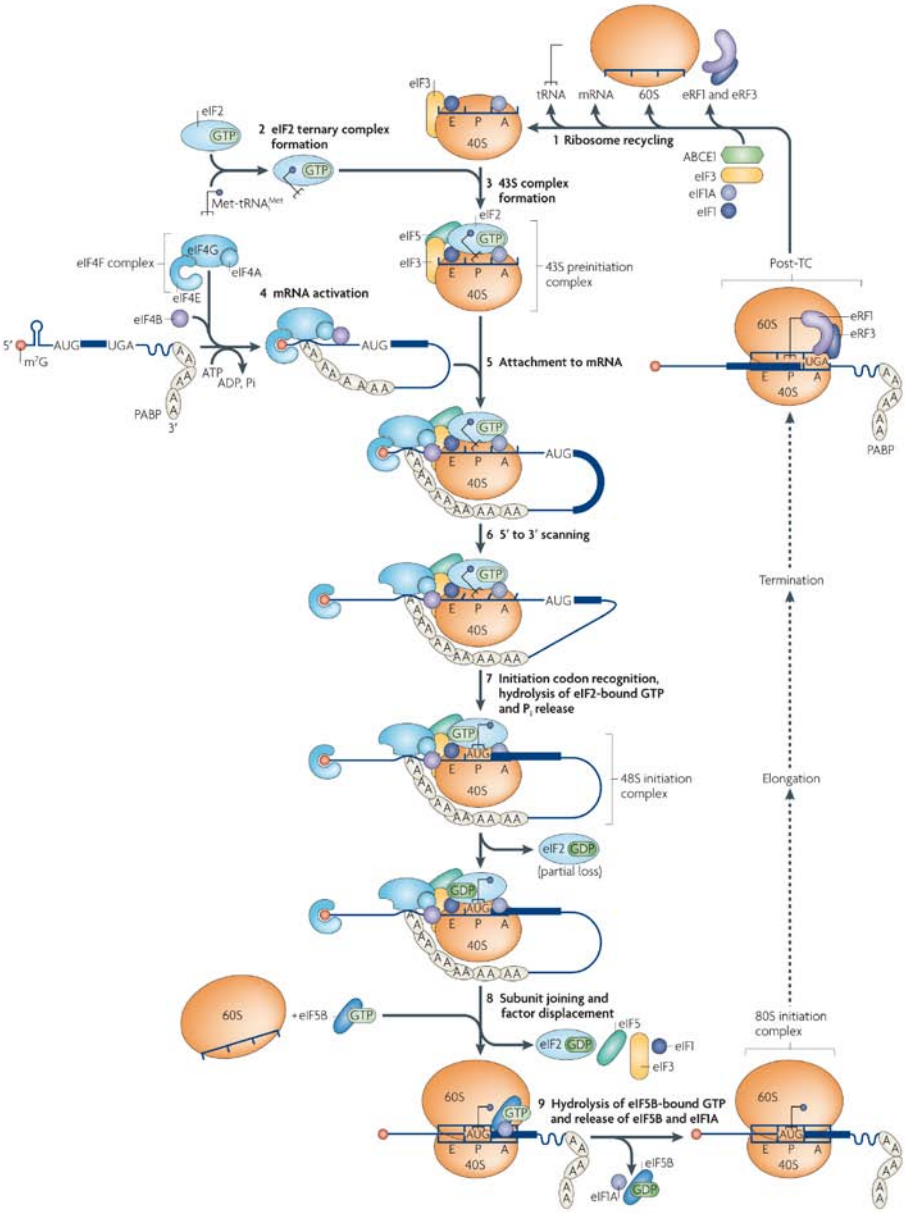

Fig. 1-2. Model for eukaryotic translation initiation pathway This eukaryotic translation initiation that is comprised of 8 steps performs after the recycling of post-termination complexes (post-TCs). (1) Formation of eIF2 ternary complex, (2) Formation of a 43S preinitiation complex involving a 40S subunit, eIF1, eIF1A, eIF3, eIF2-GTP-Met-tRNA$^{Met}_{i}$ and eIF5, (3) mRNA activation (unwinding of cap-proximal region of mRNA) by an eIF4F complex; (4) Interaction of 43S complex and mRNA: (5) Scanning of 5’UTR in mRNA from 5’ to 3’by 43S complex: (6) Formation of 48S complex by start codon recognition of tRNA$^{Met}_{i}$; (7) Joining of 60S subunit to 48S complex; (8) Formation of 80S complex by releasing eIF5B and eIF1A (4)

complex, GTP-eIF2 is hydrolyzed to GDP-eIF2 and Pi by eIF5 (a GTPase activation protein), then to GDP-eIF2 (the inactive state to initiate translation).

Finally, the 60S subunit ribosome is assembled with 40S ribosomal subunit catalyzed by eIF5B·GTP. It is GTP hydrolysis that forms the 80S ribosomal subunit to start the elongation step (143). At this point, the other eIFs (eIF1,eIF3, eIF4B, eIF4F and eIF5) are released from the ribosome by eIF5B (144,145). To restart translation initiation, GDP in eIF2 needs to be exchanged for GTP. This exchange from GDP to GTP is performed by eukaryotic initiation factor 2 B (eIF2B) (146) (Fig. 1-2).

1.5. ER stress pathways

In eukaryotic cells, the ER is the main compartment responsible for the synthesis, modification and delivery of proteins to the proper target sites within the secretory pathway and the extracellular space (147). In the ER, proteins are folded into their native conformation with post-translational modifications, including asparagine-linked glycosylation (148,149), and the formation of intra and intermolecular disulfide bonds (150). The folded proteins undergo quality-control, and then only properly folded proteins are exported to the Golgi-complex. Improperly folded proteins are retained in the ER to complete the folding process or to be targeted for degradation (151). Any kind of disruption in these processes cause accumulation of unfolded proteins and induce ER stress. There are several reasons why unfolded proteins accumulate. These are associated with the increase in translational processes exceeding the normal capacity of the ER, improper

processing of newly synthesized protein folding or accumulation of mutant proteins (152-154). Furthermore, amino acid deprivation and accumulation of lipid and cholesterol also induce ER stress by causing the accumulation of unfolded proteins (155,156). Artificially, several chemical agents result in ER stress. Thapsigargin, one of the most used ER stress inducers is an inhibitor of ER Ca^{2+} ATPase (SERCA). SERCA maintains a high Ca^{2+} concentration in the ER lumen by pumping cytosolic Ca^{2+} into the ER. Thapsigargin blocks SERCA Ca^{2+} transportation, and thus leads to increased cytosolic Ca^{2+} concentration while reducing Ca^{2+} in the ER lumen (157). Ca^{2+} homeostasis in the ER has particularly vital roles to maintain ER structure and proper folding of most of ER-localized chaperones that require Ca^{2+} binding. Ca^{2+} depletion in the ER causes misfolding of ER-localized chaperones, leading to the chaperones' inability to function. This results in accumulation of unfolded protein causing ER stress (158,159). Tunicamycin is another most used ER stress inducer that inhibits enzyme GlcNAc phosphotransferase (GPT). GTP catalyzes the transfer of N-acetylglucosamine-1-phosphate to dolichol phosphate in the first step of glycoprotein synthesis. Tunicamycin blocks the synthesis of all N-linked glycoproteins (N-glycans) and leads to accumulation of unfolded protein causing ER stress (160,161). Under conditions where unfolded proteins accumulate in the ER lumen, the cell responds by activating several down-stream signaling pathways, called the Unfolded Protein Response (UPR) (162). The UPR consists of three ER-localized transmembrane signal transducers, PERK (double stranded RNA-activated protein kinase-like ER kinase), IRE1 (inositol requiring kinase 1) and ATF6 (activating

transcription factor 6) which are activated by ER stress (163-166). The UPR is an adaptive cellular response against misfolded or unfolded protein accumulation in the ER lumen and requires regulation at all levels of gene expression including transcription, translation, translocation and ER associated degradation (ERAD) (Fig. 1-3). Regulation of all of these processes by UPR leads to cell survival by restoring proper folded proteins and ER homeostasis.

However, if accumulation of misfolded or unfolded proteins is not resolved during ER stress, chronic activation of UPR occurs and eventually apoptosis (programmed cell death) is induced (167-170). PERK is a type I transmembrane protein in the ER with a cytosolic serine/threonine kinase domain. The luminal domain of PERK is homologous with IRE1 (171). In ER stress, PERK senses the accumulation of unfolded proteins in the ER lumen. This causes PERK dimerization and *trans*-autophosphorylation to occur. This has an activation effect on Perk's eIF2α kinase function which phosphorylates eIF2α at serine 51, resulting in the inhibition of global protein synthesis (162,163,172,173). The PERK/eIF2α signaling pathway during ER stress has two main purposes, first to inhibit newly synthesized protein loading to the ER. This alleviates accumulation of unfolded proteins in the ER. Second, to reprogram gene expressions via translational up-regulation of certain mRNAs for adapting to the new environment (153,173-175) (Fig. 1-3). One of the mRNAs is activating transcriptional factor 4 (ATF4), a 39 kDa bZIP transcription factor that induces transcription of many stress response genes encoding ER chaperones such as the 78 kDa glucose-regulated protein (GRP78) and the 94 kDa glucose-regulated

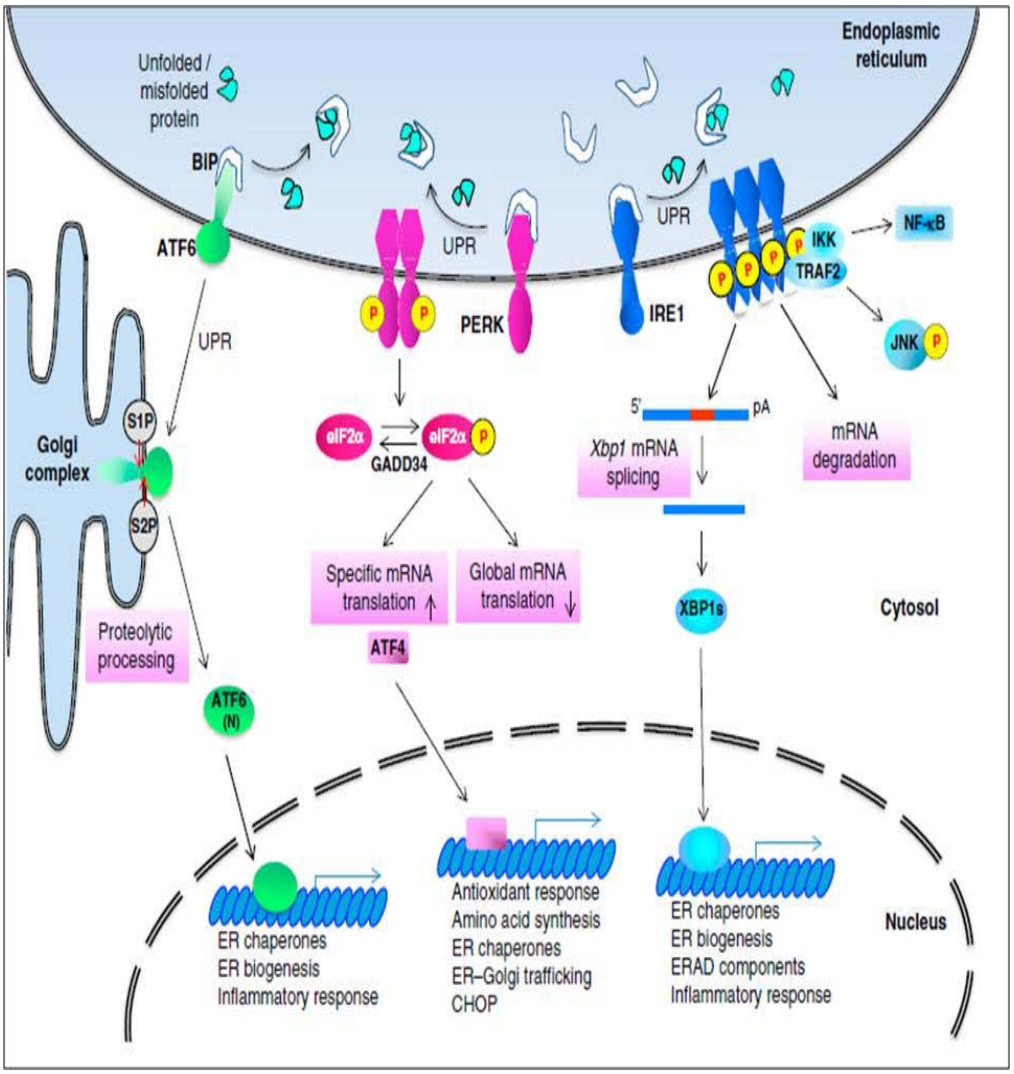

Fig. 1-3. Three signal transduction pathways by ER stress PERK/eIF2α pathway attenuates global protein synthesis and translationally up-regulates specific mRNAs. IRE1, endoribonuclease, activated by ER stress increases the expression of XBP-1 and various ER stress related proteins, and leads to pro-inflammatory pathways through NF-κB and pro-apoptotic pathways through JNK. ATF6, a transcription factor, translocates to Golgi, and is activated by site-1 and -2 protease (S1P/S2P) (2).

protein (GRP94) UPR-associated transcription factors including X-box binding protein 1 (XBP1), growth arrest and DNA damage 34 (Gadd34), CAAT/Enhancer binding protein (C/EBP) homologous protein (CHOP) and intracellular trafficking machinery for ER-to-Golgi trafficking of activating transcriptional factor (ATF6)

during ER stress (172-176). Especially, CHOP is a transcriptional factor which induces apoptosis by activating transcription of several genes, including Gadd34, ER oxidoreductin 1 (ERO1), death receptor 5 (DR5), Tribbles homolog 3 (TRB3), and carbonic anhydrase VI that may be related to apoptosis (162,177). Gadd34 forms a complex with protein phosphatase 1 (PP1) to dephosphorylate eIF2α which leads to recovery of overall translation from global protein synthesis inhibition by eIF2α phosphorylation (178). This translational recovery is highly critical at certain times after ER stress because a lot of stress-induced genes in either transcriptional or translational pathways need to be synthesized to counteract the stress (177,179).

In murine and human cells, there are two homologues of yeast IRE1, which are IRE1 α and IRE1 β. IRE1 α is expressed in all tissues, however, IRE1 β expression is restricted to gut epithelial tissue (180,181). IRE1 α is a type 1 ER-resident transmembrane protein, which contains a luminal domain sensing the protein folding environment in the ER and cytoplasmic portion including a protein kinase domain (182,183). During accumulation of unfolded proteins, IRE1 α is oligomerized and *trans*-autophosphorylated. IRE1 α has only one substrate for IRE1 α itself. Unlike PERK, *trans*-autophosphorylation of IRE1 α kinase domain activates its endoribonuclease function that causes endonucleolytic cleavage of a mRNA of X-box binding protein 1 (XBP1) in metazoans (184). Therefore, IRE1 α is a bifunctional enzyme as both a protein kinase activity and a site-specific endoribonuclease that is regulated by its intrinsic kinase module (185). IRE1 α cuts an intron of the precursor XBP1 mRNA then 5’ and 3’ mRNA fragments are

ligated. The ligated mRNA encodes a transcription factor working as an UPR target gene activator, whereas the precursor of XBP1 mRNA encodes protein repressing UPR target gene (Fig. 1-3) (186). Moreover, IRE1 α is associated with signaling functions as well as nucleolytic activity by activating Jun N-terminal kinase (JNK) or interacting with caspase-12 which is related to ER stress-induced apoptosis (187,188).

ATF6 is an ER type 2 transmembrane protein which includes an N-terminal cytoplasmic region containing a Basic Leucine Zipper Domain (bZIP), a DNA transcription activation domain, and a C-terminal luminal region that senses protein folding environment in the ER lumen and is normally tethered to the ER membrane as an inactive precursor (189). In activation of UPR by ER stress, ATF6 is transported from ER membrane to Golgi apparatus. ATF6 is cleaved by Golgi-resident proteases which are site 1 protease (S1P) and site-2 protease (S2P). The cleaved portion, which is called the ATF6 fragment (ATF6f) and contains the cytosolic bZIP and DNA transcription activation domain, is released. The released ATF6f reaches the nucleus to activate UPR related gene expression such as chaperones and ERAD proteins (190) (Fig. 1-3).

1.6. Effects of eIF2alpha phosphorylation in translation initiation

The eIF2 that consists of α, β, γ subunits is phosphorylated at residue serine 51 of α subunit of eIF2 during many different types of stress such as ER stress, oxidative stress, hypoxia, viral infection, heat shock and amino acid

starvation. The exposure of cells to stress induces adaptive responses including stress-response gene expression which regulates cell survival, apoptosis, cell-cycle progression and differentiation (191). Primarily, these stress conditions result in inhibition of global protein synthesis via eIF2α phosphorylation to save cellular energy and prevent the synthesis of misfolded or unfolded proteins that may disrupt cellular response during stress conditions. Phosphorylated eIF2 inhibit the activity of eIF2 by inhibiting eIF2B activity that exchanges of GDP-eIF2 for GTP-eIF2. Usually, the binding affinity of phosphorylated eIF2 at serine 51 with eIF2B is 150 times higher than non-phosphorylated eIF2 (192). Therefore, phosphorylated eIF2 preferably binds with eIF2B and functions as a competitive inhibitor of eIF2B by reducing the recycling chance of GDP-eIF2 to GTP-eIF2 (192,193). Thus, the formation of active ternary complex (eIF2·GTP·Met-tRNAi) is reduced by eIF2α phosphorylation (194,195). The eIF2α phosphorylation finally prevents the translation of most mRNAs. However, paradoxically, eIF2α phosphorylation increases translation of selective mRNAs related to adaptation to stress such as ATF4, CHOP, and Gadd34 (Fig. 1-5).

1.7. The effect of upstream open reading frame during cellular stresses

During ER stress, global protein translation is reduced by P-eIF2α, but paradoxically, a subset of mRNAs is translationally up-regulated. The mechanism of how these mRNAs are up-regulated can be explained by upstream open reading frames (uORF) in the 5’ untranslated region (5’UTR) of mRNA. Most of the

mRNAs regulated by uORF are oncogenes, growth factors, and cellular receptors (196). Interestingly, around 50% of human and mouse transcripts include uORFs, which are evolutionarily conserved in different species (197-199). In general, uORFs function as barriers to the scanning ribosome that leads to inhibition of translational initiation of main coding region (200). For example, GCN4 mRNA in *Saccharomyces cerevisiae*, which encodes a transcription factor for activating amino acid biosynthetic genes for survival in times of amino acid starvation, is translationally up-regulated during amino acid starvation through an increase in P-eIF2α. This translational up-regulation of GCN4 is regulated by four uORFs in its 5'UTR. The uORF1, uORF2 and uORF3 in GCN4 can be scanned by the ribosome, but when ribosomes translated uORF4, it is dissociated from mRNA GCN4, thereby represses translation of mRNA of GCN4. Therefore, ribosomes are not successful in reaching the GCN4 start codon (201). By contrast, elevated P-eIF2α during amino-acid starvation results in the reduction of ternary complexes. This leads to a delay in the formation of 43S ribosomal complexes, and thereby the frequency of reinitiation at the uORF2, uORF3 and uORF4 is reduced. This facilitate the ribosome in reaching the GCN4 ORF (141) (Fig. 1-4). ATF4 that is a transcription factor is also translationally up-regulated by elevated P-eIF2α during ER stress. The mRNA of ATF4 includes two uORFs in 5'UTR. uORF1 allows ribosomes to reinitiate after scanning, but the uORF2 has an inhibitory effect on ribosome scanning, thus ribosomes are dissociated after scanning the uORF2. This leads to a repressed ATF4 translation during non-stress conditions (202). However, during ER stress, elevated P-eIF2α accompanied by

reduced GTP-eIF2 level delays ribosome scanning to reinitiate translation. This delayed reinitiation results in the ribosome skipping the inhibitory uORF2 region,

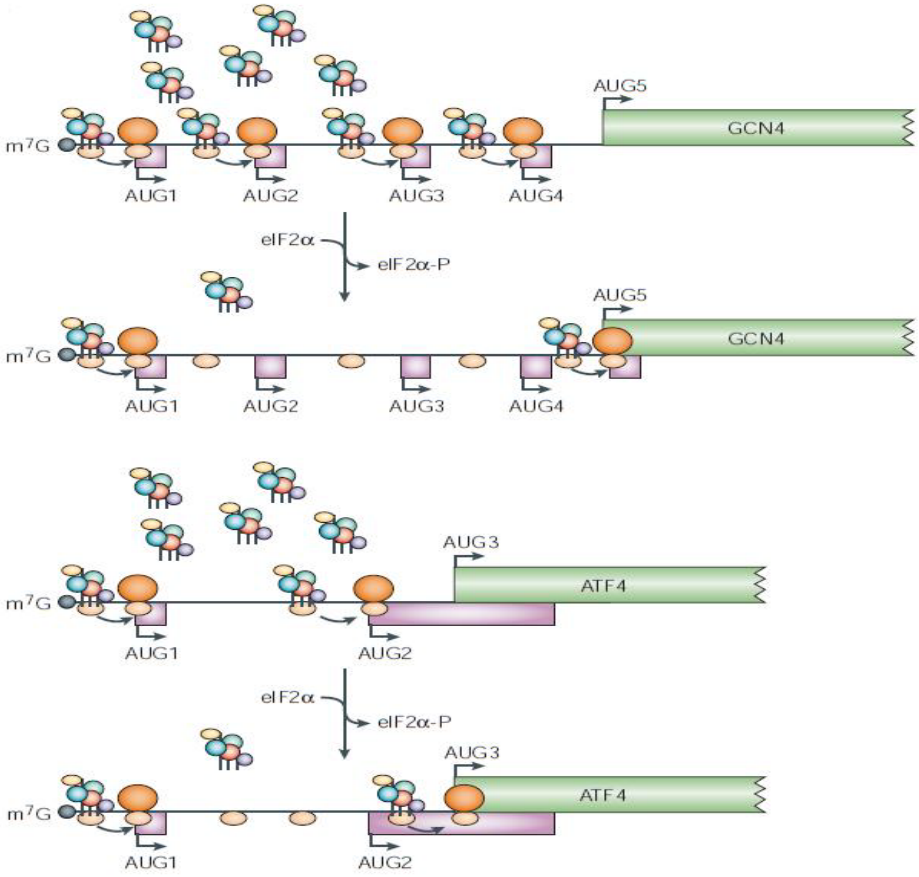

Fig. 1-4. Translational regulation by upstream open reading frame in 5'UTR of mRNA Translation of GCN4 that includes four uORFs is regulated by the upstream open reading frame in 5'UTR of the mRNA. At high levels of ternary complexes caused by lower P-eIF2α during the non-stress condition, ribosomes initiate the scanning of uORF1 and reinitiate uORF2, uORF3 or uORF4. After scanning uORF4, the ribosome can resume the scanning of GCN4 ORF, which leads to reduction of translation of GCN4. Translation of the activating transcription factor-4 (ATF4) is regulated by two uORFs. Likewise, when the ternary complex is abundant during the non-stress condition, the ribosome initiates uORF1 and easily reinitiates uORF2. This has an inhibitory effect on ribosome scanning, whereby the ATF4 translation is suppressed. However, during the ER stress condition, the ternary complexes are reduced by elevated P-eIF2α, and the ribosome has a high likelihood of initiating at the ATF4 ORF, which leads to increase in translation of ATF4 (138).

thereby reinitiating at the ATF4 coding region. The translationally increased ATF4 plays crucial roles in repairing cellular stress (176) (Fig. 1-4). For CHOP, high levels of eIF2-GTP (low P-eIF2α) in non-stress condition results in binding

of ribosomes to the 5'-end of the CHOP mRNA, which translates the uORF, leading to inhibition in translational elongation or termination of CHOP coding region. However, during stress condition, high levels of P-eIF2α leads to a reduction in translational initiation at the uORF, which causes the scanning ribosome to bypass the inhibitory uORF and instead translate the CHOP coding region (203).

1.8. The kinases for eukaryotic initiation factor 2alpha

The phosphorylation of eIF2α at serine 51 during many cellular stresses is regulated by at least four kinases, which include PERK, protein kinase RNA (PKR), haem-regulated inhibitor kinase (HRI) and general control non-dereperssible-2 (GCN2) which sense distinct cellular stresses (204). All of these kinases have a conserved eIF2α kinase domain that has 25-37% amino acid sequence identity. During cellular stress, eIF2α kinases sense distinct stresses with their regulatory domain and dimerize to be auto-phosphorylated. This elicits their kinase activity (204). PERK is activated by ER stress induced by accumulation of unfolded proteins (175) as previously explained. The double-stranded RNA (dsRNA)-activated protein kinase R (PKR) is activated by double-stranded RNA and functions as an anti-viral component (Fig. 1-5). HRI is activated by several types of stress such as low heme concentration, arsenite treatment, heat shock and osmotic shock (translational control in stress and apoptosis 47). GCN2 is activated by amino-acid starvation and UV irradiation

(205). All these kinases, which are activated by several types of cellular stress, phosphorylate eIF2α. This causes a general reduction of translation but induction of selective mRNAs translation (Fig. 1-5). PKR plays very pivotal roles in anti-viral action to limit viral replication (206). PKR includes two dsRNA binding motifs, which recognizes 30 to 85 bp of dsRNA (204,207). PKR has a low expression level in normal tissue, but during viral infection, the transcriptional level of PKR is increased by type I interferon (alpha/beta interferon [IFN-α/β]), which is produced from viral infected tissues (208) and binds with dsRNA produced from viral replication. It induces conformational changes in PKR, leading to dimerization and autophosphorylation of the kinase. Finally, PKR has kinase activity for a substrate of eIF2α (204,209-211). eIF2α phosphorylation by PKR during viral infection leads to the inhibition of viral replication by shut-off f

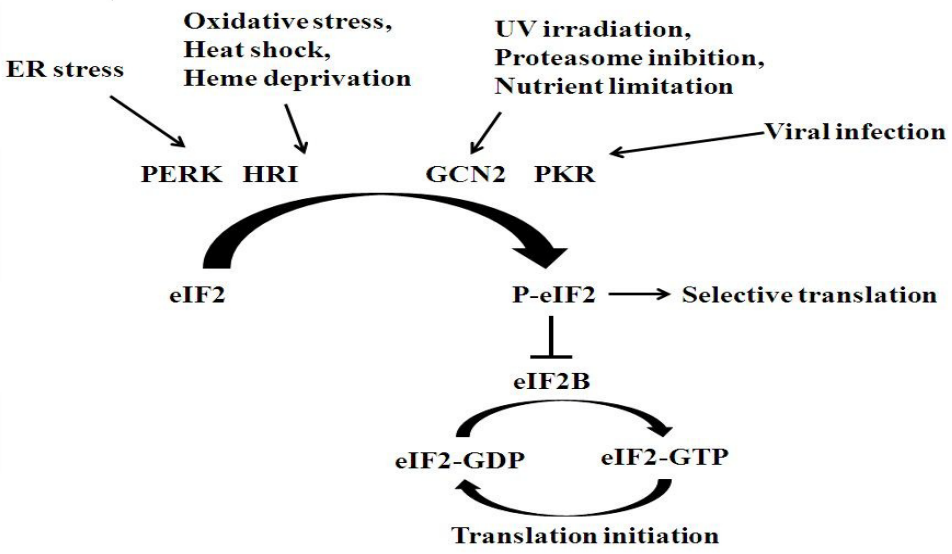

Fig. 1-5. eIF2 kinases PERK, HRI, PKR and GCN2 are activated by different stresses and regulate the level of GTP-eIF2 through eIF2 phosphorylation. The eIF2B, guanine nucleotide-exchange factor, catalyzes GDP-bound eIF2 to GTP-bound eIF2. Phosphorylated eIF2 (P-eIF2) competitively inhibits eIF2B and increases the level of GDP-eIF2 (3).

protein synthesis. Therefore, PKR is required for resistance against a number of viruses (212). For example, the PKR knock-out mice are highly susceptible to vesicular stomatitis virus (VSV) (213).

The heme-regulated inhibitor (HRI) is found in reticulocytes, which are a precursor of erythrocyte under the conditions of heme and iron deficiencies. Generally, for erythroid differentiation and maturation, 3 components of hemoglobin such as α-globin, β-globin, and heme are essential with 2:2:4 ratio ($\alpha_2\beta_2$ hemoglobin complexed with 4 heme molecules). Proper balance of these three components is important because each component has cytotoxicity to mature red blood cells (RBCs) and their precursors (214). Therefore, the amount of heme must be balanced with the amount of globin in order to produce the proper amount of haemoglobin as well as protect the RBCs (215). During deprivation of heme in reticulocytes, HRI senses intracellular heme concentration and is activated by auto-phosphorylation of HRI, which elicits shutoff of protein synthesis by phosphorylating eIF2α and causing low production of globin (216-220). In addition to the deficiency of heme, HRI is also activated by arsenite-induced oxidative stress, osmotic shock and heat shock (221).

Another eIF2α kinase is general control nonderepressible 2 (GCN2), which is activated by amino acid deficiency, and UV irradiation, thus mediating slower growth (204). GCN2 kinase activity is regulated by its domain interactions such as that at its carboxyl-terminal domain (CTD), a domain similar to histidyl-tRNA synthetase (HisRS), and a protein kinase domain. The physical interactions of these domains prevent GCN2 kinase activity during non-stress conditions (222),

but during amino acid depletion, uncharged tRNAs accumulate in the cell and binds with the CTD+HisRS domains, which cause inhibition of its domain interaction, eliciting GCN2 dimerization and autophosphorylation to obtain its kinase activity inducing eIF2α phosphorylation (204,223).

1.9. PKD2L1

PKD2L1 is a novel member of the transient receptor potential (TRP) superfamily of cation channel that is cloned from human retina EST. PKD2L1 has two isoforms, PKD2 and Pkd2L2 (224,225). PKD2L1 functions as a Ca^{2+}-induced, Ca^{2+} permeable non-selective cation channel, especially in *Xenopus* oocytes overexpressed with PKD2L1 (226). Unlike the PKD1 and PKD2, PKD2L1 has not been shown to be mutated in ADPKD, but its murine orthologue is deleted in *krd* (kidney and retinal defect) mice with renal and retinal defects. PKD2L1 has been mapped to 10q24 by radiation hybrid mapping and fluorescent *in Situ* hybridization (224). The loci of several other inherited diseases also have been assigned to this region such as urofacial syndrome (227), infantile-onset spinocerebellar ataxia (228) and partial epilepsy (229).

1.9.1 Structure of PKD2L1

PKD2L1 is composed of 805 amino acids and has 50% sequence identity and 74% similarity to PKD2. In addition to amino acid sequence similarity,

PKD2L1 has similarity in domain organization and/or membrane topology with PKD2, other TRP channels as well as the α subunits of voltage-gated Ca^{2+}, Na^{+} and K^{+} channels (1). PKD2L1 consists of six trans-membrane spanning domains and one pore region (Fig. 1-6). PKD2L1 has long extracellular loops in between the first and second putative trans-membrane segment. Likewise, PKD2 also has a long extracellular loop in the first and second trans-membrane segment, which shares a high homology with PKD2L1. PKD2L1 also has a putative $Ca2^{+}$ binding

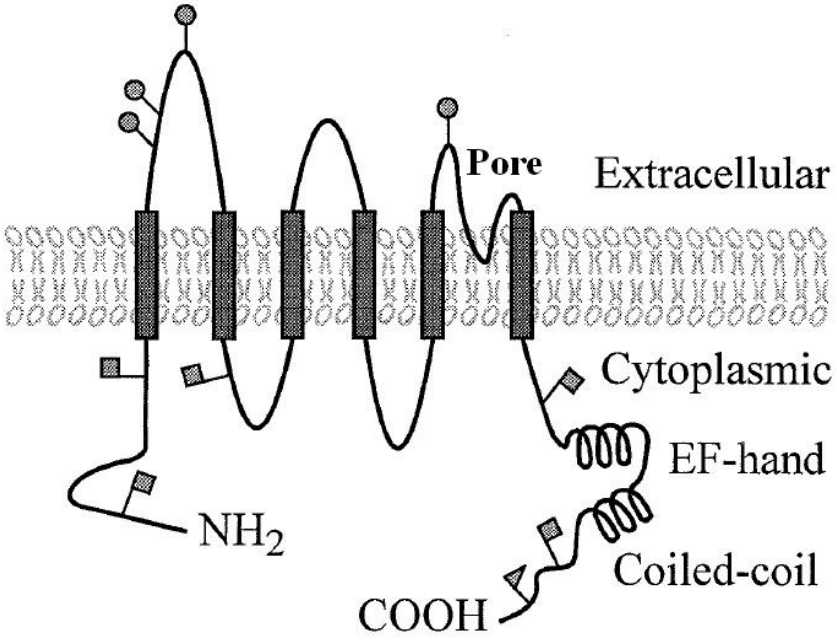

Fig. 1-6. Schematic representation of PKD2L1. Putative six transmembranes spans in the membrane. Circles represent N-glycosylation sites, triangles represent PKA-phosphorylation sites and squares represent PKC-phosphorylation sites. Pore, EF-hand and coiled-coil are shown as indicated (5)

EF-hand motif that involves two helices and a loop (Fig. 1-6). The helix region of EF-hand in PKD2L1 also partially overlaps with predicted coiled-coil region that potentially facilitates its interaction with a number of molecules. PKD2L1 has three positively charged residues in a transmembrane fourth segment, whereas a

voltage-gated channel requires five or eight positive-charged residues to be a voltage sensor. However, it is still unclear whether the three positive charged residues functions as a voltage-sensor because PKD2L1 has very weak dependency on voltage. PKD2L1 has several putative phosphorylation sites in the N- and C-terminus. For example, the C-terminal region of PKD2L1 has one cAMP, two PKC, and four casein kinase II phosphorylation sites, and the N-terminus has two other putative PKC phosphorylation sites (230). It is possible that the phosphorylation sites in PKD2L1 play a crucial role in the process of PKD2L1 channel gating and functions.

1.9.2. Kidney tissue and subcellular localization of PKD2L1

The expression of PKD2L1 is detectable at E16 in the inner medulla of mouse kidney, which simultaneously occurs with the maturation of the inner medullary collecting ducts. PKD2L1 is only expressed in one type of nephron segment which does not correspond to the wide spread of cystic lesions in ADPKD, whereas PKD1 and PKD2 are expressed in all round nephron segments. Interestingly, PKD2L1 has been shown to co-localize with PKD1 in the apical region of collecting ducts in adult mouse kidney.

PKD2L1 is mostly expressed in the ER, and its distribution is commonly overlapped with PKD1. Thus, PKD2L1 is associated with PKD1 in primary cilia. A reciprocal co-IP study found that PKD2L1 interacts with PKD1 in the centrosome. Centrosomes have been known to function as microtubule organizing

structures, which leads to the control of cell cycle initiation/progression, regulation of gene transcription, and protein recycling. This allow us to investigate an involvement of PKD2L1 in cell proliferation (54,231). A previous report showed that PKD1 and PKD2 are coexist in the same complex and modulate intracellular Ca^{2+} levels in response to fluid flow in primary cilia (56). However, the influence of the PKD1-PKD2L1 complex in primary cilia is still unknown. Possibly, appropriate functions of PKD2L1 may require divergent contexts depending on different subcellular compartments, and the localization of channel proteins in non-membrane regions (eg, centrosome) of the cell provides a possibility that polycystins have unexpected functions.

1.9.3. Physiological roles of PKD2L1

The murine orthologue of human PKD2L1 interacts with PKD1, therefore it co-localizes with PKD1. In the reciprocal co-IP experiments, it has been shown that PKD2L1 is associated with PKD1 in the same complex in both subconfluent and confluent inner medullary collecting duct (IMCD) cells (231). The co-localization or coexpression of murine PKD2L1 with PKD1 facilitates transportation of PKD2L1 to the cell surface, whereas on the other hand, the expression of PKD2L1 alone is retained in the ER in cultured human embryonic kidney 293 cells (HEK293) (232). Therefore, the interaction of PKD2L1 and PKD1 plays a crucial role in trafficking of PKD2L1 to the cell surface. The coiled-coil domain of the C-terminus in PKD2L1 is essential for the trafficking of

PKD2L1 by PKD1 to the cell surface and the interaction of PKD1 and PKD2L1. Since PKD1 also interacts with PKD2 at their C-terminal cytoplasmic tails, which has high homology to PKD2L1, the C-terminal fragment of PKD1 can modulate Ca^{2+} permeable cation channels. It is likely that the interaction between PKD2L1 and PKD1, and the trafficking of PKD2L1 by PKD1, may be critical procedures to form a functional channel. However, it is still unclear how the interaction of PKD1 with PKD2L1 influences PKD2L1 physiological functions.

Huang at al (2006) reported a new physiological role of PKD2L1 as a candidate mammalian sour taste sensor (233). It is shown that PKD2L1 is localized in a subset of taste receptor cells in the tongue, where it may have functions in sour taste, and sensing acids however, PKD2L1 is not expressed in other taste receptor cells responsible for sweet, bitter and Umami taste (15,233,234). Moreover, PKD2L1 is also found in neurons surrounding the central canal of the spinal cord where it may play an important role in the long-sought mechanism of proton-dependent regulation of action potential (233), suggesting that PKD2L1 may function to trigger initial cation influx by low pH at the taste pore. It may then lead to activate local voltage-gated cation channels through membrane depolarization, which induces an action potential.

In the tongue, PKD2L1 is localized in all of the taste parts, such as circumvallate, foliate, fungiform and palate, but PKD1L3 is only localized in circumvallate and foliate (233). Moreover, PKD2L1 is also concentrated on the apical membrane of bipolar cells in taste buds (15,233). PKD2L1 was reported to coexpress with PKD1L3 in a subset of the taste receptor cells in specific taste area

(15). The cells expressing PKD2L1 and PKD1L3 are distinguished from taste receptor cells for sweet, bitter and Umami. Very interestingly, PKD2L1 interacts with PKD1L3, which facilitate PKD2L1 targeting to cell space expression. Only various acids, but not other classes of tastants, are capable of activating cells expressing both PKD2L1 and PKD1L3. Therefore, it is suggested that a hetero-complex of PKD2L1 and PKD1L3 may play a crucial role in acid sensing or sour tasting in the tongue.

It is particularly intriguing to note that PKD2L1 has been shown to be found in various cell types such as epithelial, neuronal and endothelial cells. The distribution of PKD2L1 in manifold subcellular compartments appears to depend on cell types. The reason for the varied distribution of PKD2L1 depending on cell type is still unclear but may be a result of alternative splicing of PKD2L1 in different cells. In turn, this may play a crucial role in the diverse function and diverse targeting of PKD2L1 in several cell types (230).

1.10. RACK1

1.10.1 Gene, promoter and expression of RACK1

The Receptor for Activated C kinase 1 (RACK1) is encoded by the gene, *GNB2L1* [guanine nucleotide binding protein (G-protein), β polypeptide 2-like 1]. The gene is comprised of 8 exons and 7 introns and encodes for a protein with 317 amino acids known as a 36-kDa protein (235,236). The promoter region of *GNB2L1* has two alternative start sites of transcription for cardiac/smooth muscle

specific NF-κB (235,237). Normally, RACK1 is ubiquitously expressed in most of the tissues of human and higher mammals such as the brain, liver, and spleen (238,239) and its expression is tightly regulated. For example, expression changes of RACK1 are significantly related to cancer and brain pathologies (240-242), supported by observations that RACK1 inhibits the growth of normal fibroblasts and can be up-regulated in the transformed cells and inhibition of insulin-like growth factor 1 receptor (IGF-1R), which plays an important role in the growth of, over-expressed cells (243-245). Furthermore, RACK1 has 80 binding partners and has effects on the function and subcellular distribution of numerous proteins which leads to the regulation of wide-spread signaling pathways (246,247).

1.10.2. Subcellular localization and translocation of RACK1

RACK1 is localized in versatile cellular compartments including cytosol, endoplasmic reticulum and the nucleus. Especially, RACK1 is found in cell bodies and dendrites but not axons in the neurons (248,249). RACK1 also can be translocated to another subcellular location where it acts in response to a variety of cellular pathways induced by a stimulus. For example, active PKCβII induces the interaction with RACK1, which causes RACK1 to shuttle PKCβII to its active site (250). In hypoxia or heat shock, RACK1 is concentrated into cytoplasmic stress granules, and then prevents the activation of a stress-responsive MAPK pathway, thereby leads to cell survival (251). Moreover, activation of the cAMP/PKA pathway in several cells leads to the translocation of RACK1 to the

nucleus even though RACK1 does not contain the sequence of specific organelle localization motifs for nucleus entry and nucleus export (251-254). There is no conclusive evidence of post-translational modification of RACK1 to be translocated. Therefore, the translocation of RACK1 may be influenced by a group of partner proteins interacting with RACK1.

1.10.3. Roles of RACK1 as a multifunctional protein

RACK1 is a member of the tryptophan-aspartic acid repeat (WD-repeat) family of proteins which is found in prokaryotes and all eukaryotes and is highly conserved (255,256). The WD repeats of RACK1 forms a seven-bladed β-propeller structure (WD40: The WD40 repeat is a structural motif of approximately 40 amino acids, often terminating in a WD dipeptide), with each blade comprised by β-sheets and shares high homology to β subunit of G-proteins (Gβ). Generally, this WD-repeat family of proteins causes numerous molecular interactions (257-260). Each WD40 repeats of RACK1 interacts with several other signaling proteins and therefore, regulates several signal pathways.

Scaffold functions of RACK1

RACK1 functions as a scaffold protein and is well known to interact with multiple protein members of several signaling pathways by tethering them into complexes. Thereby it regulates a lot of signal transductions and properly

organizes signal complexes in specific areas of the cell such as the plasma membrane, the cytoplasm, the nucleus, the Golgi, endosomes, and the mitochondria. Moreover, RACK1 induces positive or negative effects on the activity of many kinases and insulation of signal proteins from phosphatases (261). RACK1 representatively functions as a receptor for activated protein kinase Cβ (PKCβ), PKCδ, and PKCμ and increases the activity of these PKCs as well as shuttle the activated PKCs to proper cellular areas (250,262-264). In contrast to the effect on PKCs, RACK1 inhibits Src kinase activity, which is involved in a family of non-receptor tyrosine kinases and regulates cell growth and adhesion (243). The cytoplasmic domain of the β-subunit of the integrin receptor (β1, β2, β3, and β5 subtypes), which is a membrane spanning receptor, interacts with RACK1 after activation of PKC. This receptor of integrin plays an important role in focal adhesions of the extracellular matrix (243,265,266). RACK1 also binds with another WD-repeat protein Gβ. The formation of RACK1-Gβ heterodimer facilitates cross-talk of signal transduction regulated by G Protein Coupled Receptors GPCRs (267-270). By virtue of RACK1 homodimerization, RACK1 can spread out a range of its binding partners. For example, the formation of RACK1 homodimer allows Fyn, Proto-oncogene tyrosine-protein kinase, to interact with NR2B subunit of the *N*-methyl-D-aspartate receptor (NMDAR) which is a glutamate receptor and is formed as a heterotetramer with two NR1 and two NR2. Both Fyn and NR2B share an identical binding site on RACK1 (252,268-274).

In addition to interacting with various binding partners, RACK1 also has crucial roles in modulating the enzyme activity of its binding partners. For example, RACK1 binds with tyrosine kinases such as Fyn and Src and inhibits their kinase activity before triggering dissociation of RACK1 complexes (252,269,271,273-276). In contrast, RACK1 maintains steady-state condition of protein phosphatase 2A (PP2A) and active conformation of PKCβII. Therefore, RACK1 performs a lot of important roles by stabilizing the active and inactive condition of its binding partners (245,250,262,277,278).

1.11. Objective, hypotheses and rationale

Objectives

The long-term objective of this study is to elucidate the molecular mechanism of ADPKD pathogenesis. For this, my specific objectives are 1) to investigate how PKD2 regulates cell proliferation through the PERK-eIF2α pathway, 2) to investigate molecular mechanisms underlying the regulation of PKD2 protein translation by cellular stress and its 5'UTR of mRNA, and 3) to study physical and functional interaction between PKD2L1 and scaffolding protein RACK1.

Hypotheses

1) that PKD2 enhances the kinase efficiency of PERK in phospharylating eIF2α thereby repressing cell proliferation, 2) that cellular stresses and P-eIF2α translationally up-regulates PKD2 protein expression through acting on the 5'UTR uORF sequence of its mRNA, and 3) that RACK1 inhibits PKD2L1 channel function through physical binding to the N-terminus of the channel.

Rationale

To elucidate the molecular mechanisms underlying ADPKD pathogenesis it is essential to understand how dysfunction of polycystins results in cellular abnormalities such as over-proliferation, apoptosis and de-differentiation. Thus, it is necessary to investigate how normal and dysfunctional PKD2 impact cellular pathways and components that regul**a**te cell proliferation and apoptosis. In general, regulation of protein expression occurs transcriptionally and translationally. Human, monkey and mouse PKD2 has a uORF in the 5'UTR of its mRNA and its roles in regulating PKD2 expression remains unknown. And because too high or too low PKD2 expression is all pathogenic, it is thus important to determine the roles of the uORF. Cultured HEK293T cells, HeLa cells, MDCK and MEF cells with deficient PERK ($PERK^{-/-}$) are very suitable models, in combination with gene over-expression/knock-down, chemical treatments. For this we employed several interdisciplinary techniques, including WB, in vitro and in vivo protein-protein interaction methods, immunofluorescence, polysome analysis, and luciferase assays, among others. These techniques are particular-appropriate for cultured mammalian cells. We are aware of limitations of cultured cells, so

current studies in Dr. Chen's laboratory include the use of more in vivo models such as PKD2 and PKD2L1 KO mice for future studies.

PKD2L1 is a homolog of PKD2 but PKD2L1 is not related to ADPKD. PKD2L1 was reported as a Ca^{2+}-modulated nonselective cation channel. However, it is still unclear how PKD2L1 is regulated by Ca^{2+} and interacting partners, which is critical for subsequently elucidating its cellular and physiological roles. PKD2L1 mainly targets to the intracellular ER membrane in common cultured mammalian cells such as HEK, HeLa and IMCD cells. However, in *Xenopus* oocytes it is substantially targeted to the PM. Oocyte is therefore a suitable model for studying its function and regulation through measuring channel activity by electrophysiology. We also use other techniques, suitable for oocytes, such as mutagenesis, WB, in vitro and in vivo protein-protein interaction, biotinylation, immunoflouroscene.

1.12. REFERENCES

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CHAPTER 2

RESULT #1

Polycystin-2 down-regulates cell proliferation via promoting PERK dependent phosphorylation of eIF2α

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Genqing Liang#, JungWoo Yang#, Zuocheng Wang, Qiang Li, Yan Tang and Xing-Zhen Chen. (2008) Polycystin-2 down-regulates cell proliferation via promoting PERK-dependent phosphorylation of eIF2α. ***Human Molecular Genetics*, 17, 3254–3262, 2008.** # Co-first author

2.1 ABSTRACT

ADPKD is characterized by the formation of renal, hepatic and pancreatic cysts and by non-cystic manifestations such as abnormal vasculature and embryo left–right asymmetry development. Polycystin-2 (PC2), in which mutations account for 10–15% in ADPKD, was previously shown to down-regulate cell proliferation, but the underlying mechanism was not elucidated. Here, we demonstrate that PC2, but not pathogenic mutants E837X and R872X, represses cell proliferation through promoting the phosphorylation of eukaryotic translation initiation factor eIF2α by PERK. ER stress is known to enhance eIF2α phosphorylation through up-regulating PERK kinase activity (assessed by phosphorylated PERK). During ER stress, PC2 knock-down also repressed eIF2α phosphorylation but did not alter PERK phosphorylation, indicating that PC2 facilitates the eIF2α phosphorylation by PERK. PC2 was found to be in the same complex as PERK and eIF2α.

Together, we demonstrate that PC2 negatively controls cell growth by promoting PERK-mediated eIF2α phosphorylation, presumably through physical interaction, which may underlie a pathogenesis mechanism in ADPKD and indicates that PC2 is an important regulator of the translation machinery.

2.2. INTRODUCTION

ADPKD is the most common form in PKD and occurs in 0.1–0.2% of adults (1-3). Renal pathogenic polycysts in ADPKD are fluid-filled epithelial-lined cavities arising from glomeruli, nephron tubules and collecting ducts (4,5). ADPKD can also result in cerebral and intracranial aneurysms, cardiovascular abnormalities (e.g. cardiac valve disorders), aberrant embryonic left–right asymmetry development and hypertension (6,7). The majority (95%) of the ADPKD cases are caused by mutations in the *PKD1* or *PKD2* gene that encodes polycystic kidney disease 1 (PKD1 or PC1) or PKD2 (PC2) (8,9). PKD1 is an integral membrane glycoprotein and acts as a G-protein-coupled receptor. PC2 is a non-selective cation channel and is mainly localized on the ER membrane as a Ca^{2+} release channel (10,11).

The mechanisms of how ADPKD results in overproliferation of renal epithelial cells are not clearly understood, and different pathways have been reported to be implicated (12-15). PKD1 regulates cell growth of renal tubular epithelial cells through p53 induction and JNK activation (15). PC2 was also reported to suppress cell growth and branching morphogenesis in kidney epithelial cells, but the underlying mechanism remains unknown (13). Bhunia et al. (12) showed that PKD1 and PC2 together regulate cell cycle through inducing p21 and activating the JAK-STAT signaling pathway. On the other hand, Li et al. (14) demonstrated that PC2 undergoes PKD1-dependent phosphorylation, which

enhances its interaction with the helix–loop-helix inhibitor ld2, to regulate cell growth and differentiation.

The ER is critical to the synthesis, modification, folding and quality control of both secretory and membrane protein (16). Conditions disrupting ER homeostasis can cause unfolded protein accumulation that constitutes a fundamental threat to all living cells and triggers unfolded protein response (UPR), which is currently termed ER stress (17). During the ER stress response or UPR, ER-resident molecular chaperones and foldases are induced to enhance the folding capacity of the ER, and translation is attenuated to reduce the biosynthetic load of ER (17,18). Current studies of the UPR mechanism in mammalian cells have identified three branches of the signaling pathway, represented by three types of ER-transmembrane proteins: pancreatic ER eIF2α kinase (PERK) leading to phosphorylation of eIF2α that causes translational repression, activating transcription factor 6 (ATF6) which induces the expression of chaperones such as GRP78, and inositol requiring 1 (IRE1) which initiates spliceosome-independent splicing of XBP1 mRNA, leading to the activation of ER chaperone genes.

Type I ER membrane protein PERK was recently reported to be involved in controlling cell growth (19,20). PERK contains a luminal ER stress-sensing N-terminal domain and a large C-terminal cytoplasmic kinase domain (18,21). During ER stress, PERK is activated by autophosphorylation via homodimerization. The phosphorylated (or activated) PERK (P-PERK) subsequently binds and phosphorylates its subunit eIF2α (21,22), which inhibits global protein synthesis and cell-cycle factors such as cyclin D1, and results in the

repression of cell proliferation (19,20,23). We demonstrate here that PC2 negatively regulates cell proliferation through enhancing PERK-dependent eIF2α phosphorylation. Our study suggests that PC2 associated ADPKD pathogenesis is due at least in part to dysregulation of PERK-dependent cell growth.

2.3. MATERIALS AND METHODS

Cell culture, DNA constructs and gene transfection

IMCD, MDCK and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (high glucose; Invitrogen) containing 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin at 37 ℃ and 5% CO_2. $PERK^{+/+}$ and $PERK^{-/-}$ MEF cells were maintained as described previously (24). Plasmids pEGFP-PC2, pEGFP-R872X, pEGFP-E837X, pEGFP-PC2ΔC, pEGFP-PC2ΔN and pEGFP-PC2ΔNC were described previously (25,26). HEK293T cells were grown to ~70% confluency prior to transfection using Lipofectamine 2000 (Invitrogen). MDCK cells stably expressing GFP-PC2 or GFP were selected as previously described (27) and maintained using G418 (300 mg/ml).

Immunoprecipitation and immunoblotting

Protein extraction, immunoblotting and immunoprecipitation (IP) were performed, as described earlier (26). Typically, 20 and 200 μg of total cellular protein were used for immunoblotting and IP, respectively. HEK293T cells were transiently transfected with pEGFP, pEGFP-PC2, pcDNA3.1 (Invitrogen) or pcDNA3.1-PERK (20,21) for IP. At 40 h post transfection, cells were used for protein extraction and precipitation. To examine the effect of PC2 mutants on eIF2α phosphorylation, we transfected HEK293T cells with pEGFP-PC2, pEGFP-R872X, pEGFP-E837X, pEGFP-PC2ΔC, pEGFP-PC2ΔN, pEGFP-PC2ΔNC or vector pEGFP. At 40 h post-transfection, cell extraction was prepared for immunoblotting.

Immunofluorescence microscopy and quantitative analysis

Immunofluorescence microscopy was performed as described earlier (26). Briefly, HEK293T cells were transiently transfected with Myc-PC2 or siRNA, mixed at the 20:80 ratio after 24 h of transfection, and then grown on cover slips. At 48 h post-transfection, cells were subject to Tg treatment for 1 h before fixation. P-eIF2α and PC2 antibodies were used for double staining. Fluorescence images were captured on a motorized Olympus IX81 microscope with a CCD cooling RT SE6 monochrome camera (Diagnostic Instruments). Final composite images were made using Image-Pro Plus 5.0 (Media Cybernetics). The quantification of expression density was performed using Image J. The area of each cell was manually defined for the analysis of both green and red signal densities. A cluster of confluent cells was defined as one cell for density calculation. For each image that contains multiple cells, the average density for red or green color was normalized to 100 after background subtraction. The correlation coefficient was obtained from linear regression using Sigmaplot 10 (Systat Software Inc.).

Antibodies

Rabbit antibodies against eIF2α and P-eIF2α (S51), and mouse anti-Myc antibody were purchased from Cell Signaling Technology. Human anti-PERK and anti-P-PERK antibodies were from R&D Systems and Biolegend, respectively. β-actin antibody was from Sigma-Aldrich, Canada. Goat anti-GRP78, rabbit anti-

ATF6α/ATF4 and mouse anti-PERK were purchased from Santa Cruz. GFP antibodies were a gift of Dr Luc Berthiaume (University of Alberta; also available at www.eusera.com). Mouse monoclonal anti-PKD antibody (1A11) was described as before (26,28). Secondary antibodies were from Amersham or Promega.

PERK and PC2 knock-down by small interfering RNA (siRNA)

PERK Stealth siRNA (Invitrogen, EIF2AK3-HSS114058, -059 and -060) and control siRNA (Invitrogen, Cat# 46-2002) were used to transfect HEK293T cells using Lipofectamine 2000 reagent following the manufacturer's instructions. PC2 knockdown was described previously (28). The efficiency of the siRNA knockdown was assessed by immunoblotting.

Cell proliferation assays

HEK293T cells were transfected with plasmid pEGFP-PC2 or PC2 siRNA in 35 mm dishes (Fig. 2-6B and C). At 24 h post-transfection, cells were split and seeded into eight separate wells of a 96-well plate. After incubation for another 30 h, luminescence activity was measured using a cell viability assay kit (Promega, Cat# G7571) and an illuminometer (Fluoroskan Ascent FL, Thermo Labsystems). The remaining cells in the 35 mm dishes were further cultured for immunoblotting. For testing the effect of PERK knock-down on PC2-regulated cell proliferation (Fig. 2-6D), HEK293T cells were transfected with pEGFP- PC2 or vector pEGFP using 35 mm dishes. At 6 h post-transfection, cells were split

into two equal fractions for subsequent transfection with PERK or control siRNA at 24 h post-transfection. Forty-eight hours after pEGFP- PC2 transfection, cells were cultured in a 96-well plate for the cell growth assay described above. MDCK stable cell lines were serum-starved overnight and then grown in the presence of serum for 48 h in a 96-well plate before measuring luminescence activity. The efficiency of siRNA knock-down and PC2 expression were assessed by immunoblotting and/or immunofluorescence microscopy.

Metabolic labeling

To measure protein translational rates, HEK293T cells were transfected with plasmid pEGFP- PC2 or vector pEGFP. At 40 h post-transfection, cells were starved for 1h in the prelabeling medium (L-methionine and L-cystin-depleted DMEM with 10% FBS and penicillin/streptomycin) (Invitrogen), followed by pulse-labeling with 50 μCi of [35S] methionine/cystine (35S-Protein Labeling Mix, Perkin Elmer) for 10 min. Cell extracts were applied for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Data analysis

Student's t-test was used for statistical significance analyses. Data were expressed in the form of mean+SE (N), where SE is the standard error and N is the number of experiment repeats. Probability values (P) of less than 0.05 and 0.01 were considered significant and very significant, respectively.

2.4. RESULTS

PC2 facilitates the eIF2α phosphorylation by PERK

We recently reported that Herp, a regulator of ER-associated degradation (ERAD) (29-32), interacts with PC2 and regulates its expression (26). Here, we started to test whether PC2 is involved in the regulation of UPR activated during ER stress (17,18). Immunoblotting experiments revealed that PC2 over-expression increases the phosphorylated eIF2α (P-eIF2α) level by 227 ± 7% ($P < 0.001$, $N = 3$) in human embryonic kidney (HEK) 293T cells (Fig. 2-1A), with quantitative analysis performed using the program Image J (v1.41c, NIH, http://rsb.info.nih.gov/ij/). Similar results were also shown in human melanoma A7 and Madin–Darby canine kidney (MDCK) cells (Fig. 2-1B and Fig. 2-2A). In the presence of ER stress induced by thapsigargin (Tg) through ER Ca^{2+} depletion, P-eIF2α substantially augmented, as expected. The combination of PC2 over-expression and Tg treatment, compared with Tg treatment alone, did not significantly further increase the P-eIF2α level (103% ± 5%, $P = 0.29$, $N = 3$), possibly because of a saturated P-eIF2α level induced by Tg. Of important note, PC2 over-expression had no effect on immunoglobulin heavy-chain binding protein (BiP, an ER chaperone protein) (Fig. 2-1A and B) and splicing of XBP-1 mRNA (Fig. 2-2B), both of which are activated during UPR (17,21), indicating that high levels of PC2 do not cause ER stress leading to UPR. This suggests that the effect of PC2 over-expression is not through triggering ER stress. It is known that increased P-eIF2α attenuates protein synthesis and selectively up-regulates

the translation of the activating transcription factor 4 (ATF4) (33). We next tested the effects of PC2 on these down-stream signals of P-eIF2α. Compared with control, GFP-expressing cells, GFP-PC2-expressing cells exhibited profound

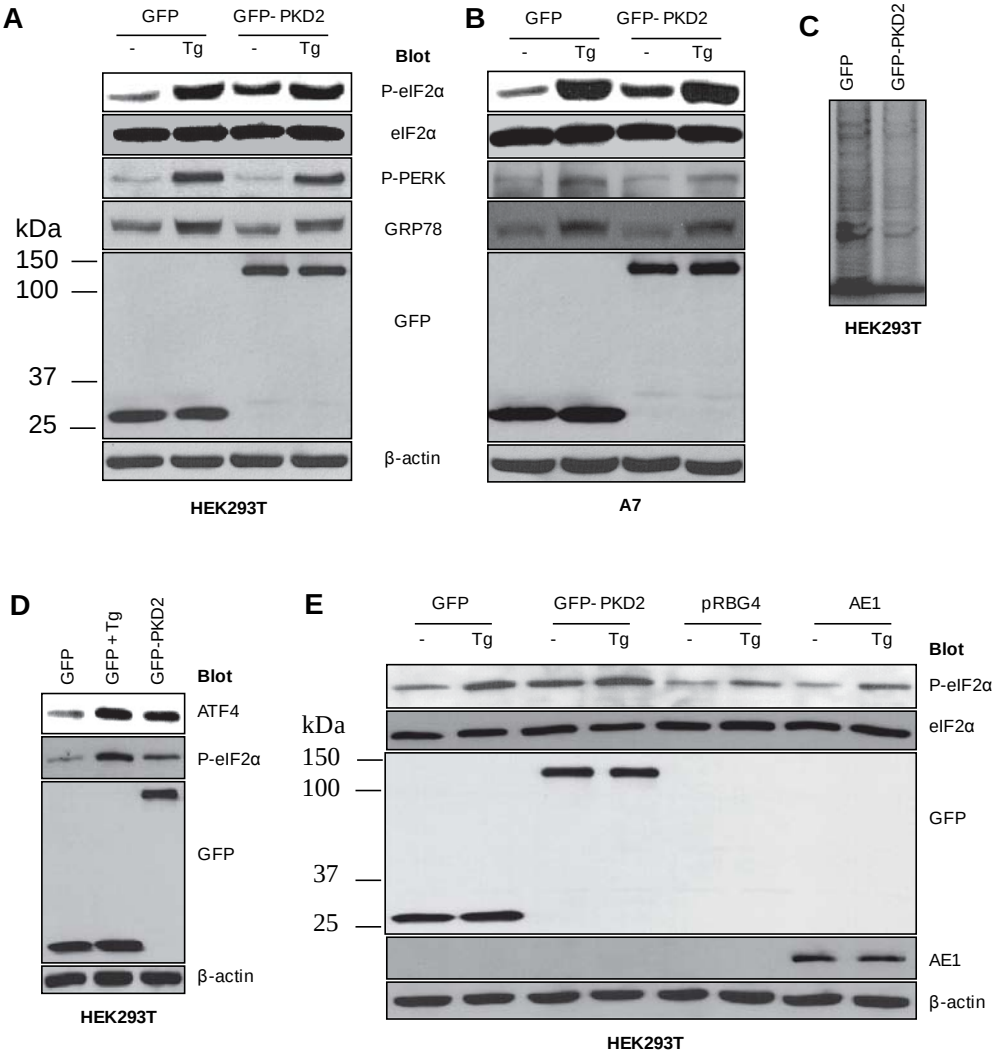

Fig. 2-1. Effects of over-expressed PC2 on eIF2α phosphorylation. (A) and (B) Effect of PC2 on eIF2α phosphorylation. Extracts of HEK293T cells transiently transfected with GFP-PC2 for 40 h (A), and A7 cells with a stable expression of GFP-PC2 (B) were used for immunoblotting with an antibody against P-eIF2α, eIF2α, GRP78 or GFP. Treatment with Tg (0.5 μM for 1 hour (h)) was used as a positive control. β-actin was used as a loading control. (C) Effect of PC2 on global protein synthesis. HEK293T cells expressing GFP-PC2 or GFP were labeled with [35S] methionine/cystine for 10 min. Whole-cell extracts were resolved by SDS–PAGE and viewed by autoradiography (upper panel). P-eIF2α and eIF2α, in the same cell extracts, were revealed by Western blotting. (D) Effect of PC2 on ATF4 translation. Extracts of HEK293T cells transiently transfected with GFP-PC2 for 40 h were used for immunoblotting with an antibody against ATF4, P-eIF2α or GFP. (E) Effect of AE1 on eIF2α phosphorylation. Extracts of HEK293T cells transfected with plasmid pEGFP, pEGFP-PC2, pRBG4 or pRBG4-AE1 were subjected to immunoblotting with a P-eIF2α, eIF2α, GFP, AE1 or β-actin antibody. Tg treatment was a positive control.

attenuation of global translation rates, as demonstrated using [35S] methionine/cystine incorporation (Fig. 2-1C). Consistently, the protein synthesis rate negatively correlated with the level of P-eIF2α (Fig. 2-1C). In addition, Western blotting showed that ATF4 expression is increased in cells expressing GFP-PC2, compared with control cells (Fig. 2-1D). Thus, these effects of PC2 are consistent with the reported down-stream signaling of eIF2α phosphorylation. Also, we found that over-expressed human anion exchanger AE1 (Gift of Dr Joe Casey, University of Alberta) in HEK293T cells has no effect on the P-eIF2 level (Fig. 2-1E), further supporting the specificity of the PC2-mediated up-regulation of P-eIF2.

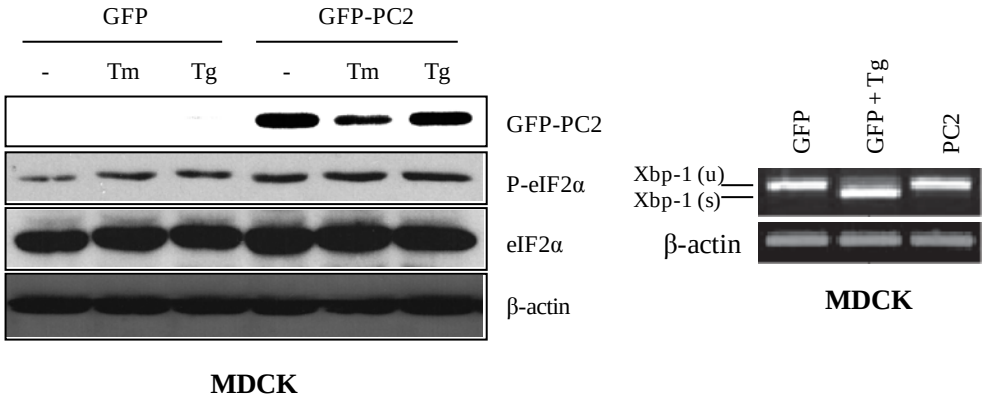

Fig. 2-2. Effect of PC2 on eIF2α phosphorylation and Xbp-1 mRNA splicing (A) Extracts of MDCK cells stably expressing GFP-PC2 were used for immunoblotting with the P-eIF2α, eIF2α or GFP antibody. Treatment with Tg (0.5 μM for 1 h) and Tm (tunicamycin, 2.4 μM for 1 h) were used as positive controls. (B) MDCK stably expressing GFP cells treated with (positive control) or without (negative control) Tg (0.5μM, 1 h), and MDCK stably expressing GFP-PC2 cell were prepared for RT-PCR.

We further tested the effect of PC2 on eIF2α phosphorylation by small interference RNA (siRNA) knock-down (26,28). PC2 siRNA caused robust reduction in the PC2 and P-eIF2α levels in HEK293T cells and mouse inner medullary collecting duct (IMCD) cells (Fig. 2-3A and B). Thus, PC2 is a

regulator of eIF2α phosphorylation. eIF2α is known to be phosphorylated by four Ser/Thr protein kinases (activated by different stressinducers): GCN2 (activated by nutrition limitation and UV irradiation), PERK (by ER stress), PKR (by double-stranded RNA and viral infection) and HRI (oxidative stress) (34).

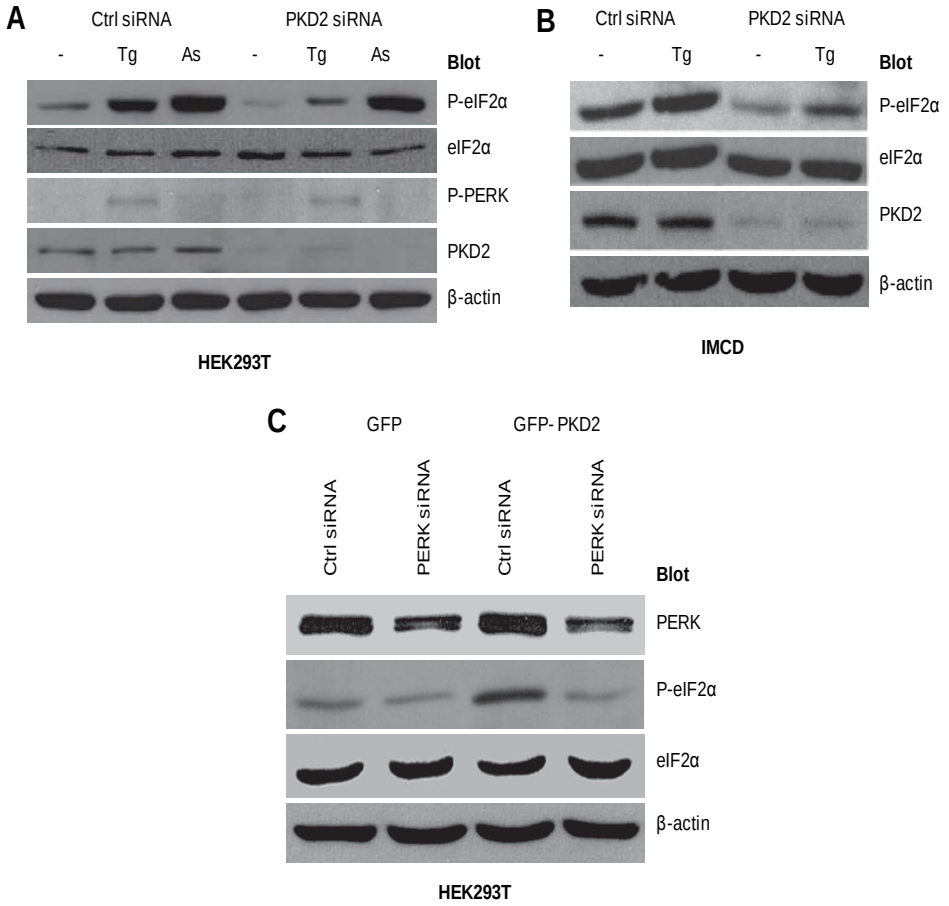

Fig. 2-3. Effects of PC2 and PERK knock-down on eIF2α phosphorylation. (A) and (B) Alteration of eIF2α phosphorylation by PC2 knock-down. HEK293T (A) and IMCD (B) cells were transfected with PC2 siRNA or control siRNA (Ctrl) (20 ml of 20 mM in 35 mm dishes) for 40 h with or without arsenite (As, 200 mM for 1 h) or Tg (0.5 mM for 1 h) before immunoblotting. (C) Effect of PERK knock-down on PC2-induced eIF2α phosphorylation. Extracts of HEK293T cells co-transfected with plasmid GFP-PC2 and PERK siRNA were subjected to immunoblotting with a PERK, P-eIF2α, eIF2α or β-actin antibody.

To identify the kinase(s) that is (are) responsible for the PC2-regulated eIF2α phosphorylation, we examined effects of different stress inducers on eIF2α phosphorylation in HEK293T cells with or without PC2 knock-down. Our data

showed that the effect of Tg (Fig. 2-3A), but not that of oxidative inducer arsenite (Fig. 2-3A), amino acid (aa) depletion or osmotic shock (data not shown), on eIF2α phosphorylation substantially diminished with the PC2 knock-down. A similar effect of PC2 knock-down was observed in IMCD cells (Fig. 2-3B). These results indicate that PC2 is crucial for ER stress-induced eIF2α phosphorylation. As PERK is known to be the major kinase phosphorylating eIF2α during ER stress (18,21), we tested whether PERK is involved. Indeed, PERK knock-down significantly repressed PC2-induced eIF2α phosphorylation in HEK293T cells (Fig. 2-3C). Together, our data showed that PC2 promotes eIF2α phosphorylation by the ER-resident eIF2α kinase PERK. In contrast, we found that PERK phosphorylation is not affected by either over-expression or knock-down of PC2 (Fig. 2-1A and B, and Fig. 2-3A), which indicates that PC2 is a crucial factor that facilitates eIF2α phosphorylation by phosphorylated (i.e. activated) PERK.

Positive correlation between the PC2 and P-eIF2α levels revealed by immunofluorescence

We performed co-immunofluorescence experiments to further document the regulation of P-eIF2α by PC2. To enlarge the range of PC2 expression levels in the same images taken by the microscope, 24 h prior to experiments, we mixed HEK293T cells transiently expressing PC2with those with PC2 siRNA knock-down at the 20:80 ratio. We found that the PC2 expression level significantly correlates with that of P-eIF2α, with a correlation coefficient of 0.82 based on 290 randomly selected cells (Fig. 2-4A and B) and that PC2 and P-eIF2α partially

colocalizes in perinuclear regions (Fig. 2-4A). Mixing native HEK293T cells with those with PC2 knock-down by siRNA at the 20:80 ratio produced similar results

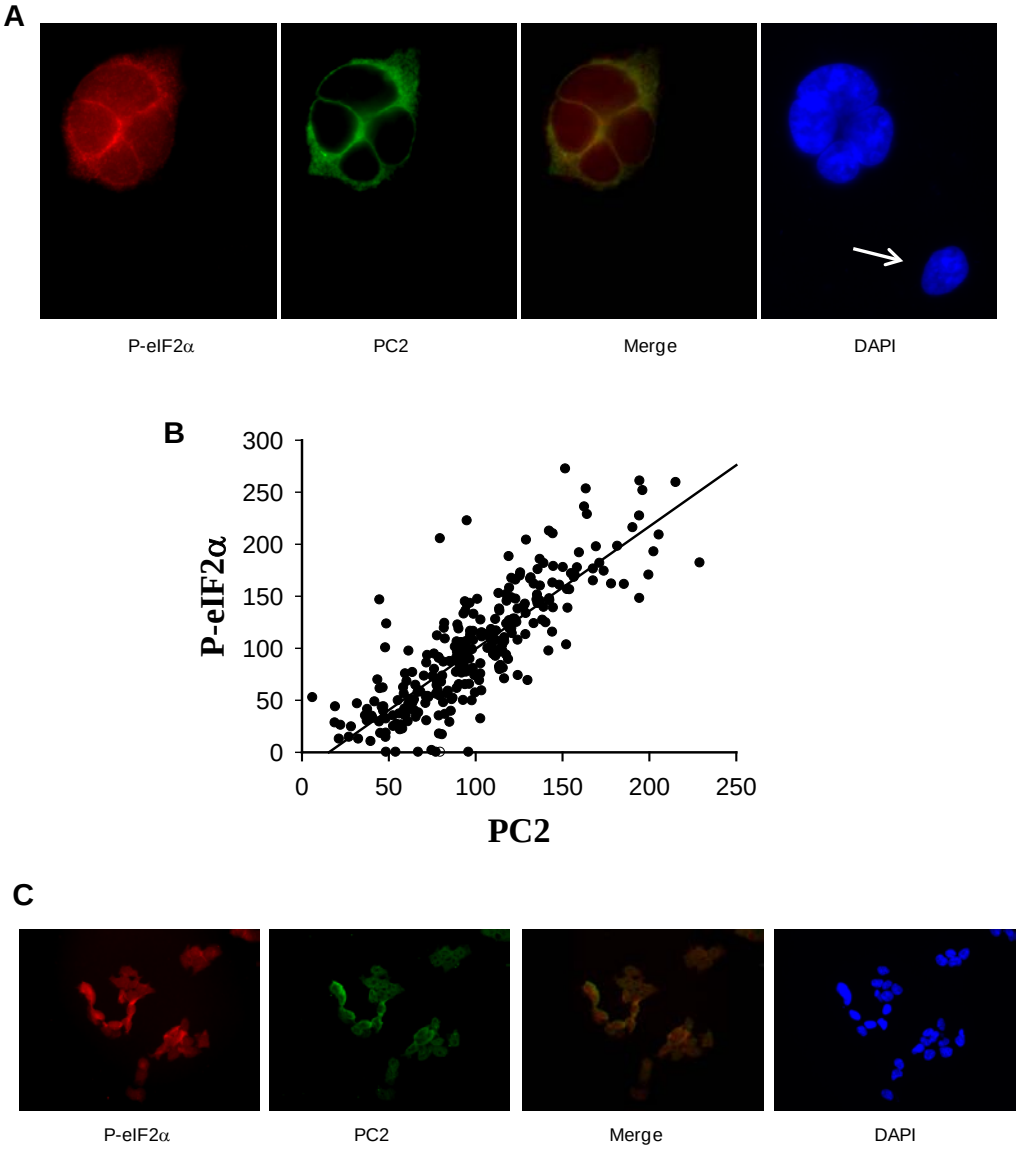

Fig. 2-4. Correlation between the PC2 and P-eIF2α levels revealed by immunofluorescence. (A) Co-immunofluorescence of PC2 and P-eIF2α from a representative image. Cells were stained with antibodies against P-eIF2α and PC2. The arrow indicates a cell with low expression of PC2 and P-eIF2α. (B) Relationship between normalized cellular levels of PC2 and P-eIF2α obtained from 290 randomly selected HEK293T cells. Cells were a mix of those expressing Myc-PC2 and those with PC2 siRNA knock-down, at the 20:80 ratio. The straight line was a linear fit using Sigmaplot 10, resulting in a correlation coefficient of 0.82. (C) Co-immunofluorescence of PC2 and P-eIF2α in a mix of HEK293T cells with PC2 siRNA or control siRNA at the 20:80 ratio. Cells were stained with the P-eIF2α and PC2 antibodies.

(Fig. 2-4C), with a correlation coefficient of 0.59 based on 107 randomly selected cells. These results support the concept that PC2 promotes the phosphorylation of P-eIF2α by the ER membrane kinase PERK, leading to the accumulation of P-eIF2α in perinuclear regions.

PERK is required for PC2-regulated cell proliferation

It was previously shown that PERK represses cell growth by inducing eIF2α phosphorylation to attenuate protein synthesis and by down-regulating cyclin D1 required for cell-cycle exit from the G1 phase (19,20,23). We thus reasoned that the reported down-regulation of cell growth by PC2 may be through the PERK-eIF2α signaling pathway. We measured cell growth rate using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) as a function of alterations in the PC2 and PERK levels. We found that an increased (or decreased) PC2 expression significantly down-regulates (or up-regulates) proliferation of MDCK and/or HEK293T cells (Fig. 2-5), consistent with the reported anti-proliferative effect of PC2 (12-14). The PC2-mediated suppression of cell growth was significantly inhibited in HEK293T cells by PERK knock-down (Fig. 2-5D), which is in parallel to the inhibition of the PC2-promoted eIF2α phosphorylation by PERK knock-down (Fig. 2-3C). In contrast, PERK knock-down slightly, but not significantly, increased the rate of cell growth in our assays. Overall, these results indicate that PERK and eIF2α play key roles in the negative control of cell proliferation by PC2.

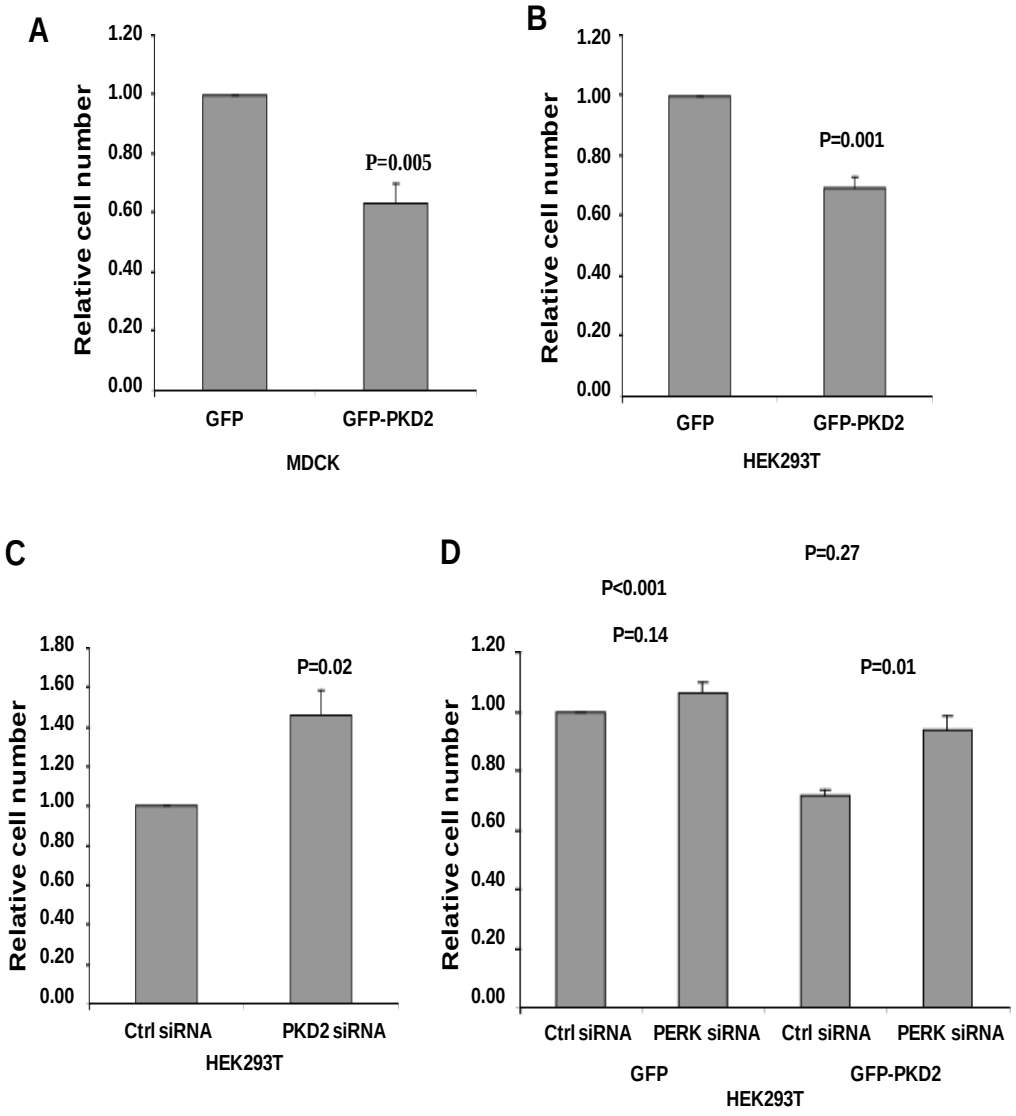

Fig. 2-5. Effects of PC2 and PERK on cell proliferation. Five thousand cells from MDCK cells stably (A) and HEK293T cells transiently (B) expressing GFP-PC2 or GFP were plated in multiple wells of a 96-well plate and grown for 48 h for cell proliferation assay (see Materials and Methods). (C) Five thousand cells from HEK293T cells with siRNA transfection for 24 h were plated in multiple wells of a 96-well plate and grown for 30 h for cell proliferation assay. (D) Five thousand cells from HEK293T cells expressing GFP-PC2 or GFP with PERK siRNA transfection for 24 h were plated in multiple wells of a 96-well plate and grown for 30 h for cell proliferation assay.

PC2 physically interacts with PERK and eIF2α

We next explored the possible association of PC2 with PERK and eIF2α. For this end, we immunoprecipitated extracts of IMCD cells using an anti-PC2

antibody (26,28). We found that PERK and eIF2α, but not the ER membrane protein ATF6a (negative control) (35), co-precipitate with PC2 (Fig. 2-6A).

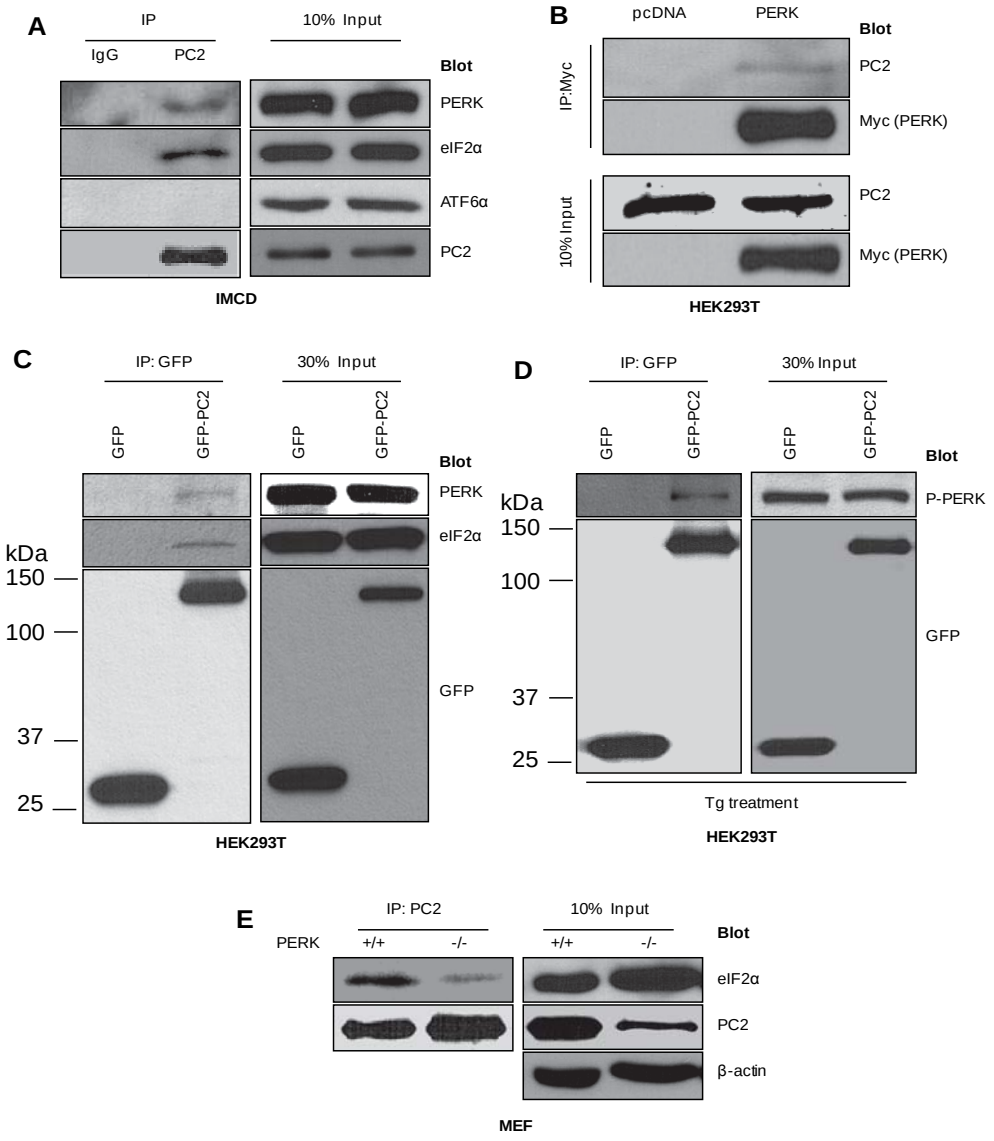

Fig. 2-6. Association of PC2 with PERK and eIF2α. (A) Extracts of IMCD cells were used for IP with an anti-PC2 antibody. The precipitates and an input (10%) were detected by immunoblotting with a PERK, eIF2α, ATF6α or PC2 antibody. (B) HEK293T cells were transiently transfected with a C-terminal Myc-tagged PERK plasmid pcDNA-PERK for 40 h, followed by protein preparation and IP with a Myc antibody. The precipitates and an input (10%) were immunoblotted by PC2 or Myc antibody. (C) and (D) Extracts of HEK293T cells transfected with pEGFP-PC2 or pEGFP (5 mg in 100 mm plates) for 40 h without (C) or with (D) Tg treatment, as described earlier, were subjected to IP with the GFP antibody. The precipitates and an input (30%) were detected by immunoblotting with the PERK, P-PERK, eIF2α or GFP antibody. (E) Role of PERK in the PC2-eIF2α interaction. Extracts of $PERK^{+/+}$ and $PERK^{-/-}$ MEF cells were used for IP with the anti-PC2 antibody. The precipitates and an input (10%) were detected by immunoblotting with the eIF2α or PC2 antibody.

Reciprocally, PC2 was immunoprecipitated by PERK in HEK293T cells expressing Myc-tagged PERK (Fig. 2-6B). Also, PC2 was associated with PERK and eIF2α in HEK293T cells over-expressing GFP-PC2 (Fig. 2-6C). These data demonstrate that PC2 is in the same complex as PERK and eIF2α. Because the eIF2α phosphorylation by the activated PERK is facilitated by PC2, we wanted to examine whether PC2 also interacts with the activated PERK. For this, ER stress was induced by Tg in HEK293T cells to stimulate the P-PERK level. Our data showed that PC2 indeed interacts with P-PERK (Fig. 2-6D). Finally, we examined the role of PERK in the PC2-eIF2α interaction, utilizing $PERK^{+/+}$ and $PERK^{-/-}$ murine embryonic fibroblast (MEF) cells. We observed that the amount of PC2-bound eIF2α is sharply decreased in $PERK^{-/-}$ MEF cells (Fig. 2-6E), suggesting that PERK mediates the PC2-eIF2α interaction, possibly by forming a PC2-PERK-eIF2α complex. Together, our data indicate that PC2promotes the eIF2α phosphorylation by P-PERK, presumably through physically facilitating the action of P-PERK on eIF2α. Interestingly, we found that PC2 expression is significantly reduced in PERK-deficient MEF cells (Fig. 2-6E, input). How PERK reversely regulates PC2 is unclear and may be a subject of future studies.

Pathogenic mutations in PC2 abrogate the regulation of eIF2α phosphorylation and cell proliferation by PC2

We investigated how PC2 mutations affect its effect on cell proliferation and eIF2α phosphorylation, using HEK293T cells transiently expressing one of the following GFP-tagged PC2 mutants: PC2 (positive control), R872X, E837X,

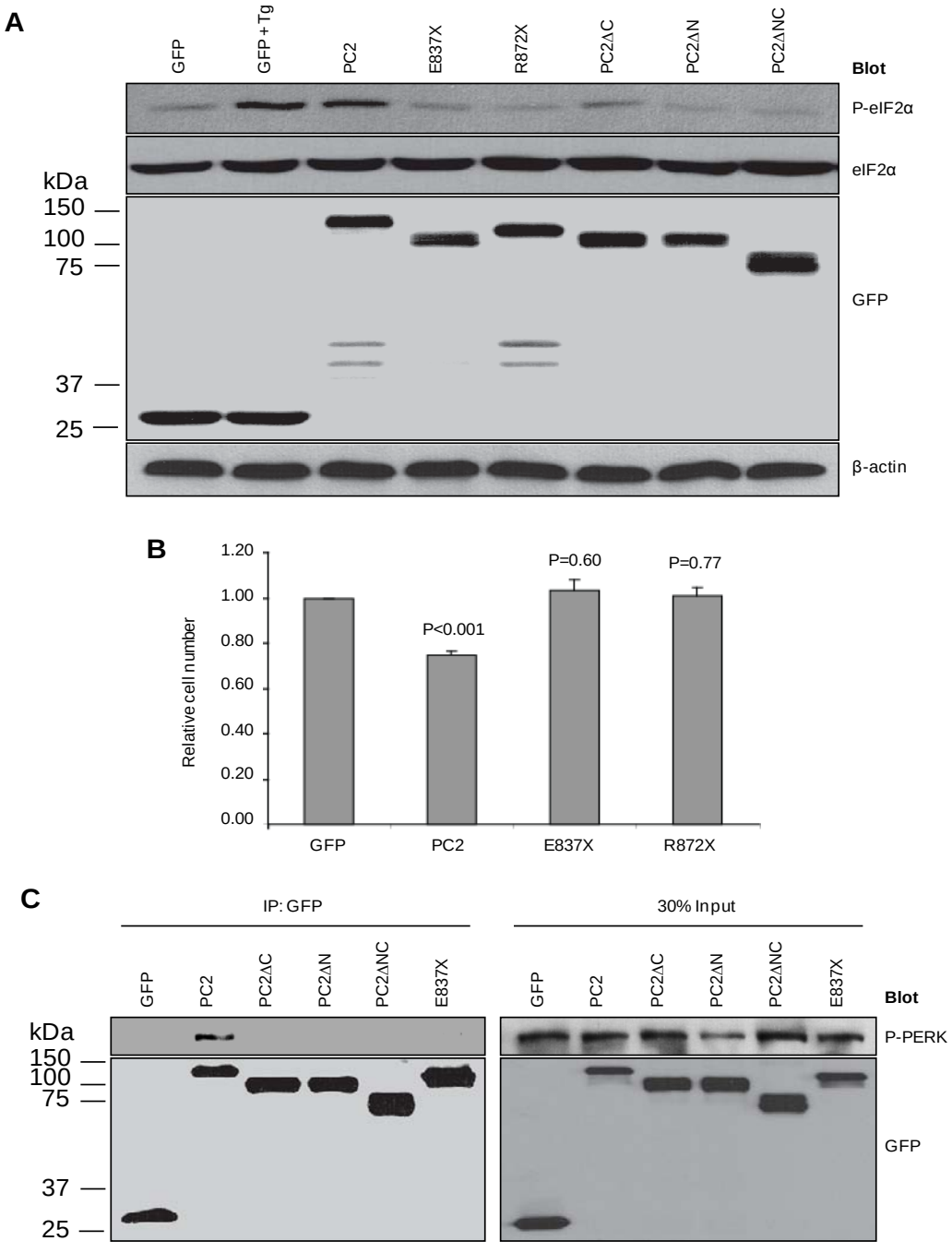

Fig. 2-7. Effects of PC2 mutations on cell proliferation and eIF2α phosphorylation. (A) Extracts of HEK293T cells transfected with GFP-PC2 or a PC2 mutant were subjected to immunoblotting with a P-eIF2α, eIF2α, GFP or β-actin antibody. Tg treatment was a positive control. (B) Five thousand cells from HEK293T cells expressing GFP, GFP-PC2, E837X or R872X were utilized for cell growth assay, as described earlier. (C) Interaction of PC2 mutants with P PERK. Extracts of HEK293T cells transfected with pEGFP, pEGPF-PC2 or a PC2 mutant for 48 h and treatment by Tg for 1 h were subjected to IP with the GFP antibody and immunoblotting with the P-PERK or GFP antibody. Cell extracts (input, 30%) were utilized for detecting transfection efficiency.

PC2ΔC (or S689X, aa 1–688, lacking the C-terminus), PC2ΔN (aa 209–968, lacking the N-terminus), PC2ΔNC (aa 209–688) and GFP (negative control) (26). We found that, unlike Tg treatment and WT PC2 (positive controls), these truncation mutants were unable to stimulate eIF2α phosphorylation (Fig. 2-7A). Using the cell viability assay described earlier, we found that pathogenic mutants R872X and E837X has no effect on the rate of cell growth (Fig. 2-7B). These data indicate that PC2 exerts its effect on eIF2α phosphorylation through physical association with PERK. Thus, the inability of PC2 pathogenic mutants in negatively controlling cell growth through stimulating eIF2α phosphorylation may lead to cyst formation. Further, PC2ΔC, PC2ΔN, PC2ΔNC and E837X lost the association with P-PERK (Fig. 2-7C), suggesting that the PC2-PERK physical interaction is critical for PC2-mediated cell proliferation and eIF2α phosphorylation.

2.5. DISCUSSION

ER stress increases the kinase activity of PERK to promote eIF2α phosphorylation, which results in translational repression and activation of down-stream signals that down-regulate cell growth (18-23). Renal cystogenesis, caused by loss-of-function mutations in PKD1 or PC2, is characterized by over-proliferation, de-differentiation and so on, but how PC2 negatively controls cell growth remains unclear. In this study, we demonstrate that PC2 regulates cell proliferation through the PERK-eIF2α phosphorylation signaling pathway.

Our data show that induction of eIF2α phosphorylation and inhibition of cell growth by PC2 are blocked in cells with the treatment of PERK siRNA, indicating that PERK plays a critical role in this event. Interestingly, among the four eIF2α kinases, only PERK is localized to the ER membrane where the ER Ca^{2+} release channel PC2 is localized. PC2, PERK and eIF2α are in the same complex, suggesting that PC2 may play a role in recruiting eIF2α to form PERK–substrate complexes for eIF2α phosphorylation. This was confirmed by the observation that repression of PC2 expression dramatically inhibits ER stress-induced eIF2α phosphorylation, which is promoted mainly by PERK (18,21). Furthermore, PC2 pathogenic mutations lose the capability of inhibiting cell proliferation and of stimulating eIF2α phosphorylation. On the basis of these data, we propose that the PERK-eIF2α pathway be part of a novel molecular mechanism underlying the PC2-associated ADPKD.

Controlling cell over-proliferation in PKD is a key principle of therapeutic treatments such as the use of antagonists of vasopressin receptors and mammalian target of rapamycin (mTOR). mTOR antagonist, rapamycin, effectively alleviates cyst development in several animal models of PKD and in human ADPKD patients (36). In fact, PKD1 interacts with mTOR to suppress the up-regulation of cell proliferation by mTOR, in which translation initiation factor eIF4E is involved (36). The current study shows that protein synthesis inhibitor P-eIF2α mediates negative control of cell growth by PC2. Therefore, the negative control of cell growth by PKD1 and PC2, either individually or as a complex (12,14) may be through the same thread, i.e. protein synthesis initiation machinery. However, it cannot be excluded that PERK, by interacting with PC2, affects PKD1-regulated cell proliferation, in which PC2 phosphorylation is required (12,14). In summary, this study shows that PC2 is an important regulator of the cellular translation machinery and may underlie a pathogenesis mechanism in ADPKD.

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CHAPTER 3

RESULT #2

Up-regulation of PKD2 protein expression by ER stress and phosphorylated eIF2α

A version of this chapter is to be submitted.

Jungwoo Yang, Wang Zheng, Qian Wang, Carlos Lara, Shaimaa Hussein and

Xing-Zhen Chen

3.1 ABSTRACT

Endoplasmic reticulum (ER) stress and the ensuing unfolded protein response are implicated in numerous human diseases but the underlying mechanisms remain poorly understood. One major UPR downstream is phosphorylation of eukaryotic initiation factor eIF2α by kinase PERK, which attenuates global protein translation and enhances translation of selected proteins. Mutations in PKD2 are associated with autosomal dominant polycystic kidney disease. We previously found that PKD2 is regulated by ER-associated proteasome degradation and enhances PERK efficiency in phosphorylating eIF2α. Here we showed that PKD2 protein expression in HEK and other cells is up-regulated by different stresses that all increase phosphorylated eIF2α (P-eIF2α). Increasing PERK activity and inducing P-eIF2α by inhibiting its de-phosphorylation or by eIF2α over-expression all regulated PKD2 expression. PCR and polysome assays showed that ER stress does not affect PKD2 mRNA but increases its binding with ribosomes, demonstrating that P-eIF2α translationally up-regulates PKD2. Mutation analysis found that the upstream open reading frame (uORF) in PKD2 mRNA 5'-untranslated region represses PKD2 translation. Thus, cellular stresses and P-eIF2α translationally up-regulates PKD2 through bypassing translation from the uORF.

3.2 INTRODUCTION

PKD2, composed of 968 amino acids, is an integral membrane protein that functions as a Ca^{2+}-permeable non-selective cation channel. Mutations in PKD2 are responsible for 10-15% of autosomal dominant polycystic kidney disease (ADPKD). Both loss- and gain-of-function of PKD2 are cystogenic (1,2) but the underlying mechanisms are not well understood. PKD2 is mainly localized on the endoplasmic reticulum (ER) membrane but is also on the plasma membrane and primary cilia membrane (3,4). Regulation of PKD1 and PKD2 expression is connected to pathways involving renal primary cilia, Ca^{2+} signaling, mammalian target of rapamycin (mTOR) and the vasopressin receptor (5). Based on these connections, anti-proliferative drugs aimed at slowing growth of cysts have been tested recently in clinical trials, of which the results have however been disappointing (6-8). Thus, it is important to explore novel pathways and mechanisms. In fact, recent studies have shown that PKD2 is involved in cell proliferation and apoptosis through cross-talk with cellular pathways related to ER stress or ER Ca^{2+} homeostasis. ER stress refers to cellular states in which misfolded or unfolded proteins accumulate in the ER, and induces unfolded protein response (UPR) to restore homeostasis, or lead to apoptosis in the presence of persistent stress (9,10). PKD2 inhibits cell proliferation and protein synthesis through promoting the phosphorylation of eukaryotic initiation factor 2α (P-eIF2α) by pancreatic ER-resident eIF2α kinase (PERK) which itself is activated by ER stress (11). It was also reported that PKD2 inhibits cell

proliferation (12). PKD2 functions as an anti-apoptotic channel protein through modulating ER Ca^{2+} homeostasis (13).

Reversely, abnormal cellular or tissue conditions regulate PKD2 expression. For example, prolonged application of tunicamycin (Tm) in culture cell lines, which induces prolonged ER stress, down-regulates PKD2 expression through ER-associated degradation (ERAD) (14). Also, PKD2 expression is significantly increased (up to 5-fold) following ischemia-reperfusion (I/R) injury in adult rat kidney and it is interesting to note that I/R-induced PKD2 expression is primarily on the ER membrane (15). In general, the I/R condition in cells simultaneously induces different cellular stresses including oxidative stress, ER stress and amino acid depletion induced stress (16). However, it is still not clearly understood how PKD2 expression is regulated and what the role of PKD2 is in cellular stress conditions. ER stress and UPR are associated with numerous human diseases, such as neuronal, cardiac and renal diseases, as well as inflammation and cancer (17,18). Indeed, ER stress is associated with a number of renal diseases, such as membranous nephropathy, glomerular disease, and tubular disease induced by ischemic injury, heavy metals or other agents (19-21).

As part of UPR, PERK kinase is activated and phosphorylates eIF2α at serine 51. As a translation initiation inhibitor, phosphorylated eIF2α (P-eIF2α) rapidly represses global protein synthesis, which prevents further accumulation of misfolded proteins in the ER (22). In mammals, eIF2α can also be phosphorylated by three other kinases, heme regulated inhibitor (HRI) induced by oxidative stress, dsRNA-induced protein kinase (PKR) induced by viral infection, and general

control nonderepressible-2 (GCN2) induced by nutrient depletion (23). The reprogramming of gene expression through P-eIF2α during several cellular stresses plays a pivotal role in determining cell survival or apoptosis (24,25). Interestingly, P-eIF2α also translationally and/or transcriptionally up-regulates some selective genes, such as activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and growth arrest and DNA damage-inducible protein (Gadd34) that are critical for efficiently coping with cellular stress conditions (26). These genes share a common feature in that in the 5' untranslated region (UTR) of their mRNAs there is an upstream open reading frame (uORF) that is inhibitory for the translation from the main ORF under normal, non-stress condition. The mechanism of inhibition is not fully understood but one possibility is that under non-stress condition the translated short peptide from uORF somehow inhibits the translation from the main ORF. Under stress conditions elevated P-eIF2α delays the initiation of scanning ribosomes, which bypasses the uORF translation and enhances the translation from the main (27,28).

In the present study, we examined how ER stress, P-eIF2α and uORF of PKD2 mRNA mediate translational up-regulation of PKD2 protein expression, using commercial mammalian culture cell lines as well as those from PKD2 knockout (KO) mouse collecting duct (MCD) cells and PERK KO mouse embryonic fibroblast (MEF) cells, in combination with Western blotting (WB), reverse transcription polymerase chain reaction (RT-PCR), real-time PCR, ribosome binding analysis, luciferase reporter, gene knockdown (KD) and over-expression.

3.3 MATERIALS AND METHODS

Cell culture, plasmid constructs and gene transfection

HeLa, MDCK and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, penicillin-streptomycin, and 10% fetal bovine serum (FBS) at 37˚C and 5% CO_2. $PERK^{+/+}$ and $PERK^{-/-}$ MEF cells and MCD D3 ($PKD2^{+/-}$) and B2 ($PKD2^{-/-}$) were maintained as described previously (29,30). Plasmids pEGFP-C2-PKD2, pcDNA3.1(+)-PERK, pcDNA3.1(+)-PERK K618A, pcDNA3.1(+)-PERK ΔC, pFLAG-CMV-2-Gadd34, pcDNA3.CD2-eIF2α and pcDNA3.CD2-eIF2α S51A were described previously (31-34). Luciferase vector BI16 is a gift from Dr. Grabczyk (Louisiana State University) and it contains both Firefly and Renilla luciferase gene sequences sharing a bidirectional promoter (35). A BamHI-NheI DNA fragment encoding the 5'UTR of the human PKD2 mRNA, along with the initiation codon of the PKD2 coding region, was inserted between the same restriction cutting sites in the BI16. Primer sequences used in this construct were as follows: sense 5'-GTCGGATCCCCGGGAAGAAAGGAACATG-3', and antisense 5'-CTGGCTAGCACCATCGCGGTCACTGGCGTC-3'. The resulting BI16-PKD2-5'UTR plasmid contains this 5'UTR sequence of PKD2 fused to the upstream of Renilla luciferase reporter gene. BI16-PKD2-ΔuORF and BI16-PKD2-ATG-to-TTG (with mutation of ATG initiation codon of PKD2 uORF to TTG) were generated using site II directed mutagenesis kit (stratagene) following the manufacturer's instruction. In order to check the effect of recognition of uORF

ATG initiation codon on the translation of downstream gene, the Kozak consensus surrounding the initiation codon was mutated to produce BI16-PKD2-KR (full-length 5'UTR: GGAAC**ATG**GCTCC, KR: GGTAC**ATG**TCTCC). All plasmids were sequenced to ensure that there were only desired changes. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) following manufacturer's recommendation to HeLa and HEK293T cells at ~70% confluency.

Antibodies

The following antibodies were used at the indicated concentrations in PBST + 3% milk unless otherwise stated: PKD2 (sc-25749, Santa Cruz Biotechnology) 1:2000; eIF2α (sc-11386, Santa Cruz Biotechnology) 1:2000; P-eIF2α (3597, Cell Signaling) 1:2000 5% BSA; PERK (AF3999, R&D SYSTEMS) 1:2000; P-PERK (631201, Biolegend) 1:1000; β-actin (sc-47778, Santa Cruz Biotechnology) 1:2000; HSP60 (sc-13115, Santa Cruz Biotechnology) 1:2000; BiP (sc-15897, Santa Cruz Biotechnology) 1:2000; ATF4 (sc-22800, Santa Cruz Biotechnology) 1:1000; c-Myc (2276, Cell Signaling) 1:2000; FLAG (sc-807, Santa Cruz Biotechnology) 1:2000; GFP (sc-9996, Santa Cruz Biotechnology) 1:2000.

RT-PCR and real-time RT-PCR

HeLa cells were treated with stress inducer Tg for 0.5, 1, 3, 6, or 12 hr, respectively, and DMSO treatment was used as a control. Total cellular RNA was

prepared using Trizol reagent (Invitrogen) according to the instruction manual. Contaminating DNA was digested with RNase-free DNase (Promega). Single strand cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PKD2, BiP, CHOP, and Herp relative mRNA levels were determined by PCR using 28 cycles which was proved to be optimal. β-actin was used as loading control. The oligonucleotide primers for each gene were as follows: β-actin (sense 5'-CCT GGCACCCAGCA CAAT-3' and antisense 5'-GGGCCGGACTCGTCATACT-3'), PKD2 sense 5'-G TATGACGGCTCACGCCTGTAATCC-3' and antisense 5'-AGAGATGGAGT T TCGCCACATTGCC-3'), CHOP (sense 5'-CCACTCTTGACCCTGCTTC-3' and antisense 5'-CCACTCTGTTTCCGTTTCC-3'), BiP (sense 5'-GAACACAGTG GTGCCTACCAAG-3' and antisense 5'-GGTGACTTCAATCTGTGGGACC-3') and Herp (sense 5'-CAATCAGAATGCTGCTCCTCAAG-3' and antisense 5'-GGTCCAATCCAACCAATCTCG-3'). Real-time PCR was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems) with the same primers. Quantitation of target genes was normalized using the reference β-actin. 7900HT Fast Real-Time PCR System software (version 2.4.1) was used to perform quantification and to generate Ct values. Values are a representation of three independent experiments, with standard errors as indicated.

Knockdown by siRNA

Knockdown of human eIF2α by siRNA was performed using oligonucleotides at 100 nM with Lipofectamine 2000 (Invitrogen) according to

the manufacturer's instructions. Oligonucleotides were purchased from Shanghai Gene Pharma Co, Shanghai, China: eIF2α (sense 5'-GGGU CUU UGAUGACA AGUATT-3', and antisense 5'-GGGUCUUUGAUGACAAGUATT-3'), its specific control siRNA (sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'). PKD2 knockdown by siRNA was described previously (31). The efficiency of the siRNA knockdown was assessed by immunoblotting.

Sucrose gradient centrifugation and polysome analysis

HeLa cells were cultured as described above and treated with 1 μM Tg or DMSO for 3 hr. 15 min prior to harvesting, cells were treated with 100 μg/ml cycloheximide. Cells were washed three times with 1 × cold phosphate-buffered saline, and harvested directly on the plate using lysis buffer (20 mM HEPES-KOH, pH 7.4, 15 mM MgCl2, 200 mM KCl, 1% Triton X-100 (v/v), 100 μg/mL cycloheximide, 2 mM DTT, and 1 mg/mL heparin). Cell lysates were passed though a 23-gauge needle three times and then incubated on ice for 10 min. Nuclei and cell debris were cleared by centrifugation at 12, 000 × g for 10 min at 4℃ and the resulting supernatant was loaded onto a 7%-47% sucrose gradient solution containing 20 mM Tris-HCl (pH 7.4), 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml cycloheximicde, 0.2 mg/ml heparin. The sucrose gradients were then subjected to centrifugation in a Beckman SW-41 rotor for 4 hr at 25, 000 rpm (69,176 x g). Fractions were collected from the top to bottom. To prevent degradation of RNA, equal volume of GulTC (6 M guanidium thiocyanate, 0.25

M sodium acetate) was added into each fraction and mixed well. Equal volume isopropanol was used to precipitate RNA, and it was then resuspended in the same volume of water for each fraction. Then the same volume of each fraction RNA was used to synthesize single-stranded cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Prepared cDNA was used to determine the relative mRNA levels for PKD2, CHOP, and β-actin with PCR. Oligonucleotide primers used in PCR were as follows: PKD2 (sense 5'-GTATG ACGGCTCACGCCTGTAATCC-3' and antisense 5'-AGAGATGGAGTTTCGCCACATTGCC-3'), CHOP (sense 5'-CCACTCTTGA CCCTGCTTC-3' and antisense 5'-CCACTCTGTTTCCGTTTCC-3') and β-actin (sense 5'-CCTGGCA CCCA GCACAAT-3' and antisense 5'-GGGCCGGACT CGTCATACT-3').

Dual luciferase assay

HeLa cells were cultured and plasmid transfections were performed as described above using BI16 (empty vector), PKD2-5'UTR, -ATG-to-TTG, -ΔuORF and -KR. Twelve hr after transfection, cells were collected and dual luciferase assays were carried out with Dual-Luciferase® Reporter Assay System (Promega) according to the instruction manual. Values are a measure of a ratio of Renilla versus firefly luciferase units (relative light units) and represent the mean values of three independent transfections, with standard errors as indicated. Firefly luciferase values did not change significantly in the dual reporter assays.

Data analysis

Signals were quantified by Gel-Pro Analyzer (Sigma-Aldrich) and data were analyzed and plotted using SigmaPlot 12 (Systat Software, San Jose, CA), and expressed as mean ± SEM (N), where SEM represents the standard error of the mean and N indicates the number of experimental repeats. A probability value (P) of less than 0.05 and 0.01 was considered significant and very significant, respectively.

3.4 RESULTS

Effects of ER stress induced by thapsigargin (Tg) or tunicamycin (Tm) on PKD2 protein and mRNA expression

It was previously reported that renal ischemia/reperfusion, which induces several cellular stress conditions among many other alterations, substantially increases PKD2 expression, notably PKD2 on the ER membrane (15,16). However, we reported in 2008 that prolonged application of Tm, for 8 or more hours (hr), results in decreased PKD2 expression due to ERAD through the ubiquitin-proteasome system (14). Thus we wanted to study how cellular stresses, notably ER stress, regulate PKD2 protein expression, using cell culture models. For this we first induced ER stress using known ER stress inducers Tg, an ER Ca^{2+} ATPase inhibitor that depletes ER Ca^{2+} stores, and Tm, an inhibitor of protein glycosylation, as they both lead to accumulation of unfolded proteins in the ER lumen (36-38). WB experiments revealed that endogenous PKD2 protein expression in human embryonic kidney 293T (HEK293T) cells treated with Tg (0.5 μM) for 1 hr increases by 81 ± 18% (P = 0.02, N = 7) (Fig. 3-1A and B). Similar results were obtained in Henrietta Lacks (HeLa) cells treated with Tg for 1 or 3 hr (Fig. 3-1C). On average, endogenous PKD2 expression increased by 86 ± 20% (P = 0.04, N = 8) following 1-hr Tg treatment (Fig. 3-1D). Tm treatment for up to 6 hr in HEK293T cells also similarly increased PKD2 expression (Fig. 3-1E). However, prolonged (6-24 hr) treatments with Tm resulted in a decrease in

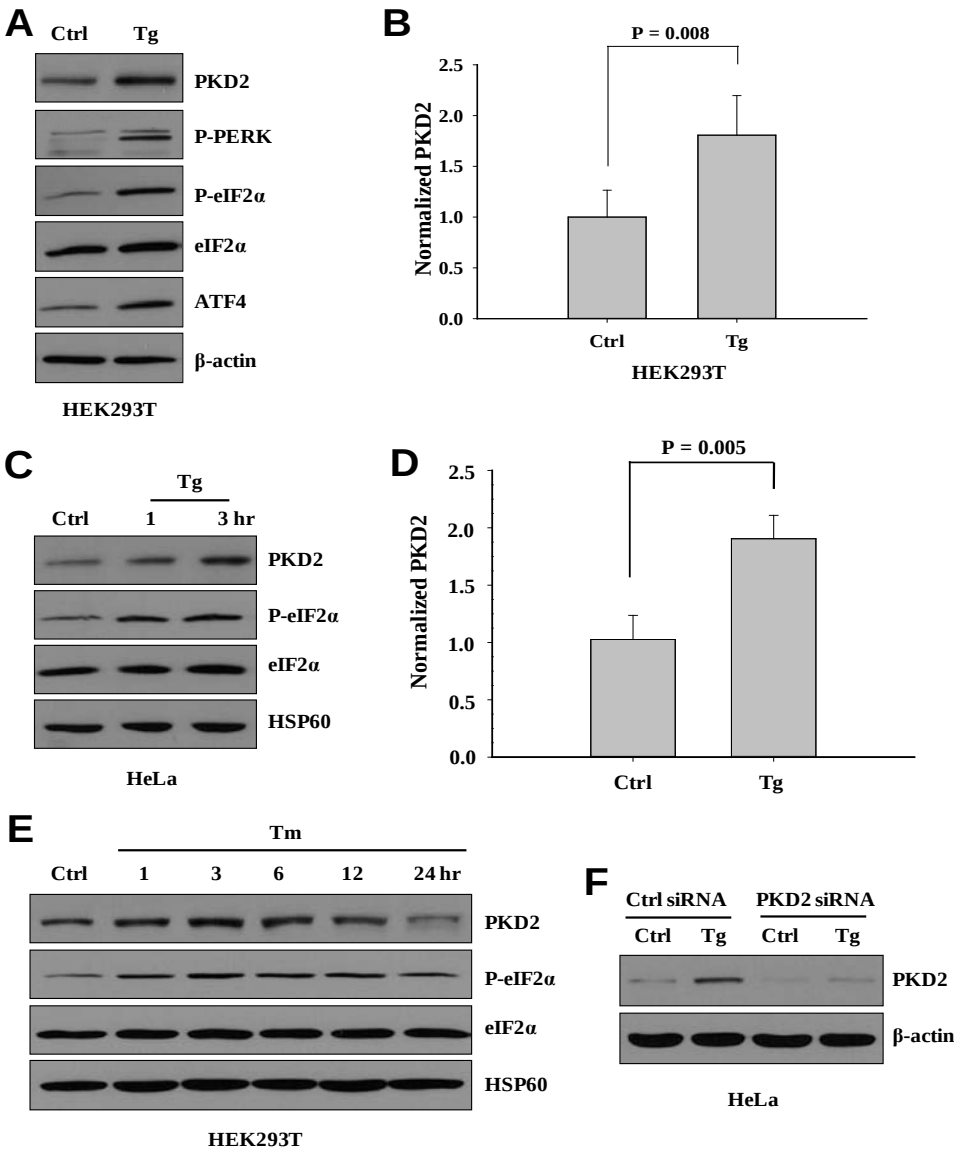

Fig. 3-1. PKD2 protein expression is induced by ER stress. (**A**) HEK293T cells were collected after treated with DMSO (Ctrl) or Tg (0.5 μM) for 1 hr. Blots were probed with the indicated antibodies and quantified by Gel-Pro Analyzer. (**B**) Data were obtained from the same conditions as in (A) and averaged to compare PKD2 protein levels between treatment of DMSO (Ctrl) and Tg (0.5 μM) for 1 hr in HEK293T cells after normalization by β-actin (N = 7). (**C**) HeLa cells were collected after DMSO (Ctrl) or Tg (0.5 μM) treatment for 1 or 3 hr. Blots were probed with the indicated antibodies. (**D**) Data were obtained from the same conditions as in (C) and averaged to compare PKD2 protein levels between treatment of DMSO (Ctrl) and Tg for 3 hr in HeLa cells after normalization with β-actin (N = 8). (**E**) HEK293T cells were collected after DMSO (Ctrl) or Tm (2.4 μM) treatment for 1, 3, 6, 12, or 24 hr. Blots were probed with the indicated antibodies. (**F)** HeLa cells transfected with PKD2 siRNA or Ctrl siRNA were treated with DMSO (Ctrl) or Tg (1 μM) for 24 hr. Cell lysates were prepared for probing PKD2 and β-actin with corresponding antibodies.

PKD2 expression from the plateau value reached at 3-6 hr, consistent with our previous report that Tm induces ERAD of PKD2 through the ubiquitin-proteasome system (14). Interestingly, PKD2 expression continued to increase even after 24-hr Tg treatment in Hela cells (Fig. 3-1F).

Because changes in the PKD2 expression are due to PKD2 protein synthesis or degradation, and one-hour Tg or Tm treatment is relatively short for significantly degrading PKD2 protein or changing PKD2 mRNA we reasoned that the induced ER stress may have enhanced the PKD2 translation rate. In fact, only about 20% PKD2 would be degraded at 1 hr (39), which cannot account for the 80% increase by Tg for one hour. Also, by RT-PCR and real-time PCR assays, we found that PKD2 mRNA remains unchanged during ER stress for up to 24 hr (Fig. 3-2), indicating that PKD2 protein expression is translationally up-regulated by ER stress.

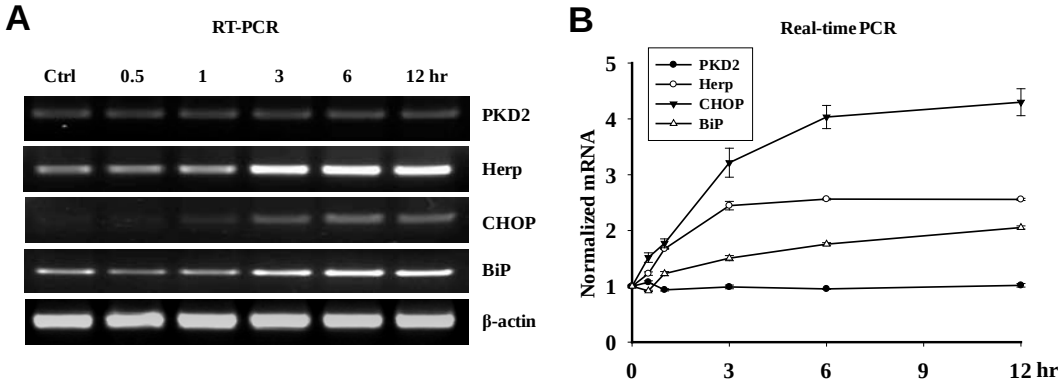

Fig. 3-2. PKD2 mRNA is not induced by ER stress. (**A**) Total RNAs from HeLa cells were prepared after DMSO (Ctrl) or Tg (1 μM) treatment for indicated durations. mRNAs levels were determined using RT-PCR, with β-actin as a loading control. (**B**) Synthesized cDNAs from (A) were also used as templates to determine the mRNAs levels using real-time PCR, with β-actin as a loading control. Shown data are averages from three independent experiments.

Effects of PERK and other kinases on PKD2 protein expression

We next wanted to examine the mechanism of how PKD2 is rapidly (ie translationally) up-regulated by ER stress which activates numerous downstream processes. In general, when it comes to rapid up-regulation of protein translation during cellular stresses, P-eIF2α is one of a few strong candidates to test. P-eIF2α was known to selectively enhance the translation of a subset of cellular factors including ATF4, Gadd34 and CHOP, which are essential for cells to cope with stress conditions. We first over-expressed PERK in HEK293T cells to increase P-eIF2α, which does not trigger ER stress (33,40), and indeed found that PKD2 protein expression significantly augments and positively correlates with an increase in P-eIF2α (Fig. 3-3A and B). Interestingly, the stimulatory effect of PERK over-expression persisted in the presence of ER stress induced by Tm or Tg. To determine whether PERK kinase activity is required for its effect we compared PERK with mutants PERK K618A and PERK ΔC (a C-terminus truncated mutant) that have no kinase activity due to loss of auto-phosphorylation (33,41). Indeed, unlike PERK, neither kinase dead mutant was able to increase the PKD2 or P-eIF2α expression (Fig. 3-3C), indicating that PERK kinase activity is required under this condition. To provide further evidence to support this, we utilized a PERK KO ($PERK^{-/-}$) MEF cell line and found that PKD2 expression in $PERK^{-/-}$ MEF cells, as opposed to WT ($PERK^{+/+}$) cells, remains unchanged by Tg treatment (Fig. 3-3D). These data together showed that PKD2 up-regulation

induced by ER stress depends on PERK kinase activity or one of its downstream factors, and that PKD2 expression positively correlates with that of P-eIF2α.

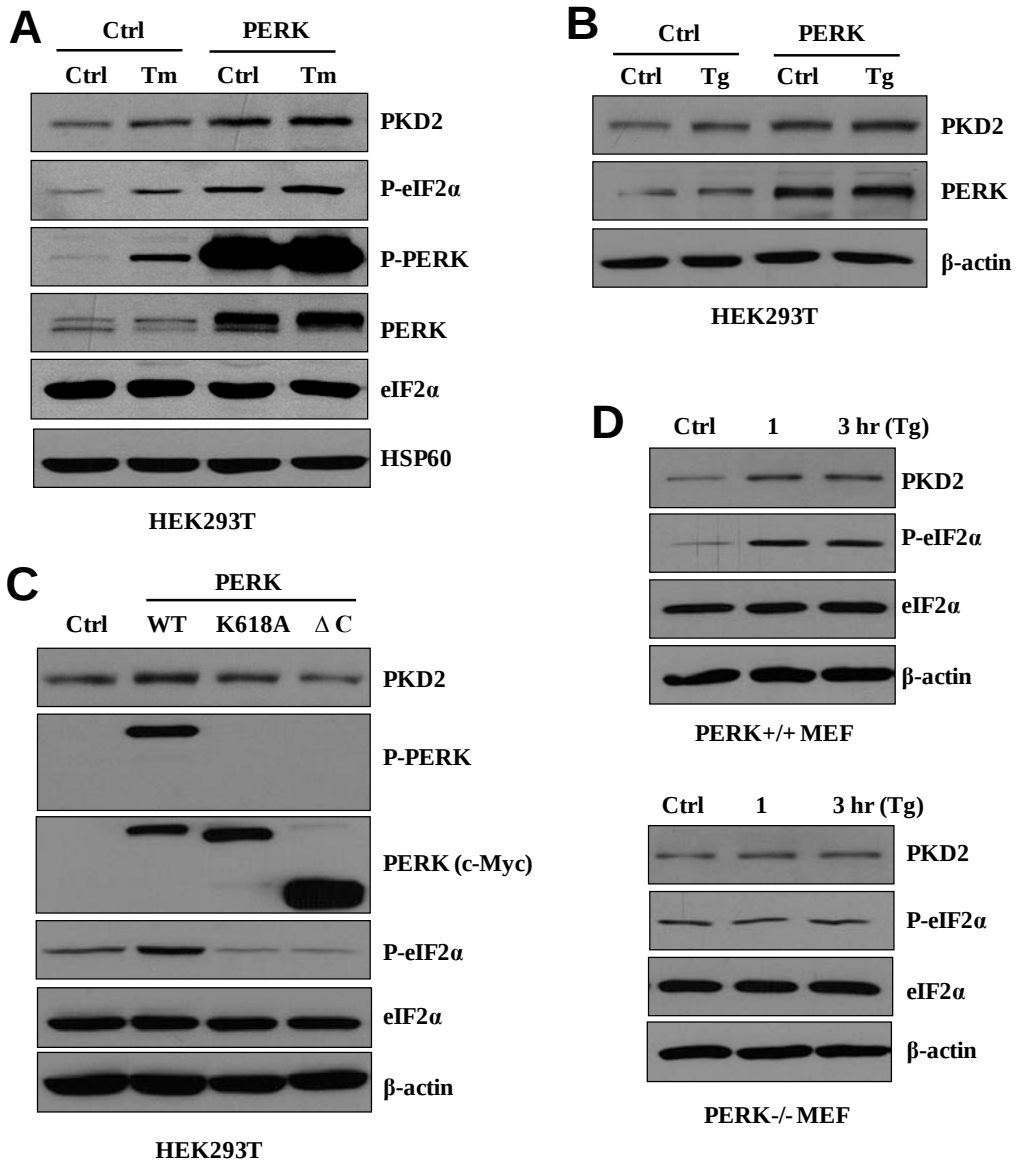

Fig. 3-3. PKD2 expression by ER stress is dependent on PERK activity. (**A** and **B**) HEK293T cells were transiently transfected with 500 ng of c-Myc-PERK fusion expression construct or empty vector (Ctrl) and treated with DMSO (Ctrl) or Tm (2.4 μM) for 4 hr (A) or Tg (0.5 μM) for 1 hr (B). After treatment, cells were collected and blots were probed with the indicated antibodies. (**C**) HEK293T cells were transiently transfected with 500 ng of c-Myc-PERK, K618A or ΔC fusion expression constructs or empty vector (Ctrl) and then cell lysates were prepared for immunoblotting with the indicated antibodies. (**D**) $PERK^{+/+}$ or $PERK^{-/-}$ MEF cells were treated with DMSO (Ctrl) or Tg (0.5 μM) for 1 or 3 hr, and then cell lysates were prepared for immunobloting with PKD2, P-eIF2α, eIF2α or β-actin antibodies.

We next examined whether and how other kinases of P-eIF2α regulate PKD2 expression. For this we activated eIF2α by arsenite (As) which induces oxidative stress and kinase HRI (42), and by polyinosinic:polycytidylic acid (poly IC) which mimics viral infection and induces kinase PKR (43). Indeed, treatment with As or poly IC up-regulated PKD2 expression in both HEK293T and HeLa cells (Fig. 3-4A and B). Furthermore, PKD2 expression in PERK$^{-/-}$ MEF cells was induced by As and poly IC but not Tg (Fig. 3-4C). These data strongly indicate that P-eIF2α, but not its kinases, mediates the regulation. In particular,

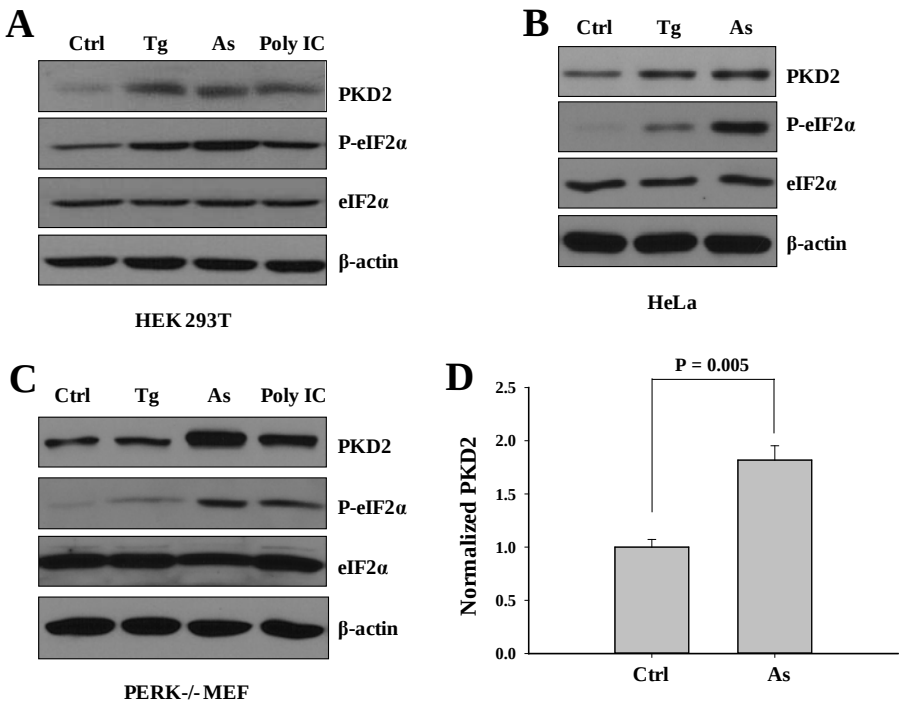

Fig. 3-4. PKD2 protein expression is induced by different stresses. (**A**) HEK293T cells were collected after DMSO (Ctrl), Tg (0.5 μM) or As (100 μM) treatment for 1 hr, or Poly IC (20 μg/ml) treatment for 18 hr. Blots were then probed with the indicated antibodies. (**B**) HeLa cells were collected after DMSO (Ctrl), Tg (0.5 μM) or As (100 μM) treatment for 1 hr. Blots were then probed with the same antibodies. (**C**) PERK$^{-/-}$ MEF cells were treated with the same procedures as HEK293T cells in (A) and detected with the indicated antibodies. (**D**) Bar graph showing the difference of PKD2 protein levels between treatments with DMSO (Ctrl) and AS for 1 hr in HeLa cells after normalization by β-actin (N = 4).

WB experiments revealed that in HeLa cells treated with As, PKD2 expression increases by 82% ± 11% (P = 0.001, N = 4) (Fig. 3-4D). Of note, putting together data obtained from using different stress conditions, we found that the P-eIF2α and PKD2 levels are not linearly correlated (see eg Fig. 3-4B), suggesting that factors other than P-eIF2α may have also contributed to regulating PKD2.

Effects of salubrinal and Gadd34 on PKD2 protein expression

Further, because different stress conditions may affect common downstream factors (other than P-eIF2α) that may have regulated PKD2, we wanted to further document the effect of P-eIF2α on PKD2 expression. For this we treated cells with salubrinal which is a chemical inhibitor of protein phosphatase 1 that is normally in complex with Gadd34 to de-phosphorylate P-

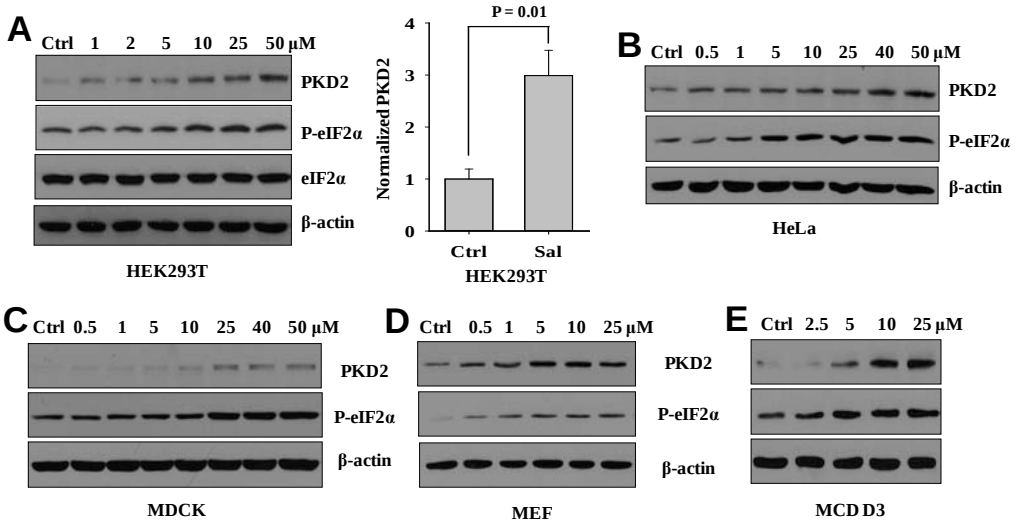

Fig. 3-5. PKD2 protein expression is induced by salubrinal. HEK293T (**A**), HeLa (**B**), MDCK (**C**), MEF (**D**) and MCD D3 (**E**) Cells were treated with DMSO (Ctrl) or salubrinal (Sal) for 40 hr at indicated concentrations. Cell lysates were then prepared and blots probed with the indicated antibodies. (**A**) Right panel, bar graph (N = 3) comparing PKD2 protein levels between treatment of DMSO (Ctrl) and salubrinal (50 μM) for 40 hr in HEK293T cells after normalization by β-actin.

eIF2α (34,44) and increase P-eIF2α without inducing cellular stress, its kinases, or other UPR target proteins such as Xbp-1, immunoglobulin heavy-chain binding

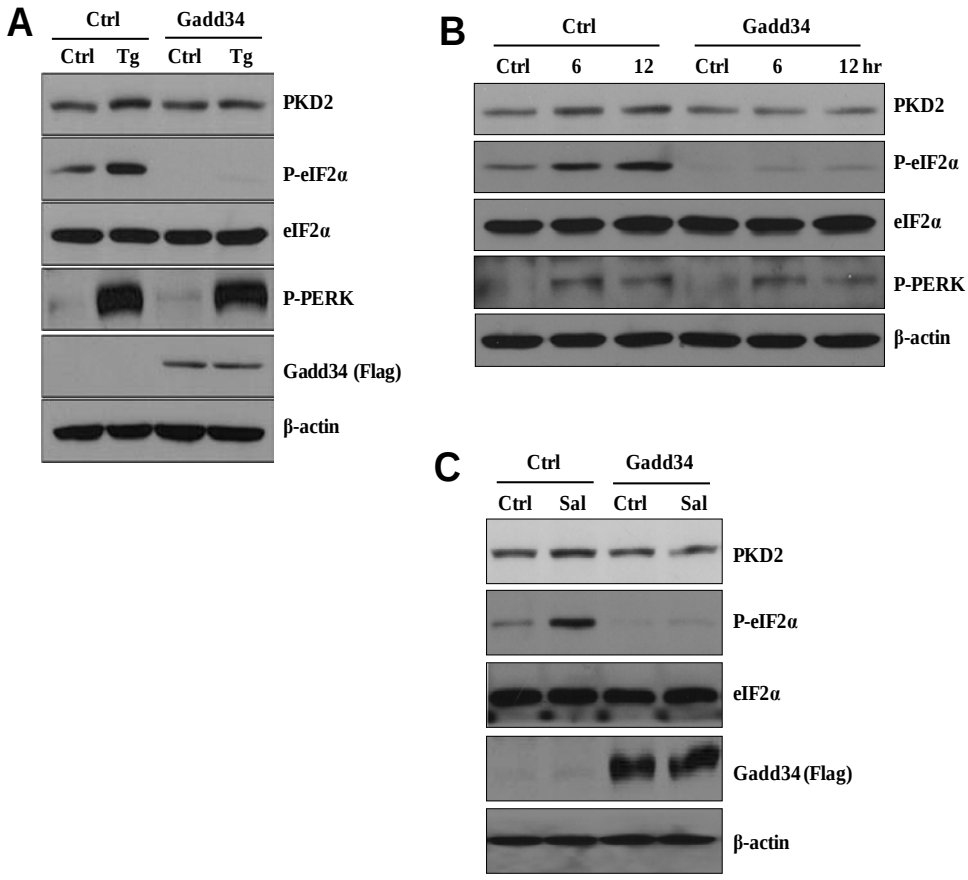

Fig. 3-6 Induction of PKD2 protein expression by ER stress is abolished by Gadd34. (**A**, **B** and **C**) HeLa cells were transiently transfected with 500 ng of Flag-Gadd34 fusion expression construct or empty vector (Ctrl). 40 hr after transfection, cells were treated with DMSO (Ctrl) or Tg (0.5 μM) for 3 hr (A), Tg (0.5 μM) for 6 or 12 hr (B), or salubrinal (50 μM) for 40 hr (C). Cell lysates were then prepared for immunoblotting with the indicated antibodies.

protein (BiP, also called GRP78) and GRP94 (45-48). In HEK293T, HeLa, Madin-Darby canine kidney (MDCK), MEF and MCD D3 cells treated with salubrinal at various concentrations, PKD2 expression augmented in a dose-dependent manner, in correlation with expected increases in P-eIF2α (Fig. 3-5).

WB experiments showed that in HEK293T cells treated with salubrinal (50 μM), PKD2 expression increases by 199 ± 55% (P = 0.01, N = 3) (Fig. 3-5A). On the other hand, we also examined the effect of Gadd34 on PKD2 expression. Over-expression of Gadd34 significantly decreased P-eIF2α and abolished ER stress- and salubrinal-induced up-regulation of PKD2, but in the absence of ER stress and salubrinal treatment, the level of PKD2 expression remained unaffected (Fig. 3-6), presumably because low P-eIF2α in this condition has two opposite effects cancelling each other: one is to up-regulate the global translation and the other is to reduce PKD2 expression.

Effect of P-eIF2α on PKD2 protein expression

So far, in all performed experiments, changes in P-eIF2α were made through indirect induction, which may result in other changes that may affect PKD2 expression. Thus we next examined the role of P-eIF2α through direct eIF2α over-expression and KD. We found that over-expression of WT eIF2α, but not the phosphorylation dead S51A mutant, increases P-eIF2α and up-regulates PKD2 in HeLa and HEK293T cells (Fig. 3-7A and B). Combination of eIF2α over-expression and Tg treatment exhibited the most pronounced increases in PKD2 expression, compared with eIF2α over-expression or Tg treatment alone (Fig. 3-7C and D). Consistently, eIF2α KD by siRNA in HeLa cells reduced PKD2 expression, in correlation with expected reduction in P-eIF2α, regardless of whether or not ER stress was induced (Fig. 3-7E).

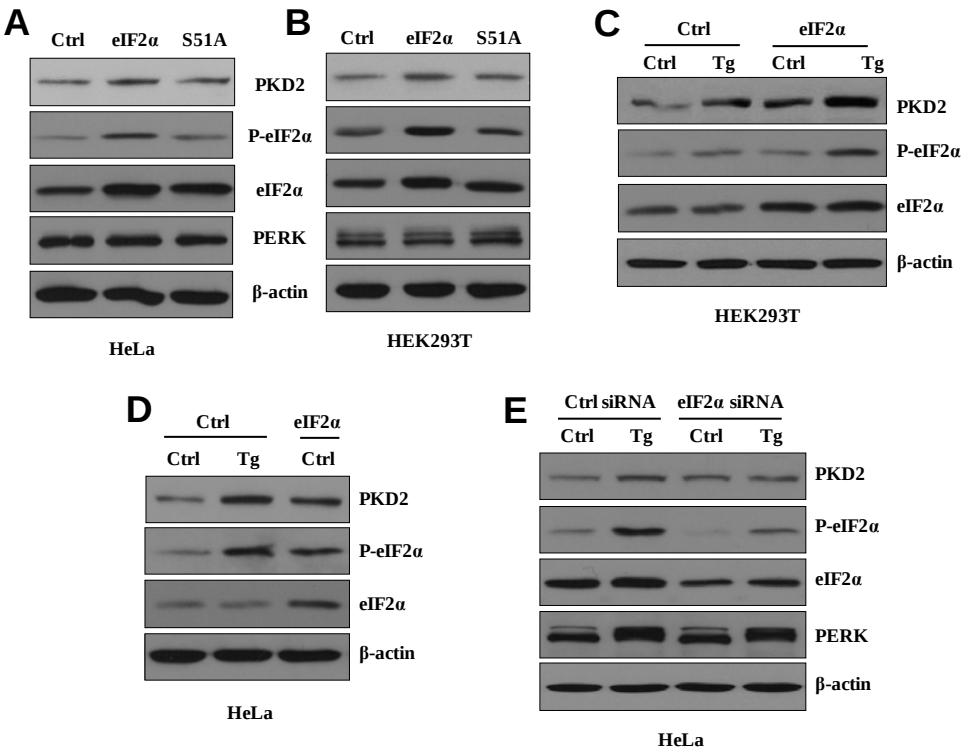

Fig. 3-7 PKD2 protein expression is regulated by phosphorylation of eIF2α. (**A** and **B**) HeLa (A) or HEK293T (B) cells were transiently transfected with 500 ng of eIF2α or eIF2α S51A expression constructs or empty vector (Ctrl). 40 hr after transfection, cells were collected for immunoblotting with the indicated antibodies. (**C** and **D**) HEK293T (C) and HeLa (D) cells were transiently transfected with 500 ng of eIF2α expression construct or empty vector (Ctrl). 40 hr after transfection, cells were treated with DMSO (Ctrl) or Tg (0.5μM) for 1 hr and then collected for immunoblotting with the indicated antibodies. (**E**) HeLa cells were transfected with eIF2α siRNA or Ctrl siRNA. 40 hr after transfection, cells were treated with DMSO (Ctrl) or Tg (0.5 μM) for 1 hr and then cell lysates were prepared and subjected to immunoblotting with PKD2, P-eIF2α, eIF2α, PERK or β-actin antibodies.

Effect of ER stress on the PKD2 mRNA binding with ribosomes

During cellular stress, mRNAs encoding proteins such as ATF4, CHOP and Gadd34 that are translationally up-regulated by P-eIF2α were found to bind more ribosomes. We thus also employed polysome binding analysis for PKD2 in the presence and absence of ER stress. For this, mRNAs within sucrose density gradient fractions were extracted and then prepared for RT-PCR. In HeLa cells treated with DMSO (non-stress condition), β-actin mRNA (as a negative control)

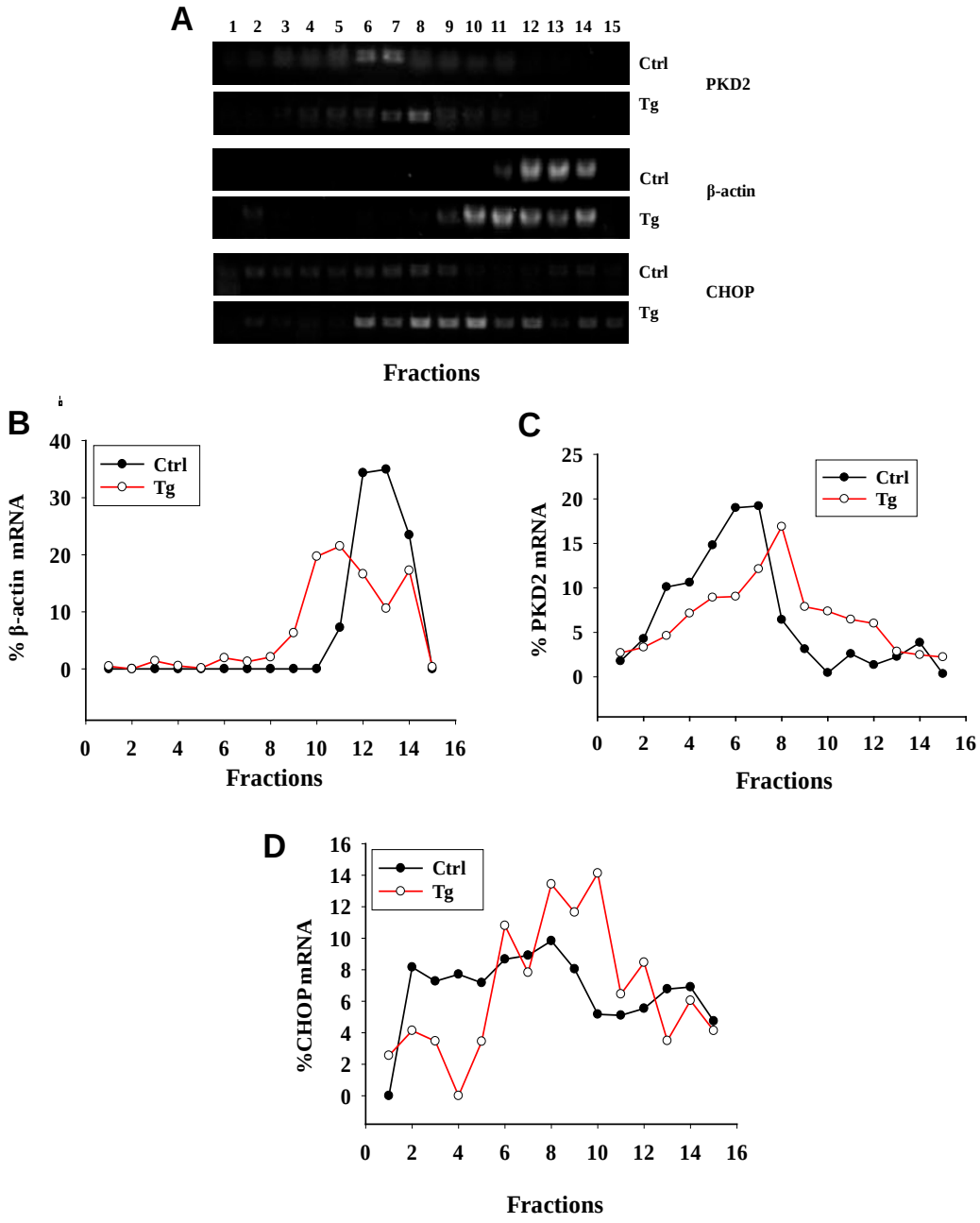

Fig. 3-8 Translation efficiency of PKD2 mRNA is enhanced by ER stress. (**A**) HeLa cells were treated with DMSO (Ctrl) or Tg (0.5 μM) for 3 hr and then cell lysates were fractionated by sucrose gradient centrifugation. Fractions were then collected from top to bottom and named from fraction 1 to fraction 15. RT-PCR was used to detect the mRNA levels of PKD2, β-actin (negative Ctrl) or CHOP (positive Ctrl). PCR products were then subjected to 1% agarose gel electrophoresis and quantified by Gel-Pro Analyzer. (**B**, **C** and **D**) Line graphs comparing polysome distribution of β-actin (B), PKD2 (C) or CHOP (D) between DMSO (Ctrl) and Tg (0.5 μM) treatments for 3 hr in HeLa cells. Each fraction is presented as a percentage of the total density from all fractions within each sucrose gradient.

primarily sedimented to the high polysome-binding fractions 12, 13, and 14, indicating that β-actin mRNAs are actively translated (Fig. 3-8A and B). However, Tg treatment resulted in a significant portion of β-actin mRNAs shifted to lower polysome-binding fractions 10 and 11, indicating that β-actin translation is repressed during ER stress. On the contrary, CHOP mRNA (as a positive control) sedimented to higher polysome-binding fractions under ER stress condition (Tg) compared to non-stress condition (DMSO) (Fig. 3-8A and D), consistent with the fact that CHOP translation is induced by ER stress and P-eIF2α (49). Similar to CHOP, by Tg treatment, significant portion of PKD2 mRNAs shifted from fractions 6 and 7 to fractions 7 and 8 (Fig. 3-8A and C), indicating that PKD2 translation is augmented by ER stress and P-eIF2α.

Role of uORF of PKD2 mRNA in the up-regulation of PKD2 translation by ER stress

A Bypass Model was recently proposed in an effort to account for how CHOP is translationally up-regulated by P-eIF2α and assumes that a uORF(s) present in the 5'UTR of a mRNA sequence mediates the translational regulation of the protein encoded by the main ORF of the mRNA by P-eIF2α and cellular stresses (64). The model further assume that, under non-stress condition, either translation of a peptide (from the uORF) inhibits the translation from the main ORF or the inhibitory function of the uORF causes dissociation of ribosomes from the mRNA at the end of uORF translation, possibly through preventing translation elongation or termination (49). When P-eIF2α is high, eg, during stress

conditions, due to delayed initiation, scanning ribosomes bypass the uORF and increase the translation from the main ORF. An analysis of annotated human, mouse, monkey and pig PKD2 mRNAs revealed one non-over-lapping uORF (Fig. 3-9A). To elucidate whether the PKD2 uORF is indeed involved in the translational up-regulation of PKD2 by P-eIF2α, we constructed DNA plasmids harboring the mouse PKD2-5'UTR DNA (with full-length 5'UTR) or PKD2-ΔuORF DNA (with deleted uORF) for transfection into HeLa cells. Indeed we found that transfection of PKD2-ΔuORF produces significantly higher PKD2 expression compared to transfection of PKD2-5'UTR (Fig. 3-9B), while their mRNA levels are similar (Fig. 3-9C). Moreover, PKD2 protein expression from PKD2-5'UTR, but not that from PKD2-ΔuORF, was responsive to Tg treatment and eIF2α phosphorylation (Fig. 3-9B). Also, mutating the uORF start codon ATG to GGG abolished the responsiveness to Tg treatment (Fig. 3-9D and E).

Further, to confirm the inhibitory effect of uORF on the translation of PKD2 found by WB experiments, we performed dual-luciferase assays with a reporter plasmid harboring both Firefly and Renilla luciferase genes, which share a bidirectional promoter. Full-length 5'UTR or 5'UTR with uORF deletion, start codon ATG-to-TTG mutation, or Kozak sequence KR mutation (GG<u>A</u>AC**ATG**<u>G</u>CTCC to GG<u>T</u>AC**ATG**<u>T</u>CTCC) was inserted upstream of the Renilla luciferase gene. Firefly luciferase served as an internal control. We transfected the reporter plasmids into HeLa cells and found that full-length 5'UTR insertion inhibits the luciferase activity while uORF deletion or ATG-to-TTG mutation significantly reduced the inhibition (Fig. 3-9F), indicating that the uORF

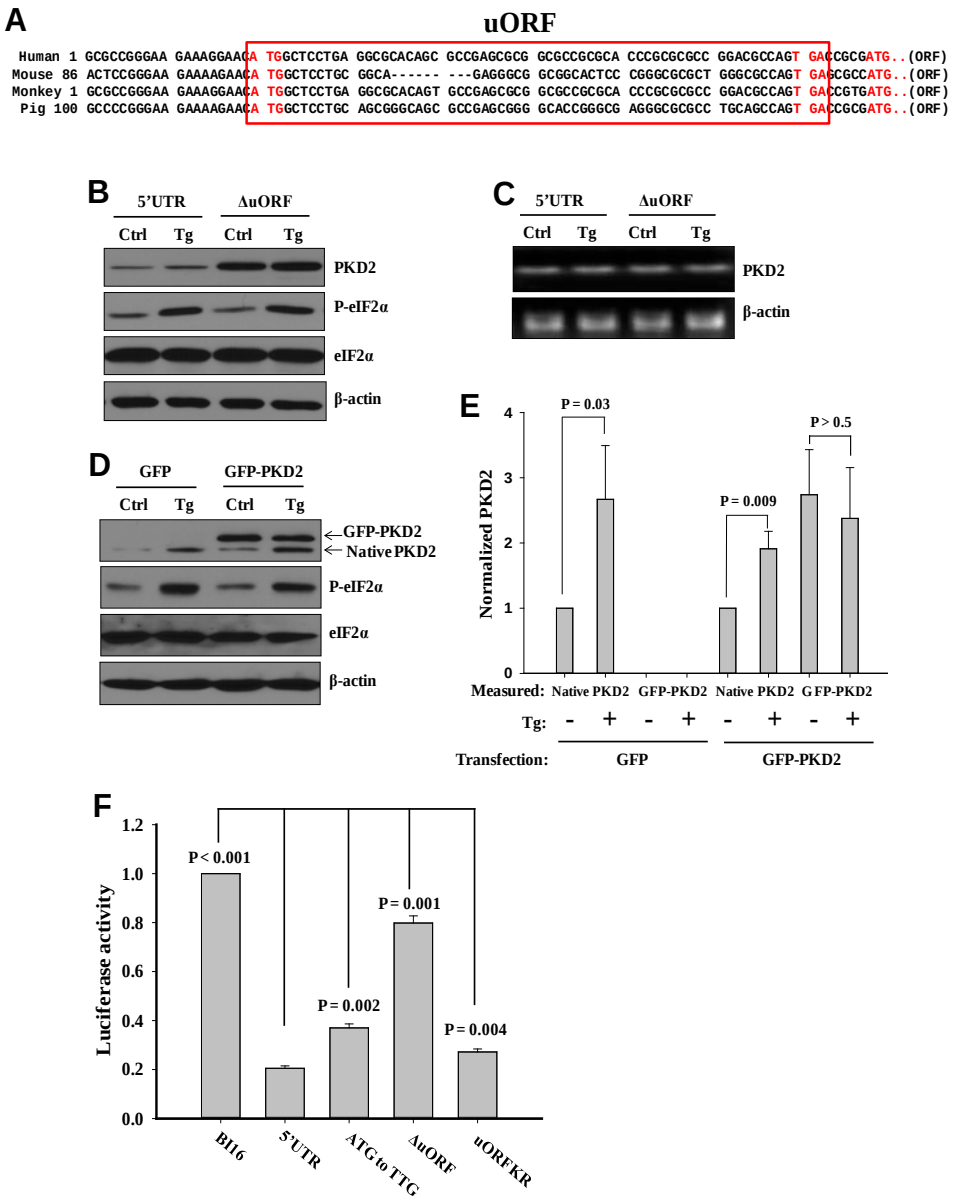

Fig. 3-9 PKD2 expression induction by ER stress is mediated by uORF in 5'UTR of its mRNA. (A) An alignment of annotated human, mouse, monkey and pig 5'UTRs of PKD2 mRNA. (**B** and **C**) HeLa cells were transiently transfected with 10 ng of mouse PKD2-5'UTR or PKD2-ΔuORF expression constructs or empty vector (Ctrl). 12 hr after transfection, cells were collected after DMSO (Ctrl) or Tg (1μM) treatment for 3 hr and subjected to immunoblot for protein detection (B) or RT-PCR for mRNA detection (C). (**D**) HeLa cells were transiently transfected with 10 ng of GFP-PKD2 fusion expression construct or empty vector pEGFP-C2. 12 hr after transfection, Cells were treated with DMSO (Ctrl) or Tg (1μM) for 3 hr, and then cells were collected for immunoblotting. (**E**) Bar graph (N = 3) showing PKD2 protein relative levels under different conditions in HeLa cells after normalization by β-actin. (**F**) HeLa cells were transiently transfected with 80 ng of BI16, PKD2-5'UTR, ATG-to-TTG, ΔuORF or uORF KR luciferase constructs. 12 hr after transfection, cell lysates were prepared and luciferase activities measured. The bar graph (N = 3) shows Renilla luciferase relative activity after normalization by firefly luciferase.

in the 5'UTR mediates the inhibitory effect on luciferase activity (ie, PKD2 protein translation). We also tested the role of the PKD2 Kozak sequence and interestingly found that the KR mutation also significantly reduced the inhibitory effect of 5'UTR (Fig. 3-9F), consistent with the expected effect of a Kozak sequence, ie, through enhancing the translation of uORF to reduce the translation of luciferase. Our data together demonstrated that ER stress and P-eIF2α up-regulates PKD2 protein translation, through bypassing the uORF in the 5'UTR of PKD2 mRNA.

3.5. DISCUSSION

In this study we have first found that endogenous PKD2 protein expression in mammalian culture cells is significantly up-regulated by ER stress. By RT-PCR and real-time PCR we then have shown that the up-regulation is not through increasing PKD2 mRNA. Then, using PERK over-expression, induction of other stress conditions, salubrinal treatment, Gadd34 and eIF2α over-expression, together with WB and polysome analyses, we have accumulated extensive indirect as well as direct data that together have firmly established that P-eIF2α mediates PKD2 up-regulation. Finally, by WB and luciferase analyses we have shown that this up-regulation by P-eIF2α is through bypassing the translation of the inhibitory uORF in the 5'UTR of PKD2 mRNA.

During ER stress conditions, global translation is rapidly repressed through phosphorylation of eIF2α in response to an accumulation of misfolded proteins in the ER, which prevents further overload (22,23). At the same time, elevated P-eIF2α allows preferential translation of a selective panel of cellular factors encoded by genes that possess one or more uORFs located in their 5'UTR, including ATF4, CHOP, and Gadd34 (27,49,50). The rapid induction of these factors is essential for cell survival because it allows them to execute protective processes as part of UPR. Thus, our current study allowed adding PKD2 as a novel member to this panel. However, it is worth to mention that ATF4, CHOP and GADD34 are both translationally and transcriptionally up-regulated during ER stress (51-53), which would allow them to have more pronounced increases,

which is in contrast to PKD2 that is only subjected to translational up-regulation (Figure 1 and 2). Consistently, PKD2 exhibited about 1.8-fold (80%) increase during ER stress (Figure 1B and D) while ATF4 and CHOP increased 4-fold under comparable conditions (54,55). Also, the ways by which PKD2 (protein and mRNA) is regulated by P-eIF2α should be consistent with the assumption that too much increase in PKD2 is harmful to cells, as suggested by the fact that both PKD2 KO and transgenic mice develop renal cysts and are associated with over-proliferation and apoptosis (1,2). However, we should bear in mind that PKD2 is likely regulated by several other cellular factors, as indicated by our observation that P-eIF2α does not linearly up-regulate PKD2 (Figure 4B and 5E).

Since our data demonstrated that PKD2 is translationally up-regulated by ER stress, one obvious question is what the function of PKD2 in ER stress is. PKD2 is mainly located on the ER membrane (56), and also present in the primary cilium (57,58) and plasma membrane (59). It was reported that ER membrane PKD2 protein expression is significantly increased following renal ischemia-reperfusion injury which simultaneously induces different cellular stresses, including ER stress and oxidative stress (16). Because PKD2 is a Ca^{2+} permeable non-selective cation channel (4,60), it is reasonable to think that increased PKD2 during ER stress may augment Ca^{2+} release from ER to the cytoplasm resulting in decreased concentration of Ca^{2+} in the ER lumen. Therefore, rapidly increased PKD2 protein level during ER stress should render cells less susceptible to apoptosis at the beginning of ER stress conditions and allow stress alleviation through UPR. In that sense, PKD2 is anti-apoptotic, in

agreement with a previous study (13). Prolonged ER stress induced by Tm, which decreased PKD2 (Figure 3-1E) because of ERAD (14), may suggest the presence of apoptosis although Tg treatment for up to 24 hr did not have a similar effect on PKD2 (Figure 3-1F). This difference between prolonged Tm and Tg treatments has however remained unaccounted for.

How ER stress and UPR are implicated in human diseases is an emerging area of research. Although it is known that they are involved in a number of human diseases, including Parkinson's and Alzheimer's diseases, inflammation, cancer and diseases in heart, kidney, liver, lung and pancreas (17-19,61), the underlying mechanisms have been poorly understood. Most reports have remained on the descriptive level. For example, ER stress and several UPR factors such as PERK, activating transcription factor 6 and inositol-requiring protein-1are induced in brain and heart ischemia, leading to CHOP-dependent neuron apoptosis and cardiomyocyte degeneration (21). Protein turnover rates are high in kidney. In fact, fractional daily protein synthesis rate in human kidney is 42% per day, compared to only 12% and 1.5% per day in an intestinal tissue and skeletal muscle, respectively (17), suggesting that renal ER is more likely to face protein misfolding or ER stress. Indeed, ER stress is involved in several renal diseases, such as membranous nephropathy, glomerular disease, and tubular disease induced by ischemic injury, heavy metal or other agents (17,19-21). ER stress markers were found to be high in some glomerulopathies, proteinuria, and diabetic nephropathy. Some pathogenic mutations such as those in nephrin and podocin, lead to misfolding and retention in the ER, which may result in ER stress,

UPR and/or ERAD (17,19). The implication of UPR in ADPKD is supported by our previous reports that prolonged ER stress induced by Tm promotes PKD2 degradation and that PKD2 up-regulates PERK-dependent eIF2α phosphorylation (11,14,18).

Homozygous BiP KO mice are lethal but reduced BiP in BiP heterozygous mice are associated with glomerulosclerosis and tubulointestitial disorders (17,19). BiP is a master regulator of UPR sensors and functions as a ER molecular chaperone that is induced by ER stress to increase the protein folding capacity (62). It was found that ER stress or cell preconditioning with ER stress exhibits protective roles against induction of renal injury (17). Thus, cytoprotective components of ER stress or UPR pathways, including BiP up-regulation and eIF2α phosphorylation, are emerging as promising and novel targets for therapeutic intervention of renal, cardiac as well as other diseases.

I/R cause several cellular stresses such as oxidative stress, ER stress and amino acid depletion. For example, renal I/R induces ER stress that activates PERK, which significantly increases P-eIF2α in tubular epithelial cells in both cortex and medulla (63). PKD2 expression is also significantly up-regulated in adult rat kidney after I/R (15). This suggested that PKD2 has important roles in the processes against cellular stresses. Interestingly, $PKD2^{+/-}$ mouse kidneys have higher tubular and interstitial proliferative responses following injury compared with WT kidneys (64), suggesting that PKD2 dosage affects cellular repair responses. However, in addition to PKD2 protein expression, I/R causes an

increase in the PKD2 mRNA level as well (64), indicating the presence of another factor or pathway that is activated under I/R to regulate PKD2 mRNA.

In ADPKD, heterozygous loss-of-function mutations in PKD2 cause cyst formation accompanied with over-proliferation, de-differentiation and increased apoptosis (65). Our previous study revealed that PKD2 up-regulates P-eIF2α through enhancing the efficiency of eIF2α kinase PERK, thereby repressing cell proliferation and protein synthesis (11). On the other hand, our current study showed that elevated P-eIF2α induced by cellular stresses increases PKD2 expression, indicating that PKD2 and P-eIF2α may form a positive feedback loop to reach equilibrium, which may be important for jointly regulating cell proliferation and maintaining cell homeostasis. Interestingly, PKD1 down-regulates cell proliferation through a mTOR pathway that involves eukaryotic initiation factor 4E (eIF4E) (66). Thus, protein translation initiation machinery may be a common thread through which PKD1 and PKD2 jointly regulates cell growth. In summary, our present study found that PKD2 is a new member of uORF-possessing proteins that are translationally up-regulated by P-eIF2a and cellular stresses. This novel PKD2 regulation sheds light on the mechanism of ADPKD cystogenesis and may allow discovering new therapeutic targets. Future studies using *in vivo* models such as mice with salubrinal injection and double heterozygous mice with PKD2 and PERK KO would provide insights into the cross-talk between PKD2 and eIF2α pathways.

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CHAPTER 4

RESULT #3

Receptor for Activated C Kinase 1 (RACK1) inhibits function of transient receptor potential (TRP)-type channel PKD2L1 through physical interaction

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Jungwoo Yang, Qian Wang, Wang Zheng, Jagdeep Tuli, Qiang Li, Yuliang Wu, Shaimaa Hussein, Xiao-Qing Dai, Shiva Shafiei, Xiao-Gai Li, Patrick Y. Shen, Jian-Cheng Tu, and Xing-Zhen Chen. Receptor for Activated C Kinase 1 (RACK1) Inhibits Function of Transient Receptor Potential (TRP)-type Channel PKD2L1 through Physical Interaction. ***JOURNAL OF BIOLOGICAL CHEMISTRY*, 287, 6551–6561, 2012.**

4.1. ABSTRACT

PKD2L1 is a non-selective cation channel permeable to Ca^{2+}, $Na^{+,}$ and K^{+} and is activated by Ca^{2+}. It is also part of an acid-triggered off-response cation channel complex. We previously reported roles of the PKD2L1 C-terminal fragments in its channel function, but the role of the N-terminus remains unclear. Using a yeast two-hybrid screen, we found that the PKD2L1 N-terminus interacts with RACK1, a scaffolding/anchoring protein implicated in various cellular functions. This interaction requires the last two Trp-Asp (WD) motifs of RACK1 and fragment Ala^{19}–Pro^{45} of PKD2L1. The interaction was confirmed by GST pulldown, blot overlay, and co-immunoprecipitation assays. By ^{45}Ca tracer uptake and two-microelectrode voltage clamp electrophysiology, we found that in *Xenopus* oocytes with RACK1 overexpression PKD2L1 channel activity is abolished or substantially reduced. Combining with oocyte surface biotinylation experiments, we demonstrated that RACK1 inhibits the function of PKD2L1 channel on the plasma membrane in addition to reducing its total and plasma membrane expression. Overexpressing PKD2L1 N- or C-terminal fragments as potential blocking peptides for the PKD2L1-RACK1 interaction, we found that PKD2L1 N-terminal fragment Met^{1}–Pro^{45}, but not Ile^{40}–Ile^{97} or C-terminal fragments, abolishes the inhibition of PKD2L1 channel by overexpressed and oocyte-native RACK1 likely through disrupting the PKD2L1-RACK1 association. Taken together, our study demonstrated that RACK1 inhibits PKD2L1 channel function through binding to domain Met^{1}–Pro^{45} of PKD2L1. Thus, PKD2L1 is a

novel target channel whose function is regulated by the versatile scaffolding protein RACK1.

4.2. INTRODUCTION

PKD2L1 is a homologue of PKD2 with 54% sequence identity (1). Both proteins are Ca^{2+}-modulated cation channels permeable to Ca^{2+}, Na^{+}, and K^{+} (2-4). PKD2 and PKD2L1 share high similarity in membrane topology to the transient receptor potential (TRP) 2 superfamily of cation channels and have been termed TRPP2 and TRPP3, respectively, as members of the TRP polycystin (TRPP) subfamily (5). Mutations in the *PKD1* or *PKD2* gene account for most cases of autosomal dominant polycystic kidney disease with an incidence of 0.1–0.2% worldwide. We previously reported that PKD2L1 channel is activated by application of extracellular Ca^{2+} followed by an ensuing inactivation (3) and is inhibited by amiloride analogues and large monovalent cations such as tetrapentylammonium and tetrabutylammonium, which led to an estimation of its channel pore size of ~7 Å (6,7).

PKD2L1 channel activity is modulated by membrane potential, pH, and cell volume (3,8,9). We also reported that PKD2L1 physically interacts with α-actinin, an important component of the actin filament, in brain and other tissues and is functionally stimulated by α-actinin *in vitro* (10). Although overexpressed PKD2L1 is targeted to the plasma membrane (PM) of *Xenopus* oocytes, it mostly localizes in intracellular membranes of mammalian cells when expressed alone (10-12). PKD2L1 co-localizes with PKD1 in the centrosome and may function in the cell cycle (13). Interestingly, co-expression of PKD2L1 with PKD1 in human embryonic kidney (HEK) 293 cells resulted in PKD2L1 trafficking to the PM

where PKD2L1 seemed to mediate Ca^{2+} entry in the presence of a hypo-osmotic extracellular solution (12). PKD1L3, a homologue of PKD1, is critical in the PM trafficking of PKD2L1 because knock-out of PKD1L3 resulted in internalization of PKD2L1 in mouse taste bud neurons (11). How Ca^{2+} triggers the activation remains unknown. So far it is known that EGTA preinjection abolishes this activation (3), indicating that an increase in the intracellular Ca^{2+} concentration is required for the activation and that deletion of the PKD2L1 C-terminal domain Thr^{622}–Ser^{805} does not abolish Ca^{2+}-induced activation (14).

PKD2L1 localizes to tongue taste receptor cells together with PKD1L3, which is possibly involved in sour tasting (15-17), and to central canal neurons of the spinal cord, which are possibly involved in proton-dependent regulation of action potentials (15). Mouse PKD2L1 and PKD1L3 together mediate pH-dependent cation conductance (16), which was later described as the off-response (*i.e.* the channel is activated only after the low extracellular pH is removed) in a report proposing that the PKD2L1-mediated off-response may work together with an on response mediated by other acid receptors to account for acid sensing at large pH ranges (18). It was shown that PKD2L1 together with a carbonic anhydrase is involved in sensing gaseous CO_2 in the tongue (19). Interestingly, in the tongue of two patients with an acquired sour ageusia (*i.e.* unresponsive to sour stimuli), the mRNA and protein of PKD2L1, PKD1L3, and acid sensing ion channels were undetectable (20). Indeed, mice with PKD2L1 knock-out (KO) have reduced responses to sour stimuli (21). However, no anomaly in tasting was observed in PKD1L3 KO mice (21,22), and responses in sour taste cells are

associated with acid-activated proton, but not Na^+, currents (23), which challenges the implication in acid sensing of PKD1L3 or PKD2L1, a Na^+-permeable channel. Chang *et al.* (23) also found that these proton currents are not affected by targeted deletion of PKD1L3, a result that is incompatible with the study by Ishimaru *et al.* (11) about PKD1L3-dependent plasma membrane trafficking of PKD2L1, which would let one argue whether these proton currents are indeed mediated by PKD2L1. Thus, whether and how PKD1L3 or PKD2L1 is involved in acid sensing and whether there is a link between Ca^{2+}- and proton-induced channel activation remain subjects of further studies. PKD2L1 is also present in ganglion cells and other retina neuron cells (24), several compartments of mouse brain (10), kidney, liver, and heart (14). The physiological role of PKD2L1 in these tissues and whether it involves Ca^{2+} and/or acid dependence of the channel remain to be determined.

RACK1 is a guanine nucleotide-binding protein, β2-like 1 (also called GNB2L1) and a conserved scaffolding protein that regulates a range of cell activities, including cell growth, shape, and protein translation (25,26). RACK1 was originally identified as an anchoring protein for activated protein kinase C and a scaffolding protein that recruits PKCs (and other proteins) into a signaling complex (27,28). RACK1 is a 36-kDa protein composed of seven repeating units of Trp-Asp 40 (WD40) motifs forming an overall β-propeller structure (27). These WD40 motifs represent structural domains formed by four anti-parallel β-strands and are thought to mediate protein-protein interaction. In the last decade, several of its new cellular functions have been discovered (29). In fact, RACK1

acts as a scaffolding/anchoring protein for or regulates the expression/function of a number of binding partners, ranging from signaling proteins such as protein kinase C (27), Src tyrosine kinase (30), protein-tyrosine phosphatase PTPμ (31), cAMP-specific phosphodiesterase PDE4 D5 (32), and planar plan polarity protein Vangl2 (33) to receptor/channel proteins such as TRPM6 and TRPC3 (34,35), integrin β subunit (36), type 1 IFN receptor (37), NMDA receptor (NMDAR) NR2B subunit (38), $GABA_A$ receptor β subunits (39), G protein-coupled receptor for thromboxane A2 (40), and inositol 1,4,5-trisphosphate receptors (41). In addition, recent data suggest that the binding of RACK1 plays a role in gene expression, translation, and ribosome assembly and activation (25,26).

RACK1 mediates the regulation of NMDAR by ethanol (25,42) and together with PKC is an integral part of the circadian feedback loop (25,43). RACK1 is ubiquitously expressed in the tissues of higher mammals and human, including brain, liver, and spleen, suggesting that it has an important functional role in most if not all cells (29,38). Therefore, RACK1 is a versatile protein that regulates various biological functions.

In the present study, we first examined the physical interaction of PKD2L1 with RACK1 using different protein-protein interaction approaches. We then investigated the effect of RACK1 on channel function and expression of PKD2L1 and the importance of their physical interaction in the regulation of the channel by RACK1.

4.3. MATERIALS AND METHODS

Plasmid Constructs

We cloned a RACK1 cDNA from a human kidney cDNA library (Clontech) through a standard PCR approach with a pair of specific primers spanning the entire open reading frame: forward, 5'CTCTAGAGGATCC ATGACTGAGCAGATGACCC, and reverse, 5'CCATATGCTA GCGTG TGCC AATGGTCACC. XbaI, BamHI, and NdeI sites (underlined) were engineered into the primers to facilitate subsequent cloning. The authenticity of all constructs was confirmed by sequencing. Human PKD2L1 and RACK1 were subcloned into vectors pCDNA3.1 and pEGFPKD2, respectively, for mammalian cell expression and vector pTLN2 (44) for *Xenopus laevis* oocytes expression. His-tagged PKD2L1 N- and C-terminal fragments tagged with 5' His were subcloned into vector pCHGF for oocyte expression. The pCHGF vector was generated by deleting the enhanced GFP fragment and part of the CMV promoter in vector pEGFP-C2 using restriction enzymes NheI and SmaI and then ligating in (by the same enzymes) the β-globin 5' and 3' untranslated regions generated by PCR from vector pTLN2.

Preparation of mRNAs and Microinjection into Oocytes

PKD2L1 and RACK1 cDNAs in vector pCHGF were linearized with MluI followed by phenol/chloroform purification and ethanol precipitation. Linearized DNAs were used to *in vitro* synthesize capped mRNAs using the

mMessageMachineTM kit (Ambion, Austin, TX). Stage V-VI oocytes were isolated from *X. laevis*. Defolliculation of oocytes was performed through incubation in Ca^{2+}-free Barth's solution (14) containing collagenase (2 mg/ml) at room temperature (RT) for 2-2.5 h. Oocytes were then incubated at 18 °C in Barth's solution for at least 3 h before injection of 50 nl of H_2O containing variable amounts of mRNAs. An equal volume of H_2O was injected into control oocytes. Injected oocytes were incubated at 16–18 °C in Barth's solution supplemented with antibiotics for 2–4 days prior to experiments.

Cell Culture

HEK293, mouse inner medullary collecting duct-3 (IMCD3), and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells of less than 25 passages were cultured to full confluence before collection.

Yeast Two-hybrid Analysis

Human PKD2L1 N-terminus (PKD2L1N; amino acids Met1–Ile97) was generated by PCR and subcloned in-frame into the GAL4DNAbinding domain vector pGBKT7 (Clontech). A yeast two-hybrid assay using yeast strain AH109 and PKD2L1N as bait to screen a human kidney cDNA library constructed in vector pACT2 (Clontech) was performed according to protocols reported previously (45,46).

GST Pulldown

Precleared mammalian cell lysates containing HA-PKD2L1N were incubated with 2 μg of purified GST-RACK1 protein in binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$). The mixture was incubated at RT for 1 h with gentle shaking followed by another hour of incubation after addition of 100 μl of glutathione-agarose beads (Sigma-Aldrich). The beads were then washed several times with 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5, and the remaining proteins were eluted using 10 mM glutathione, 50 mM Tris, pH 8.0. The protein samples were resolved by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Bio-Rad). The filters were then blocked with 3% skim milk powder, immunoblotted with HA antibody (Cell Signaling Technology, Danvers, MA), and visualized with enhanced chemiluminescence (Amersham Biosciences).

Blot Overlay Assay

Purified proteins were resolved by SDS-PAGE (10%) and transferred to a nitrocellulose membrane. The membrane was then incubated at 4 °C overnight with lysates of NIH 3T3 cells in blocking buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 1% BSA), washed three times (10 min each) in blocking buffer containing 0.05% Tween 20, detected with a RACK1 antibody (1:1500; catalog number 610178, BD Biosciences) and a horseradish peroxidase-coupled secondary antibody (1:1500; Chemicon International, Temecula, CA), and visualized with enhanced chemiluminescence (Amersham Biosciences).

Co-immunoprecipitation (co-IP) and immunoblotting

Co-IP experiments using *Xenopus* oocytes co-expressing PKD2L1 and RACK1 were performed using a modified protocol (47). Briefly, each sample contained 10 healthy oocytes that were injected with PKD2L1 and/or RACK1 mRNAs and harvested in 500 μl of ice-cold CelLyticMlysis buffer (Sigma-Aldrich) 3 days after injection. Total proteins from the postnuclear supernatant were incubated on ice for 2 h either with anti-PKD2L1 antibody PR71 or anti-RACK1 antibody (B-3, catalog number sc-17754, Santa Cruz Biotechnology for mammalian cells or BD Biosciences for oocytes) followed by another 2 hr incubation with gentle shaking upon addition of 100 μl of protein A (for the anti-PKD2L1 sample; Sigma-Aldrich) or protein L (for the anti-RACK1 sample; Pierce)-Sepharose. The immunoprecipitates absorbed to protein A/L-Sepharose were resuspended in 40 μl of Laemmli sample buffer, and a 20 μl aliquot of the extract was subjected to SDS-PAGE followed by immunoblotting. Co-IP using native tissues and mammalian cultured cells was conducted as described recently (48).

^{45}Ca Uptake Measurements

$^{45}CaCl_2$ (Amersham Biosciences) at 30 μM was added to uptake solution (100 mM *N*-methyl- D-glucamine, 2 mM KCl, 1 mM MgCl2, 10 mM Hepes, pH 7.5) plus 1 mM non-radioactive $CaCl_2$ and 1:1000 radiolabeled $^{45}Ca^{2+}$with a specific activity of 2 μCi/μl (GE Healthcare, Montreal, QC, Canada). Ten oocytes

of each sample were incubated in 0.5 ml of uptake solution for 30 min, and the incubation was terminated by washing oocytes through six wells containing an ice-cold NaCl-containing solution (7). Individual oocytes were then dissolved in 250 μl of 10% SDS and mixed with 2.5 ml of scintillation mixture prior to scintillation counting. Data were compared using the paired or unpaired Student's *t* test and expressed as means ± S.E. $p < 0.05$ and $p < 0.01$ were considered statistically significant and very significant, respectively.

Electrophysiology

Current and voltage signals were measured using the conventional two microelectrode voltage clamp technique with a commercial amplifier (TEV-200A, Dagan, Minneapolis, MN). Electrodes were fabricated from borosilicate glass (Warner Instruments, Hamden, CT) by a micropipette puller (P-87, Sutter Instruments, Novato, CA) and filled with 3 M KCl with a typical tip resistance of 0.5–3 megaohms. The Digidata 1320A converter and pClamp 8 (Axon Instruments, Union City, CA) were used for data acquisition and analysis, respectively. SigmaPlot 11 or 12 (Jandel Scientific Soft-ware, San Rafael, CA) was used for further data analysis, including data plotting, *t*-test, analysis of variance, and regressions. In experiments using a ramp or gap-free protocol (3), current/voltage signals were digitized at 0.2 or 200 ms/sample.

Immunofluorescence

Xenopus oocytes expressing PKD2L1 alone or PKD2L1 plus RACK1 were embedded in a cryostat medium and shock frozen, and 10-μm sections were

sliced on a cryostat and mounted onto Superfrost Plus microscope slides (Fisher). Sections were dried for 30 min at RT and then fixed in phosphate-buffered saline (PBS) plus 2% paraformaldehyde for 10 min. After rinses in PBS, sections were blocked in 2% normal rabbit serum and 0.1% Triton X-100 in PBS for 5 min and incubated at RT for 1 h with anti-PKD2L1 rabbit polyclonal antibody PR71. Sections were then rinsed and incubated with a secondary FITC-conjugated anti-rabbit antibody (1:100; Chemicon International) for 1 h. After washing, sections were mounted in Vectashield (Vector Laboratories, Burlington, Ontario, Canada) for fluorescence detection with a Zeiss 510 confocal laserscanning microscope.

4.4. RESULTS

Interaction between PKD2L1 and RACK1 Revealed by Yeast Two-hybrid Analysis

Our previous study showed that the C-terminal EF-hand motif Glu^{637}–Leu^{665} acts as a negative regulator of Ca^{2+}-induced PKD2L1 channel activation and that truncation mutant T622X still exhibits channel function, indicating that the domain C-terminal of Thr^{622} is not essential for channel function (14). In the present study, we utilized the N-terminus (PKD2L1N; Met^{1}–Ile^{97}) as bait to screen for interacting partner proteins by the yeast two-hybrid approach. For this, PKD2L1N was fused into vector pGBKT7 as bait to screen a kidney cDNA library in yeast strain AH109. The C-terminus of RACK1 (Lys^{139}–Arg^{317}; named RACK1C) was identified from the library as a PKD2L1N binding partner (Fig. 4-1A). The physical association between the two protein fragments was confirmed by an individual pair test in yeast. To further delineate the corresponding regions within PKD2L1N and RACK1 that mediate the association, we constructed a series of truncated cDNA fragments that encode peptides expressed as fusion proteins in yeast strain Y187 using vector pGBKT7. Upon examination for interaction with PKD2L1N, we found that at least two domains of RACK1, including Lys^{139}– Glu^{223} and Met^{217}–Arg^{317}, interact with PKD2L1N (Fig. 4-1A) and that on the other hand fragment Ala^{19}–Ile^{97} of PKD2L1N interacts with RACK1C (Fig. 4-1B). Furthermore, our yeast two-hybrid assays found that RACK1 also interacts with the PKD2L1 C-terminus (PKD2L1C; Glu^{566}–Ser^{805}),

indicating that more than one domain in both RACK1 and PKD2L1 are involved in their interaction.

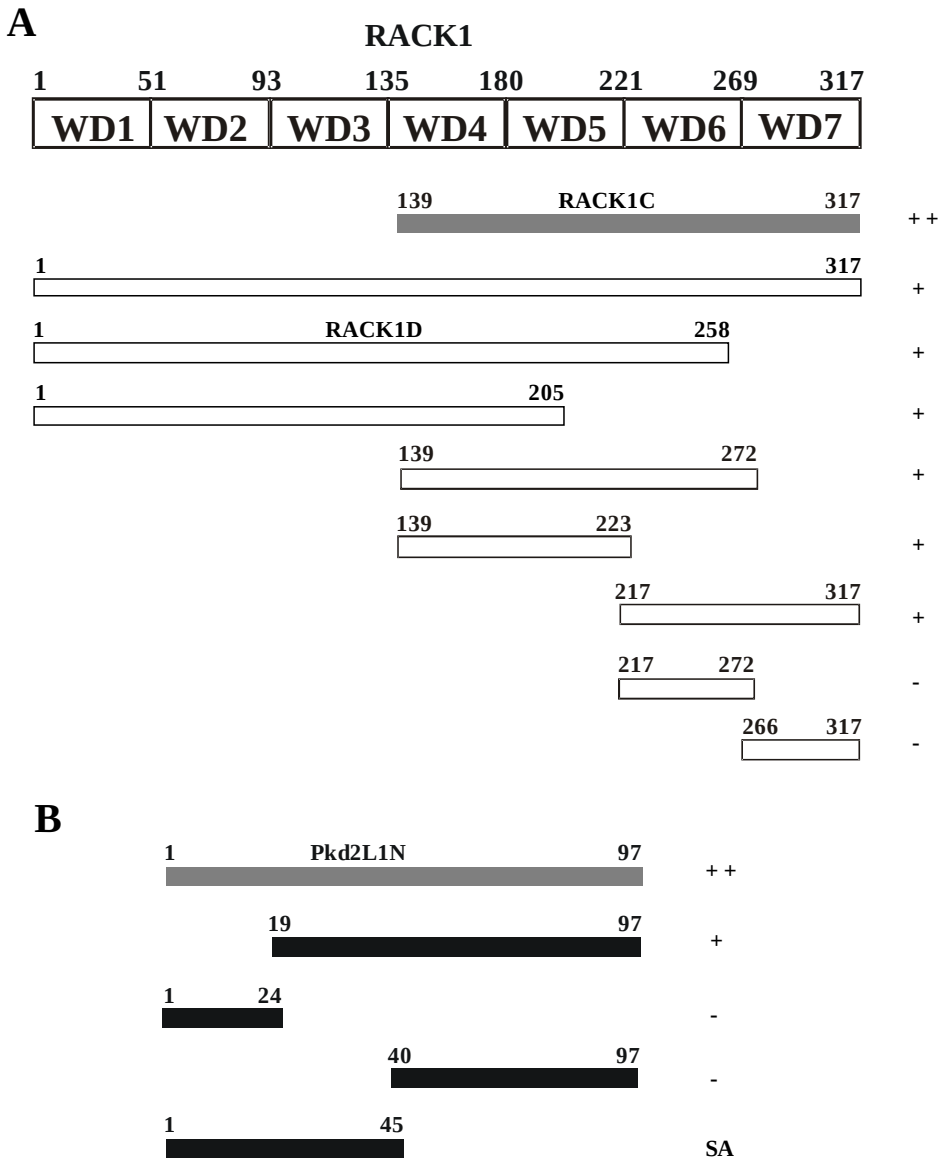

Fig. 4-1. Interaction between PKD2L1 N-terminus and RACK1 identified by yeast two-hybrid assay. The start and end amino acid residue numbers for each domain are indicated. "++", "+," and "-" indicate the presence of a strong interaction, positive interaction, and absence of interaction, respectively. **A**, RACK1 was identified from a human kidney library by yeast two-hybrid screening using the PKD2L1 N-terminal segment Met^{1}–Ile^{97} (PKD2L1N) as bait. RACK1C and several truncated fragments of RACK1 were assessed for their interaction with PKD2L1N. **B**, PKD2L1 N-terminal fragments were examined for interaction with RACK1C. Domain Met^{1}–Pro^{45} exhibited self-activation (SA) and was not used to assess the interaction with RACK1.

Interaction between PKD2L1 and RACK1 under in Vivo and in Vitro Conditions

We performed other assays to further document the interaction between PKD2L1 and RACK1. By GST pulldown, we showed that HA-PKD2L1N

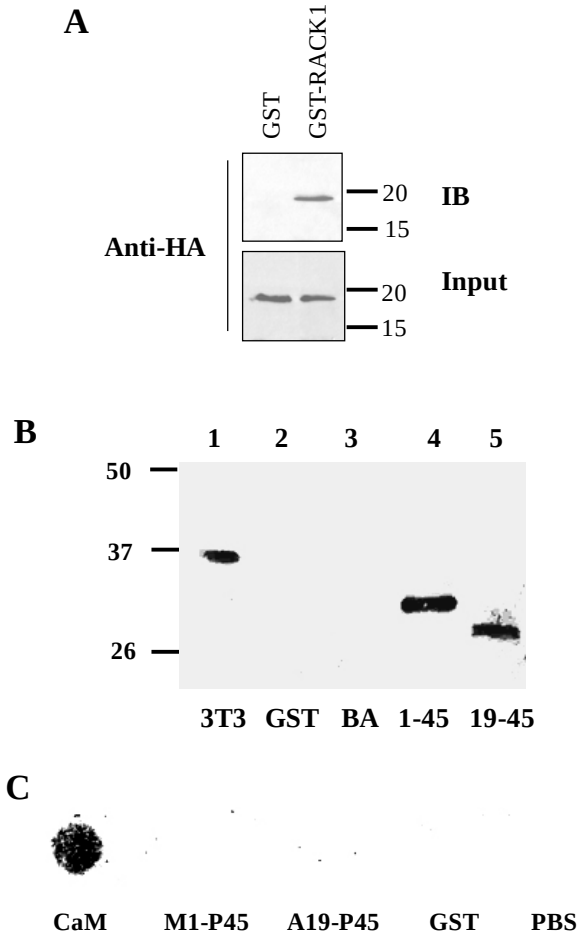

Fig. 4-2. Interaction between PKD2L1N and RACK1 by GST pulldown and blot overlay. A, GST pulldown. Precleared HEK293 cell lysates containing HA-PKD2L1N were incubated with purified GST-RACK1 protein (2 μg) in binding buffer. **B**, blot overlay for interaction between a PKD2L1 N-terminal fragment and endogenous RACK1. A nitrocellulose membrane containing purified GST-fused PKD2L1 N-terminal fragments was incubated with total protein lysates of NIH 3T3 cells and immunoblotted (IB) by the anti-RACK1 antibody. NIH 3T3 cell lysates were used as a positive control (lane 1). **C**, Ca^{2+} binding of PKD2L1 N-terminal domains Met^{1}–Pro^{45} and Ala^{19}–Pro^{45}. GST fusion polypeptides purified from E. coli were spotted on the nitrocellulose membrane strips, overlaid with ^{45}Ca in PBS buffer, and autoradiographed. Calmodulin (CaM) served as a positive control, whereas GST alone and bovine albumin (BA) served as negative controls.

expressed in HEK293 cells is able to interact with GST-RACK1 purified from Escherichia coli (Fig. 4-2A) with full-length human RACK1 generated by PCR. We also used a blot overlay assay to verify this interaction in vitro. Purified fusion proteins, including GST-PKD2L1N, GST-Met^{1}–Pro^{45}, and GST-Ala^{19}–Pro^{45}, were resolved using SDS-PAGE and transferred to a nitrocellulose membrane followed by incubation with NIH 3T3 cell lysates. Immunoreactive protein bands from probing with a RACK1 antibody were observed only in the presence of GST-PKD2L1N, GST-Met^{1}– Pro^{45}, or GST-Ala^{19}–Pro^{45} but not with GST alone or bovine albumin (Fig. 4-2B), which is indicative of specific binding between PKD2L1N and RACK1. Furthermore, we in vitro transcribed complementary RNAs of PKD2L1 and RACK1 and injected them into *Xenopus* oocytes. After 3 days, total protein extracts were used to perform co-IP assays between the two proteins. Precipitation with anti-PKD2L1 antibody PR71 resulted in the pulldown of RACK1, and reciprocally, anti-RACK1 antibody was also able to co-precipitate PKD2L1 in oocytes (Fig. 4-3A), demonstrating that the two proteins are in the same complex in oocytes. We further substantiated the interaction between PKD2L1 and RACK1 by co-IP using native HEK293 and IMCD3 cells and mouse kidney and heart tissues (Fig. 4-3B), indicating that the two proteins interact with each other in vivo. It is well known that RACK1 is abundantly expressed in brain and interacts with brain proteins such as NMDA and GABA receptors (38,39,49). On the other hand, PKD2L1 was also recently found in several brain compartments such as hippocampus, thalamus, and spinal cord, although its physiological roles there remain unclear (10,15). We thus tested their

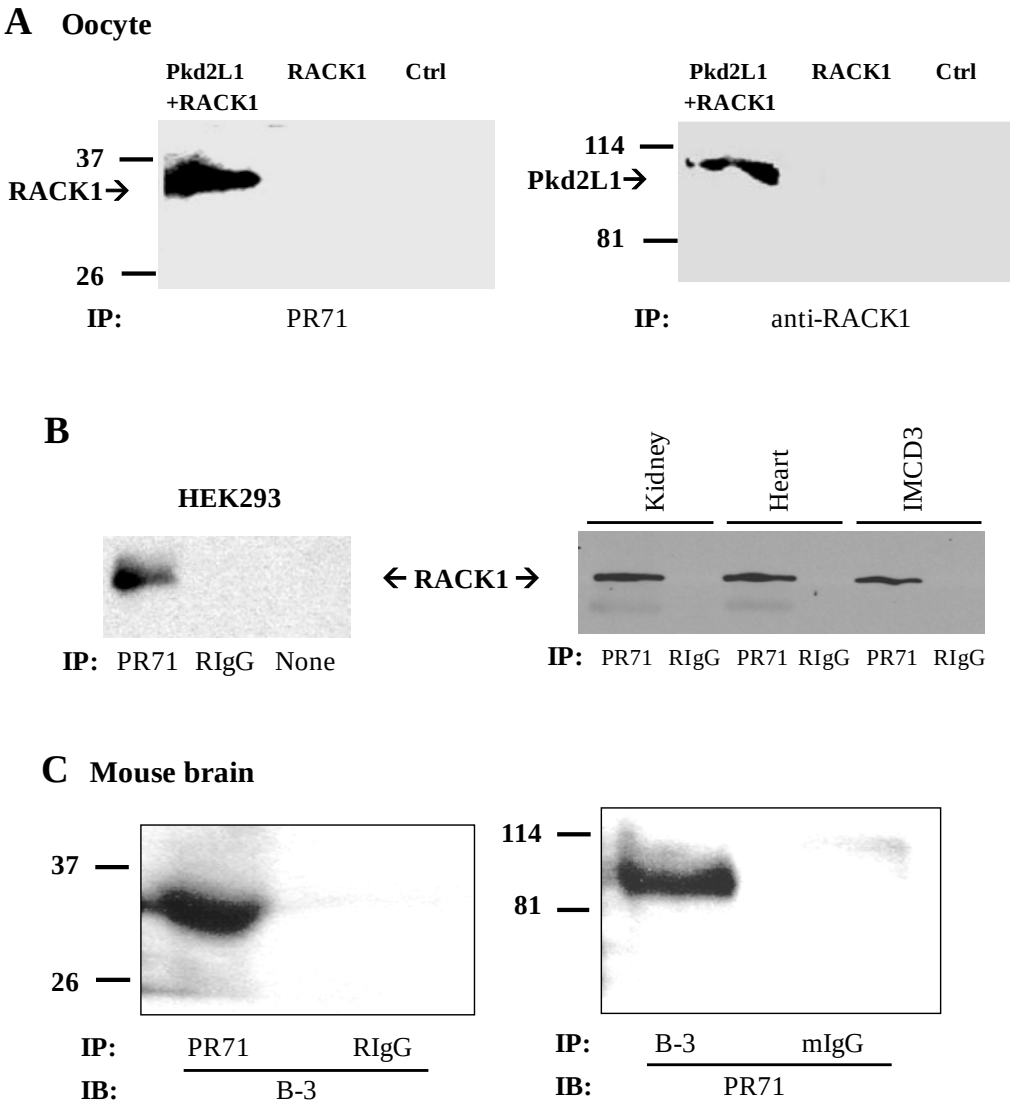

Fig. 4-3. Interaction between PKD2L1 and RACK1 by co-IP. A, *Xenopus* overexpressing PKD2L1+RACK1 or RACK1 and control (Ctrl) oocytes were used for reciprocal co-IP using anti-PKD2L1 (PR71) or anti-RACK1 antibody for IP or immunoblotting (IB) as indicated. **B**, left panel, cell lysates from native HEK293 cells were precipitated with PR71, non-immune rabbit IgG serum (RIgG; as control), or no antibody (None; as control). Immunoprecipitated proteins were analyzed by WB using the anti-RACK1 antibody (BD Biosciences). Right panel, representative data obtained from similar co-IP assays using native IMCD3 cells and mouse kidney and heart tissues with anti- PKD2L1 and -RACK1 antibodies for IP and immunoblotting, respectively. **C**, interaction between PKD2L1 and RACK1 in mouse brain by reciprocal co-IP. Left panel, cell lysates from mouse brain were precipitated with PR71 or rabbit IgG serum (RIgG). Immunoprecipitated proteins were analyzed by WB using the anti-RACK1 antibody. Right panel, reciprocal co-IP experiments were performed using RACK1 antibody B-3 or non-immune mouse IgG serum (mIgG) for IP and PR71 for immunoblotting.

interaction in mouse brain tissues by co-IP. Indeed, we found that PKD2L1 and RACK1 precipitate with each other and reciprocally (Fig. 4-3C). Taken together, our data demonstrated the interaction between PKD2L1 and RACK1 under in vitro and in vivo conditions.

Inhibition of PKD2L1 Channel Activity by RACK1

We next tested whether RACK1 has any effect on PKD2L1 channel function. By co-expressing PKD2L1 and RACK1 in *Xenopus* oocytes, we found that overexpression of RACK1 abolishes the Ca^{2+} transport ability of PKD2L1 (Fig. 4-4A). Under voltage clamp conditions, RACK1 abolished both the basal conductance and Ca^{2+}-induced channel activation of PKD2L1 (Fig. 4-4 B and C). On average, RACK1 inhibited 65 and 87% of basal and Ca^{2+}- activated currents, respectively (Fig. 4-4D). Because *Xenopus* and human RACK1 are 95% identical and 99% similar, we assume that endogenous RACK1, if present, is detectable by our antibody and inhibits part of PKD2L1 channel activity. Indeed, our Western blotting (WB) assays detected the endogenous RACK1 and showed that the total PKD2L1 expression is reduced by overexpressed RACK1 (to 68 ± 3%; averaged from five experiments) and increased by endogenous RACK1 knock-down by siRNA (Fig. 4-5A) with sequences 5'-CAAUCAAACUGUGGAACACTT- 3' and 5'-GUGUUCCACAGUUUGAUUGTT- 3' synthesized by Shanghai GenePharma Co. (Shanghai, China). Consistently, RACK1 knock-down on average increased Ca^{2+}-induced channel activity of PKD2L1 by 79% (n = 4) (Fig. 4-5B). We then performed biotinylation assays to examine whether abolished or

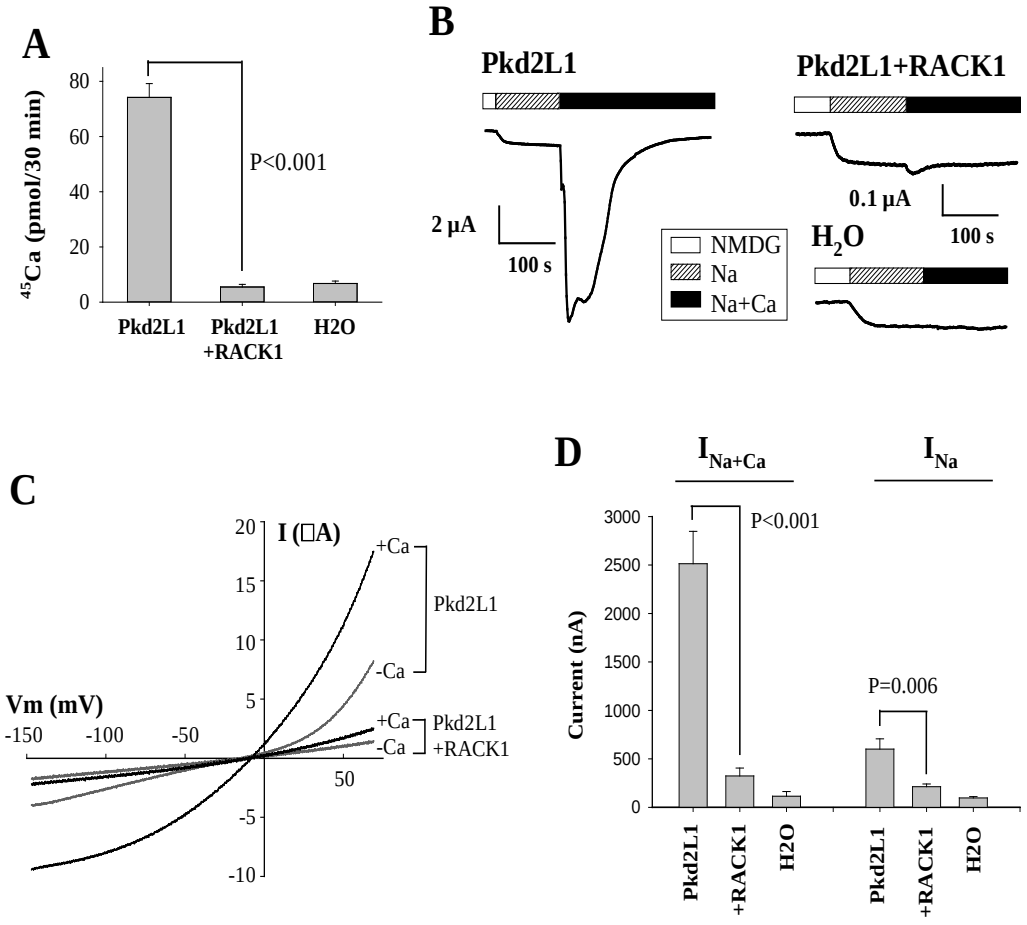

Fig. 4-4. Functional modulation of PKD2L1 channel by RACK1. A, radiolabeled ^{45}Ca uptake in oocytes expressing PKD2L1 + RACK1 or PKD2L1 alone and H_2O-injected control oocytes. Radiolabeled ^{45}Ca uptake was performed using uptake solution 3 days following RNA injection. Data were averaged from three experiments. The effect of RACK1 was analyzed by unpaired t test. **B**, representative whole-cell current tracings under voltage clamp (Vm= -50 mV) using the two-microelectrode voltage clamp technique. Different scales of current were used as indicated. Currents were measured using solution "Na" (the standard Na^+-containing solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.5) or "Na+Ca" (standard Na^+-containing solution + 5 mM CaCl2) by the two-microelectrode voltage-clamp technique with a voltage ramp protocol. **C**, representative current-voltage curves in an oocyte expressing PKD2L1 alone or PKD2L1+RACK1 in the presence of the standard Na^+-containing solution with (+Ca) or without (-Ca)5 mM $CaCl_2$ as indicated. **D**, averaged currents obtained in oocytes expressing PKD2L1+RACK1 (n = 17) or PKD2L1 alone (n = 17) and H_2O-injected control oocytes (n = 6) in the presence of Na^+-containing solution with (Na+Ca) or without (Na) addition of 5mM $CaCl_2$. Inhibition of currents by RACK1 was very significant both with and without Ca^{2+} with $p < 0.001$ and $p = 0.006$ (unpaired t test), respectively. Two-way analysis of variance found that the inhibitory effect of RACK1 is very significant ($p < 0.001$), that the stimulatory effect of Ca^{2+} is very significant ($p < 0.001$), and that the effect of RACK1 is significantly different ($p < 0.001$) whether Ca^{2+} is present or not in the solution, consistent with t test analysis. Error bars indicate S.E.

substantially reduced PKD2L1 activity by RACK1 could be accounted for by a decrease in the PKD2L1 PM expression. We found that PKD2L1 PM expression in the presence of RACK1 overexpression was decreased to 64 ± 7% (averaged from four experiments) (Fig. 4-5C). Thus, RACK1 reduces similarly the total and PM expression of PKD2L1 to about two-thirds, indicating that it does not alter the PKD2L1 trafficking. This reduction in the PM expression does not seem to be sufficient to account for the decrease in the Ca^{2+}-induced current (to 13%), indicating that RACK1 inhibits PKD2L1 channel function. Immunofluorescence data are also in agreement with the conclusion that RACK1 overexpression and knock-down, respectively, reduce and increase the PKD2L1 surface membrane expression (Fig. 4-5D). To provide further documentation, we injected oocytes with decreasing amounts of PKD2L1 mRNA to identify an amount that generates channel activity comparable with that produced by 50 ng of PKD2L1 + 25 ng of RACK1. We found that the current obtained from injection of 50 ng of PKD2L1 + 25 ng of RACK1 is comparable with that from the injection of 6.2 ng of PKD2L1 alone, but the latter has much less PM expression than the former (Fig. 4-6). In fact, although the PKD2L1 PM expression is comparable between injection of 50 ng of PKD2L1 + 25 ng of RACK1 and that of 25 ng of PKD2L1 alone, the latter produced far larger channel currents than the former. In this biotinylation assay, we used Na^+, K^+-ATPase as a PM marker control and calreticulin and β-actin as non-PM marker controls. These data demonstrated that RACK1 substantially inhibits PKD2L1 channel function.

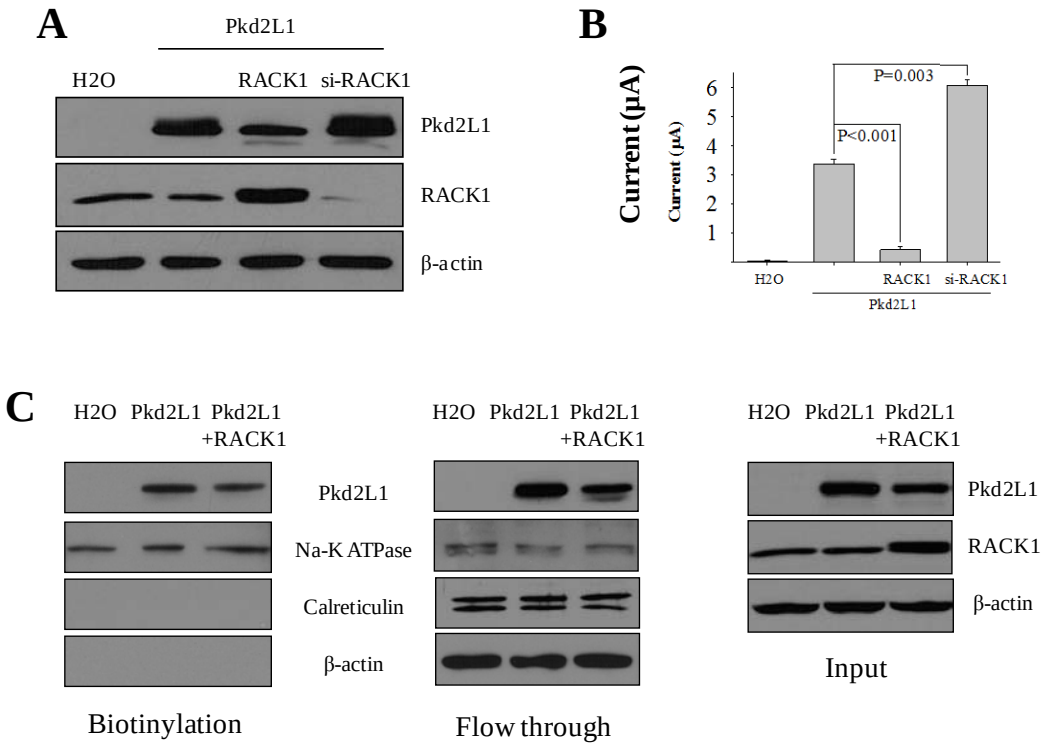

Fig. 4-5. Effects of RACK1 on channel activity and total and PM expression of PKD2L1 expressed in *Xenopus* oocytes. A and **B**, oocytes were injected with PKD2L1 + RACK1, PKD2L1 + si-RACK1, PKD2L1 alone, or water (negative control). **A**, representative data on expression of PKD2L1 and RACK1 by WB. **B**, Ca^{2+}-activated currents were measured at -50 mV. Shown are averaged values from four oocytes per group with p values from a paired t test. Error bars indicate S.E. **C**, left panel, representative data on the PM expression of PKD2L1 by biotinylation. Na^{+},K^{+}-ATPase (PM marker) and calreticulin and β-actin (non-PM markers) were used as controls of the biotinylation assay. Middle and right panels, flow-through and WB input data, respectively, from the same experiment. **D**, representative data on subcellular distribution of PKD2L1 and RACK1 by immunofluorescence.

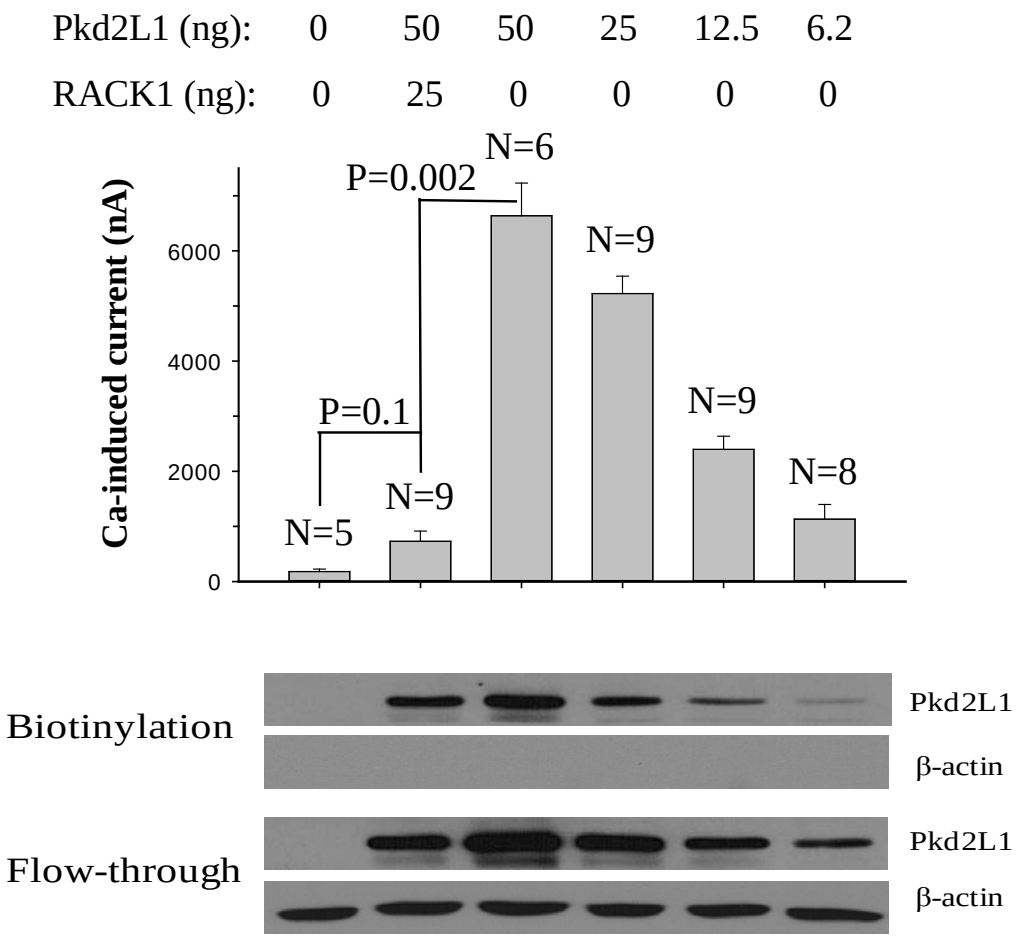

Fig. 4-6. Inhibition of PKD2L1 channel function by RACK1. Top, *Xenopus* oocytes were injected with various amounts of PKD2L1mRNAwith or without co-injection of RACK1. Shown are averaged Ca^{2+}-activated currents measured at -50 mV from different numbers of oocytes as indicated. p values obtained from an unpaired t test are indicated. Furthermore, linear and Michaelis-Menten regression analyses (current versus mRNA amount) indicate that current very significantly ($p < 0.001$) increases with the amount of injected mRNA. Bottom, the same batches of oocytes as in the top panel were used for biotinylation assays. Shown are representative data on the PM expression of PKD2L1 with β-actin as control.

Effects of Mutations in RACK1 and PKD2L1 on PKD2L1 Channel Function

Although both fragments Met^{1}–Ser^{205} and Met^{217}–Arg^{317} of RACK1 bind PKD2L1 (Fig. 4-1A), whether they are sufficient to exhibit inhibition of PKD2L1 channel function remains unknown. To test this, we co-expressed each of various fragments of RACK1 with PKD2L1 in oocytes and performed the two-electrode

voltage clamp experiments. We found that, similar to the full-length RACK1, fragments Met^{1}–Asp^{220} and Met^{1}–Ile^{258} significantly reduce PKD2L1 channel activity, whereas Met^{217}–Arg^{317} has no significant effect (Fig. 4-7A). This indicates that, although Met^{217}–Arg^{317} binds PKD2L1N, the binding is not essential for the channel inhibition. We also found that two missense mutants of RACK1, Y228F and Y246F, have inhibitory effects similar to those of WT RACK1, suggesting that the phosphorylation of RACK1 at Tyr^{228} or Tyr^{246} by Src tyrosine kinase (50) is not involved in its inhibition of PKD2L1 channel function. We also conducted PKD2L1 transport activity by means of ^{45}Ca radiotracer uptake. The results are in agreement with our data obtained from electrophysiology experiments (Fig. 4-7B).

Because our yeast two-hybrid experiments revealed that RACK1 binds fragment Ala^{19}–Pro^{45} of PKD2L1 and RACK1 was reported to modulate the function of target channels through regulating their phosphorylation (35,38), we made point mutations at potential phosphorylation sites within this fragment to determine whether these sites are important for channel function or inhibition by RACK1. The NetPhosK online program predicted that Thr^{34}, Thr^{39}, Ser^{41}, and Ser^{42} in PKD2L1N are candidate phosphorylation sites. We thus changed these residues to alanine and found that the resulting mutants still retain channel function comparable with that of WT PKD2L1 (Fig. 4-7C). Furthermore, RACK1 overexpression inhibited the channel function of these mutants as well (Fig. 4-7C). This result suggests that phosphorylation within Ala^{19}–Pro^{45} is not critical to PKD2L1 channel function or to its inhibition by RACK1.

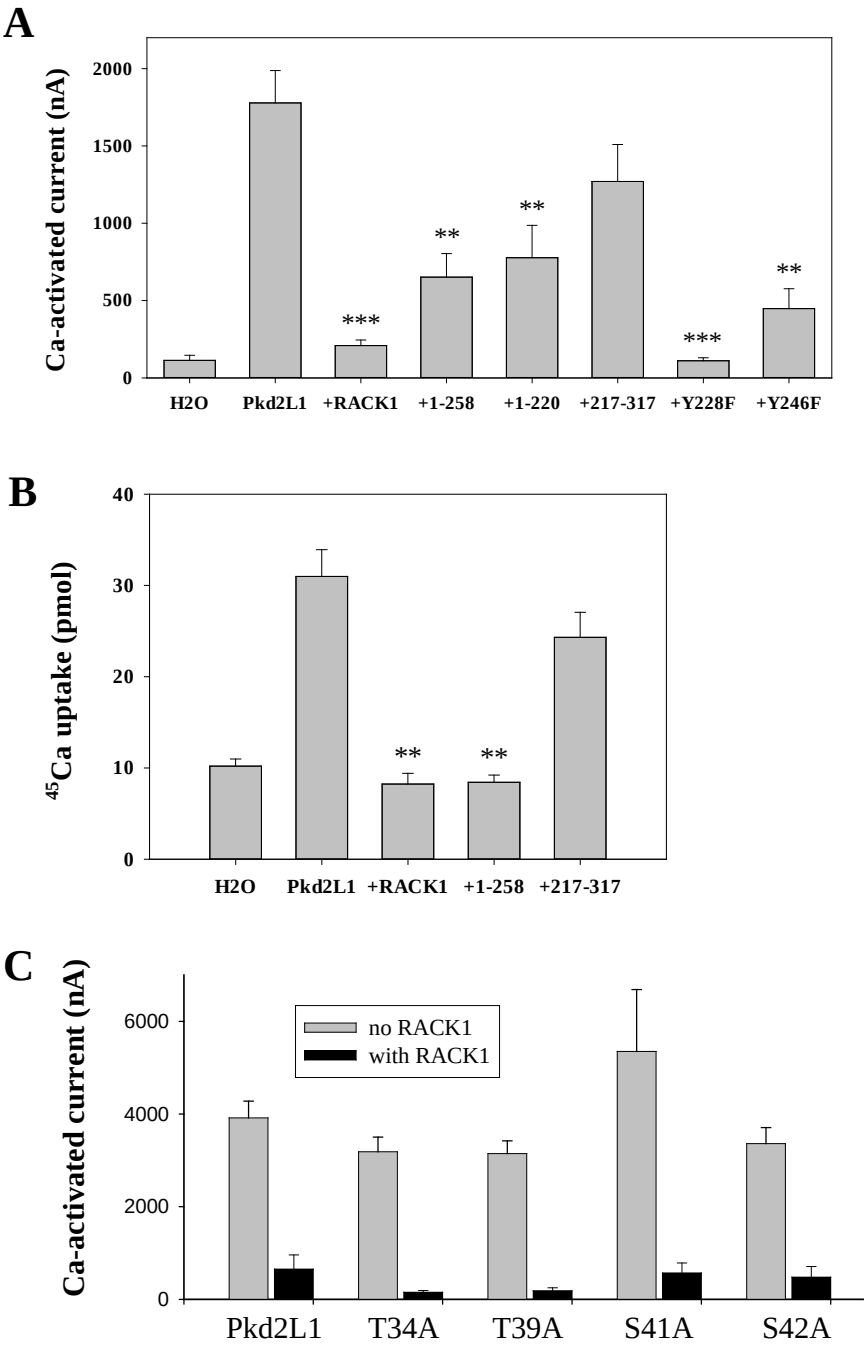

Fig. 4-7. Effects of mutations in RACK1 and PKD2L1. A and **B**, PKD2L1 was expressed in oocytes with or without co-expression of a RACK1 mutant. A, averaged Ca^{2+}-activated currents measured at -50 mV with each group from 5 to 16 different oocytes. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively. **B**, averaged ^{45}Ca tracer uptake from three independent experiments using uptake solution. Error bars indicate S.E. **C**, effect of RACK1 on the function of PKD2L1 mutants. PKD2L1 missense mutants were expressed in oocytes with or without co-expression of RACK1.Shown are averaged Ca^{2+}-activated currents measured at -50 mV with each group from 7 to 11 different oocytes.

Role of PKD2L1 Fragment Met^{1}–Pro^{45} in Mediating PKD2L1 Channel Inhibition by RACK1

To determine whether the physical interaction between PKD2L1 and RACK1 through the Met^{1}–Pro^{45} fragment of PKD2L1 is important for the inhibition of PKD2L1 channel function by RACK1, we co-expressed PKD2L1 and RACK1 with His-tagged Met^{1}–Pro^{45} peptide that should serve as a blocking peptide through competing with PKD2L1 for binding RACK1. Indeed, expression of His-Met^{1}– Pro^{45} rescued the channel function of PKD2L1 otherwise inhibited by RACK1 (Fig. 4-8A). In contrast, the remaining part of the N-terminus (His-Ile^{40}–Ile^{97}) and C-terminal fragments (His- tagged Glu^{566}–Thr^{622} and Gln^{617}–Ser^{805}) had no rescuing effects (Fig. 4-8B). By WB, we verified that the functional rescue by Met^{1}–Pro^{45} is not through decreasing RACK1 overexpression (Fig. 4-8C). Consistently, we found by co-IP that Met^{1}–Pro^{45} indeed substantially decreases the PKD2L1-RACK1 interaction (Fig. 4-8D). In negative control experiments, we verified that Met^{1}–Pro^{45} expressed alone has no channel function (data not shown). Taken together, our data support that binding between PKD2L1 and RACK1 through its N-terminal fragment Met^{1}–Pro^{45}, but not PKD2L1C or the remaining part of PKD2L1N, is required for inhibition of PKD2L1 channel function by RACK1.

Because oocytes possess significant endogenous RACK1 levels, we reasoned that Met^{1}–Pro^{45} should block the inhibition of PKD2L1 channel function by endogenous RACK1. Indeed, we found that overexpression of blocking peptide Met1–Pro45 roughly doubles the Ca^{2+}-induced current (at -50 mV) from

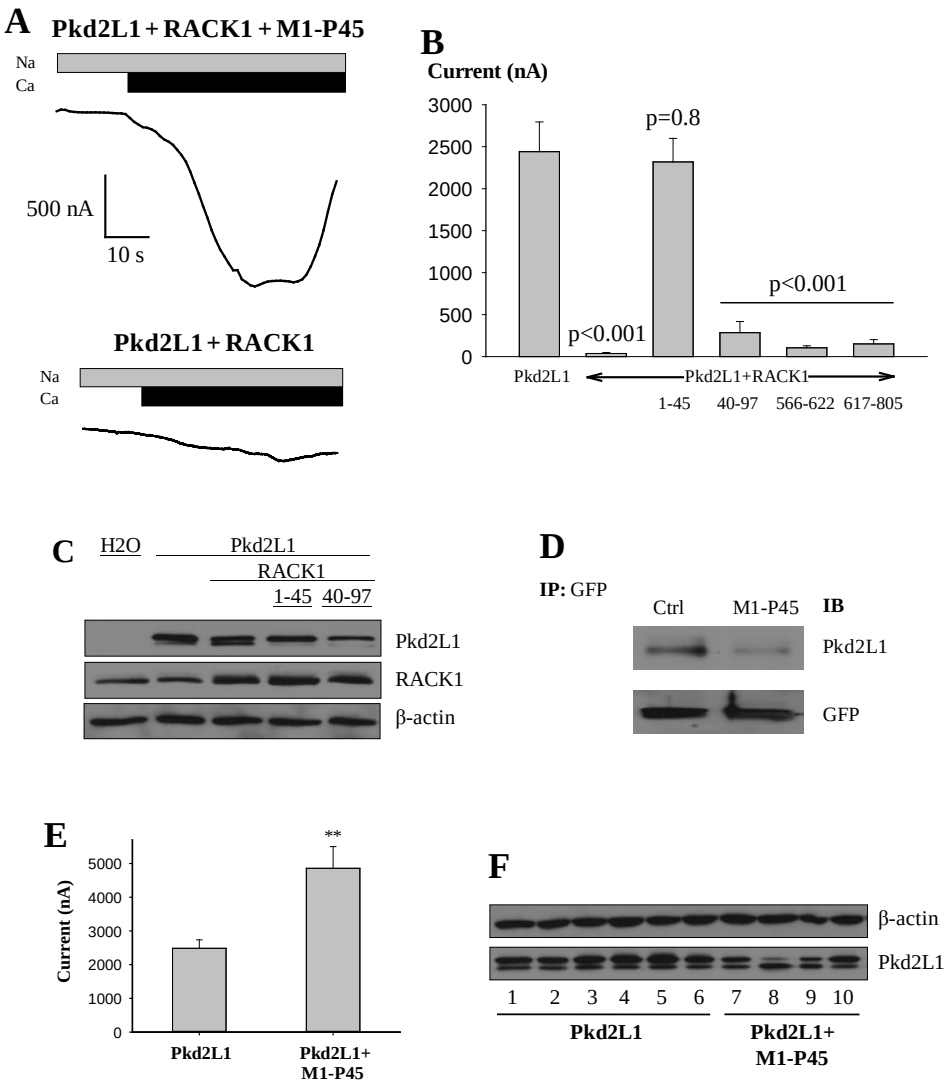

Fig. 4-8. Effects of PKD2L1 N- and C-terminal fragments on inhibition of PKD2L1 by RACK1 and on PKD2L1-RACK1 interaction. A, representative recordings of currents measured at -50 mV in oocytes expressing PKD2L1 and RACK1 with or without co-expression of Met^{1}–Pro^{45} in the presence of Na^{+}-containing solution ±5 mM Ca^{2+} as indicated. **B**, averaged Ca^{2+}-induced currents measured at -50 mV in oocytes expressing PKD2L1 alone (n = 15), PKD2L1+RACK1 (n = 12), or PKD2L1+RACK1+a blocking peptide (Met^{1}–Pro^{45}, n=14; Ile^{40}–Ile^{97}, n=11; Glu^{566}–Thr^{622}, n=9; and Gln^{617}–Ser^{805}, n=6). P values obtained from comparison with PKD2L1 alone (by unpaired t test) are indicated. **C**, expression of RACK1 and PKD2L1 with or without co-expression of Met^{1}–Pro^{45} in oocytes assessed by WB. **D**, the PKD2L1-RACK1 interaction assessed by co-IP with or without (control (Ctrl)) co-expression of Met^{1}–Pro^{45}. PKD2L1, GFP-RACK1, and/or His-Met^{1}–Pro^{45} were expressed in oocytes. Shown are representative data in which GFP antibody was used for IP and PKD2L1 and GFP antibodies were used for immunoblotting (IB) as indicated. **E**, effect of overexpression of Met^{1}–Pro^{45} on PKD2L1 activity. Shown are averaged Ca^{2+}-induced currents from oocytes expressing PKD2L1 (n=24) or PKD2L1 plus Met^{1}–Pro^{45} (n=20, p=0.005; **, unpaired t test). **F**, representative data of PKD2L1 expression by WB in individual oocytes expressing either PKD2L1 (lanes 1–6) or PKD2L1 plus Met^{1}–Pro^{45} (lanes 7–10). These oocytes are part of the oocytes used in generating currents in E. Error bars indicate S.E.

an average of 2.5 ± 0.3 μA (n = 24) in oocytes expressing PKD2L1 to 4.8 ± 0.6 μA (n = 20, p = 0.005) in those expressing PKD2L1 + Met^{1}–Pro^{45}. Furthermore, in the presence of Met^{1}– Pro^{45}, the PKD2L1 expression assessed by WB was slightly decreased (Fig. 4-8F) possibly due to limited expression capability of oocytes. These data indicate that Met^{1}–Pro^{45} blocks the inhibition of PKD2L1 by endogenous RACK1 in agreement with the data obtained from using overexpressed RACK1 (Fig. 4-8, A and B).

4.5. DISCUSSION

The mechanism of Ca^{2+}- or acid-induced PKD2L1 channel activation has remained obscure. The C-terminus of PKD2L1 contains a number of motifs, including a putative Ca^{2+}-binding EF-hand and a coiled coil domain, but when these motifs were removed as in the truncation mutant T622X or when the EF hand was deleted, Ca^{2+}-induced activation was still present and even increased (14). In an effort to examine the role of the PKD2L1N-terminus in channel activation, we found in the present study that PKD2L1N associates with RACK1 and that RACK1 inhibits PKD2L1 channel function through binding to PKD2L1N. RACK1 was initially identified as a receptor for activated PKC, but recent studies showed that it is also involved in many other cellular functions. For example, RACK1 acts as a novel inhibitory scaffolding protein in complex with tyrosine kinase Fyn and its substrate, the NR2B subunit of NMDAR, and this association prevents the phosphorylation of NR2B by Fyn and inhibits NMDAR channel activity (38,42). The presence of RACK1 in CA1 hippocampal slices resulted in a 50% decrease in NMDAR-mediated current (38).

Because RACK1 reduced both the total and PM expression of PKD2L1, it remained to be determined whether RACK1-inhibited whole-cell PKD2L1 channel activity is solely due to the reduced PM level of PKD2L1 or also to the inhibited activity of individual channel molecules. First, although co-expression of RACK1 reduced the PKD2L1 PM level by 36%, it inhibited 87% of the Ca^{2+}-activated whole-cell current, indicating that RACK1 represses the activity of each

individual PKD2L1 channel. Then we found that although the PKD2L1 surface membrane expression from injection of 50 ng of PKD2L1 and 25 ng of RACK1 mRNAs, is comparable with that from injection of 25 ng of PKD2L1 mRNA alone, the whole-cell current associated with injection of 50 ng of PKD2L1 + 25 ng of RACK1 is only comparable with that associated with injection of 6.2 ng of PKD2L1 alone (Fig. 4-6). Thus, these data together showed that RACK1 substantially inhibits PKD2L1 channel function in addition to reducing its total and surface membrane expressions.

We found that two RACK1 fragments bind PKD2L1 and that RACK1 binds both the N- and C-termini of PKD2L1. It was thus unclear whether all physical bindings are important for inhibition of PKD2L1 function by RACK1. Although we could utilize truncation/deletion mutations in PKD2L1 and/or RACK1 that disrupt the PKD2L1-RACK1 interaction to examine whether a mutant RACK1 still inhibits WT channel function or whether WT RACK1 inhibits the function of a mutant PKD2L1, the information we obtain would not be sufficient to make a conclusion because the normal function of RACK1 or PKD2L1 may readily be affected by these mutations. In view of this, we utilized co-expression of PKD2L1 N- and C-terminal fragments as potential blocking peptides to break down the physical association between WT PKD2L1 and RACK1. Our data demonstrated that fragment Met^{1}–Pro^{45} is able to substantially reduce the PKD2L1-RACK1 binding and abolish inhibition by RACK1, indicating that their binding through domain Met^{1}–Pro^{45} is the strongest and critical for RACK1 to exert functional inhibition. In contrast, although PKD2L1C

also binds RACK1, overexpression of PKD2L1C was unable to significantly affect the PKD2L1- RACK1 binding, suggesting that their binding through PKD2L1C is not as strong as through Met^{1}–Pro^{45} and is not important for functional regulation. It is interesting to note that similarly, although both Met^{1}–Asp^{220} and Met^{217}–Arg^{317} of RACK1 associate with PKD2L1, only Met^{1}–Asp^{220} inhibited PKD2L1 channel activity, indicating that physical interaction of Met^{217}–Arg^{317} with PKD2L1 is not essential for the channel inhibition.

How the binding of RACK to domain Met^{1}–Pro^{45} of PKD2L1 inhibits the channel function remains unclear. Because RACK1 was known as a chaperone of protein kinases to promote the phosphorylation of target proteins, it is possible that binding of RACK1 to domain Met^{1}–Pro^{45} prevents phosphorylation of a residue within the domain. Therefore, we mutated candidate phosphorylation residues to alanine: T34A, T39A, S41A, and S42A. However, all these mutants still possessed channel function comparable with that of WT channel and were inhibitable by RACK1, indicating that these potential phosphorylation sites are not important for channel function or binding with RACK1. On the other hand, if Ca^{2+} could bind to Met^{1}–Pro^{45} of PKD2L1 then RACK1 may prevent the Ca^{2+} binding, thereby inhibiting channel activation. However, our dotblot calcium overlay assays for testing whether Met^{1}–Pro^{45} can directly bind Ca^{2+} ions using GST-fused peptides Met^{1}–Pro^{45} and Ala^{19}– Pro^{45} purified from *E. coli* revealed that GST-tagged Met1– Pro45 and Ala^{19}–Pro^{45} cannot bind Ca^{2+} (Fig. 4-2C). Thus, it is possible that Ca^{2+}-induced channel activation is mediated by an unknown protein that binds Met^{1}–Pro^{45} and that RACK1 competitively disrupts their

interaction, thereby inhibiting PKD2L1 channel function. Further studies to identify and characterize such an unknown protein would shed light on the mechanism underlying Ca^{2+}-induced PKD2L1 activation.

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CHAPTER 5

GENERAL DISCUSSION

5.1. Mechanism of how PKD2 regulates cell proliferation

PERK is an important sensor and mediator during ER stress. Above all, activated PERK by ER stress phosphorylates eIF2α that leads to inhibition of global protein synthesis that is associated with down-regulation of cell growth, and translational up-regulation of selected cellular factors (1,2). For example, elevated P-eIF2α promoted by PERK during ER stress represses the translation of cyclin D1, a regulatory subunit for cell cycle G1/S transition, which leads to ER stress-induced G1 arrest in the cell cycle (3). ADPKD caused by loss- or gain-of-function of PKD1 or PKD2 is characterized by over-proliferation, apoptosis and de-differentiation, among others.

Our study shows that, in the presence of reduced PERK, the ability of PKD2 to promote eIF2α phosphorylation and repression of cell proliferation is compromised, indicating that the regulation of eIF2α phosphorylation and cell proliferation by PKD2 is PERK-dependent (Fig. 2-6 and 8). Interestingly, the fact that among the four kinases only PERK is localized on the ER membrane and that PERK is in the same complex as eIF2α and PKD2 (Fig. 2-5) suggest that PKD2 may play an essential role in recruiting eIF2α to form PERK-substrate complexes for eIF2α phosphorylation. It suggests that PKD2 promotes the efficiency of the interaction of PERK with eIF2α (as a substrate) for PERK-dependent eIF2α phosphorylation.

Taken together, we propose that the PERK/eIF2α pathway is involved in PKD2–regulated cell proliferation. This finding will shed light on understanding

the molecular mechanism underlying the PKD2-associated cell over-proliferation in ADPKD.

5.2. Mechanism of how PKD2 expression is regulated under stress and non-stress conditions

The UPR maintains cell homeostasis during cellular stresses by facilitating the translation and transcription of selected proteins and genes to rescue the cells (4). For example, ATF4 is translationally up-regulated by elevated P-eIF2α through skipping an inhibitory uORF in the 5'UTR of ATF4 mRNA during cellular stress (5,6). Similarly, Gadd34 and CHOP also possesses an inhibitory uORF in their 5'UTR that inhibits their translation under non-stress (low P-eIF2α), and is skipped under stress conditions (elevated P-eIF2α), which leads to translational up-regulation (7,8). How uORF exerts an inhibitory effect under non-stress conditions remain not well understood but it was suggested that small peptide translated from a uORF somehow inhibits the scanning of ribosome-mediated translation of the main ORF (8). Our study using WB shows that PKD2 expression is similarly regulated by stress as ATF4, CHOP and Gadd34, ie, stays relatively low under non-stress conditions and increases during ER stress (Fig. 3-8A and B). In sucrose gradient polysomal assays, PKD2 mRNAs are associated with heavier ribosomes under ER stress, compared to the non-stress condition, supporting that PKD2 translation is actively promoted during ER stress (Fig. 3-7). We think that, similar to ATF4, CHOP and Gadd34, the uORF in PKD2 5'UTR inhibits PKD2 translation under non-stress (low P-eIF2α), and that is skipped

under stress conditions (elevated P-eIF2α), which leads to PKD2 translational up-regulation.

Because proteins regulated by P-eIF2α such as ATF4, Gadd34 and CHOP are tightly associated with UPR and play critical roles in defining cell fate during cellular stress, it is possible that PKD2 may also play an important role in cell survival and growth through acting as a downstream factor of P-eIF2α and UPR pathways.

5.3. Function and regulation of PKD2 in UPR

The UPR in response to cellular stresses has three representative pathways in the ER. One of these is for 1) reducing global translation level to prohibit further production of misfolded or unfolded proteins, which is induced by elevated P-eIF2α (9), and 2) increasing ER chaperone proteins such as GRP78, calnexin and GRP94, to improve the protein folding capacity (10), and 3) promoting degradation of misfolded proteins, called ER-associated degradation (ERAD) (11). Elevated P-eIF2α activated by PERK at the same time up-regulates the translation of selected cellular factors including ATF4, Gadd34 and CHOP, which have crucial functions in mitigating cellular stress conditions (7,8,12-16). Furthermore, PERK/eIF2α pathway is also involved in induction of cellular inhibitors of apoptosis (cIAP1 and cIAP2) that inhibits ER stress-induced apoptosis and ER lumen chaperones like GRP78 (17,18). Our study showed that the eIF2α phosphorylation by PERK during ER stress is strongly reduced in the cells with PKD2 knock-down by siRNA (Fig. 2-4), indicating that PKD2 is an

important regulator of the PERK/eIF2α pathway in UPR. This was supported by our observation that, in PKD2 KO mouse collecting duct (MCD) cells D3 ($PKD2^{+/-}$) and B2 ($PKD2^{-/-}$), cIAP2 expression that is regulated by PERK/eIF2α pathway is significantly reduced in B2 cells compared to D3 cells (data not shown). In addition, induction of GRP78 expression induced by ER stress is significantly reduced in B2 cells. Possibly, loss of PKD2 negatively influences the PERK/eIF2α pathway, which leads to dysregulation of its down-stream factors such as cIAPs and ER lumen chaperones, which are strongly related to ER stress-induced apoptosis. Thus, the effects of PKD2 on PERK/eIF2α pathways may consistently explain the mechanism of how cystic cells caused by PKD2 loss-of-function are associated with elevated apoptosis.

In addition to eIF2α, PERK also phosphorylates Nrf2 that is a crucial transcription factor for cell survival during cellular stresses. Nrf2 is associated with a cytoskeletal anchor protein, Keap1 during non-cellular stress and dissociated from Keap1 induced by Nrf2 phosphorylation that is phosphorylated by PERK during cellular stresses, which is followed by nuclear import and induction of Nrf2-dependent gene expression of phase II detoxifying enzymes (19). Here, our data showed that knock-out of PERK negatively influences PKD2 protein expression in MEF $PERK^{-/-}$ cells (Fig 2-5B). By co-IP, P-PERK was found to have higher binding affinity with PKD2 than non-phosphorylated PERK (data not shown). Possibly, PKD2 also can be one of the substrates phosphorylated by PERK, which may regulate protein stability, channel activity or other cellular functions of PKD2.

All evidence together indicates that most substrates and down-stream factors activated by PERK/eIF2α pathways, which includes PKD2, play very important roles in cell survival during cellular stress. Therefore, PERK/eIF2α pathways may be part of the mechanism underlying pathgenesis of ADPKD.

5.4. Regulation of PKD2L1 channel activity

PKD2L1 functions as a Ca^{2+}-modulated nonselective cation channel when expressed in *Xenopus* oocytes (20). The C-terminus of PKD2L1 includes an EF-hand motif that may bind Ca^{2+}, but when mutants with the EF-hand motif deletion still exhibit Ca^{2+}-modulated channel activities, indicating the motif is not critical to its channel function (21). Also, PKD2L1, in the presence of co-expression of PKD1L3, also functions as an off-response channel induced by acid in mammalian cells (22), in which it seems that PKD1L3 is required both PKD2L1 PM targeting and channel function. However, our study showed that PKD2L1 alone still exhibits acid-induced off-response using *Xenopus* oocyte expression. Therefore, whether and how PKD2L1 is activated by acid, and whether and how Ca^{2+}- and acid-induced activations share similar molecular mechanisms remain unknown. Unpublished data from Dr. Chen's laboratory indicate that truncation mutant of PKD2L1 missing the whole C-terminus retains its activation by acid but has no channel activation by Ca^{2+}, suggesting the two activation are mechanically distinct.

PKD2L1 channel activity is regulated by several interacting partner proteins. For example, the channel activity of PKD2L1 is inhibited by troponin I

through direct physical association (23) and interaction with α-actinin promotes PKD2L1 channel activity (24). In an effort to find out how PKD2L1 channel activity is regulated by Ca^{2+}, we found that PKD2L1 is associated with RACK1 protein. In general, RACK1 is known as a receptor for activated PKC, which is involved in various cellular functions. For example, RACK1 acts as a scaffolding protein in complex with α-kinase domain in the TRP melastatin (TRPM6), which leads to inhibition of TRPM6 channel activity (25), and RACK1 also inhibits NMDA receptor (NMDAR) channel activity through inhibiting the phosphorylation of NR2B by tyrosine kinase Fyn (26).

Using electrophysiology, our study showed that RACK1 dominantly inhibits PKD2L1 channel activity even though RACK1 inhibits total and plasma membrane expression of PKD2L1. N-terminal fragment Met^{1}-Pro^{45}, a binding partner of RACK1, but not the C-terminus or other parts of the N-terminus, of PKD2L1 acts as a blocking peptide and abolishes the inhibition of PKD2L1 channel activity by RACK1, indicating that Met^{1}-Pro^{45} competes with PKD2L1 for binding RACK1 thereby releasing PKD2L1 from inhibition by RACK1 (Fig. 4-8). This competitive binding is shown by our further co-IP experiments (Fig. 4-8D). However, how the RACK1-PKD2L1 binding through the M1-P45 domain inhibits PKD2L1 remains to be studied. RACK1 has been known as a scaffolding protein to transport protein kinases to target proteins. Interestingly, most of phosphorylation sites (T34, T39, S41 and S42) in PKD2L1 are localized in the N-terminus. Thus, it is possible that RACK1 binds with PKD2L1 either to facilitate the phosphorylation of the channel protein by a protein kinase or to alter the

phosphorylation state within its M1-P45 domain. We have examined some candidate phosphorylation sites in the N-terminus through mutagenesis (T34A, T39A, S41A and S42A) but so far we have not identified the site that accounts for RACK1-mediated channel inhibition (Fig. 4-7). Furthermore, the dot blot Ca^{2+} overlay assay reveals that Met^{1}-Pro^{45} of PKD2L1 is not a domain for binding with Ca^{2+} (Fig. 4-2C). Therefore, we suggest that possibly, Ca^{2+}-modulated channel activation of PKD2L1 is regulated by an unknown, Ca^{2+}-sensitive protein whose binding with Met^{1}-Pro^{45} of PKD2L1 is disrupted by RACK1, which leads to inhibition of Ca^{2+}-induced channel activation.

Taken together, although the mechanism of how PKD2L1 channel activity is regulated by Ca^{2+} is still unclear, this study has made significant progress towards elucidating the mechanism.

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