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The Structure and Function of PKN, a Protein Kinase Having a Catalytic Domain Homologous to That of PKC

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PKN is a serine/threonine protein kinase that has a catalytic domain homologous to protein kinase C (PKC) family members and a unique regulatory region containing antiparallel coiled-coil (ACC) domains. PKN is the first identified serine/threonine protein kinase that can bind to and be activated by a small GTPase Rho, and it can also be activated by fatty acids such as arachidonic acid *in vitro*. PKN is widely distributed in various organisms such as mammal, frog, fly, and starfish. There are at least three different isoforms of PKN (PKNα/PAK-1/PRK-1, PKNβ, and PRK2/PAK-2/PKNγ) in mammals, each of which shows different enzymological properties, tissue distribution, and varied functions.

Key words: fatty acid, PKN, PAK, PRK, Rho.

Abbreviations: aa, amino acid; C-terminal, carboxyl-terminal; N-terminal, amino-terminal; PKC, protein kinase C.

Structure

PKN cDNA was first isolated in 1994 from a human hippocampal cDNA library by a low stringency plaque hybridization using the cDNA fragment for the catalytic domain of PKCβII as a probe (*1*), and PKN protein was purified to homogeneity from rat testes using a specific antibody raised against recombinant PKN (*2*). Subsequent molecular cloning and enzymological analysis revealed the existence of multiple isoforms of PKN in various species. Since the sequence analysis was carried out independently in several laboratories, different nomenclatures have been proposed for the various cDNA clones, as given in Table 1. A novel protease-activated protein kinase activity had been detected in cytoplasmic extracts of rat liver using a peptide analog of ribosomal protein S6 as a phosphate acceptor in 1984 (*3*), and the presumed responsible enzyme was designated as PAK-1 (*4*) by Wettenhall and colleagues. Later, the enzyme turned out to be the product of the PKN gene (*5*). The nomenclature adopted herein places priority on the primary structure of the enzymes, designating the family of enzymes as "PKN" and the three isoforms of PKN so far isolated as "PKNα", "PKNβ", and "PRK2/PKNγ". The structures of the isoforms of PKN are shown schematically in Fig. 1. The C-terminal region of PKN contains the Ser/Thr type protein kinase domain, which shows high sequence homology to that of PKC family members. The N-terminal region of PKN contains three homologous stretches of approximately 70 amino acids, each containing a region relatively rich in charged residues, followed by a Leu zipper-like sequence. Structural analysis of the first stretch showed that two long α helices form an antiparallel coiled-coil fold (ACC finger) (*6*) and also bind to the small GTPase RhoA as described below. Hence, this stretch is called the "ACC domain" (Fig. 1). The ACC domain was previously called the "CZ region" (charged aa and Leu-zipper-like sequence) or "HR1" (*7*). ACC domains also work as a binding interface with various other associated proteins, such as anchoring protein CG-NAP (centrosome and Golgi localized PKN associated protein) (*8*). A stretch of about 130 amino acids [corresponding to HR2 (*7*)] between the ACC domain and the catalytic domain of PKN has a weak homology to the C2 domain of PKC ε and η, but its tertiary structure has not yet been elucidated. The C-terminal part of the C2-like region functions as an auto-inhibitory region, which is sensitive to arachidonic acid, one of the activators of PKN as described below (*9*). The ACC domain and the C2-like region are conserved among PKN family members isolated from different organisms such as mammals, frog (*10*), and fly (*11*), and also among the isoforms [PKNα, PKNβ (*12*), and PRK2/PKNγ (*7*)]. PRK2/PKNγ and PKNβ have one and two Pro-rich regions, respectively, containing the minimal consensus sequence for Src homology 3 (SH3) domain recognition, between the C2-like region and the catalytic domain of each enzyme. The Pro-rich region of PRK2/PKNγ and the first one of PKNβ possess the class II-like consensus sequence (*13*). The Pro-rich region is absent from PKNα. In fact, some proteins with SH3 domains have been reported to associate with PRK2/PKNγ and PKNβ: NCK (*14*) and Grb4 (*15*) with PRK2/PKNγ; and GRAFs (*16*), GTPase-activating protein for RhoA, and Cdc42Hs with PKNβ.

Distribution of PKN

PKNα mRNA is expressed ubiquitously in human and rat tissue (*1*, *17*). Antiserum against a recombinant fragment of PKNα detected an ~120-kDa protein band on SDS-PAGE in various rat tissues, and abundantly so in the spleen, thymus, and testes (*2*). Although Palmer et al.

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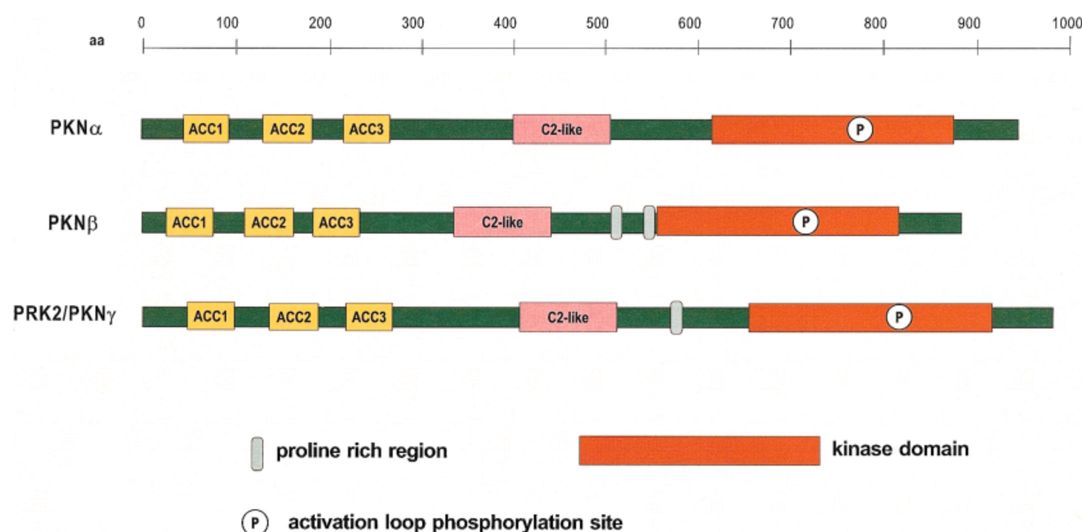

Fig. 1. **Primary structure of human PKN.**

reported that human PRK2/PKNγ mRNA was expressed specifically in a DX3 lymphoid lineage by Northern blotting and RT-PCR experiments (*7*), mouse PRK2/PKNγ mRNA was detected ubiquitously in tissues by Northern blotting (*14*) and whole body situ hybridization (personal communication). Also, endogenous PRK2/PKNγ protein was reported to be identified in rat liver (*18*, *19*), mouse keratinocytes (*20*), Jurkat cells (*21*), HeLa cells (*22*), and starfish oocytes (*23*, *24*). In contrast, PKNβ mRNA was almost undetectable in normal human adult tissues, but was detected in various cancer cell lines. Levels were especially abundant in chronic myelogenous leukemia K-562, colorectal adenocarcinoma WE480, and HeLa cells (*12*). The PKNα level in the rat brain was approximately 0.01% of total protein, and biochemical fractionation revealed that half of the total PKNα is distributed in the soluble cytosolic fraction, and one-third of it is in a post-nuclear membrane fraction (*2*). Light, electron, and confocal laser microscopy revealed that PKNα was enriched in neurons of human brains, and that the enzyme is concentrated in a subset of the endoplasmic reticulum (ER) and ER-derived vesicles localized to the apical compartment of the juxtanuclear cytoplasm, as well as late endosomes, multivesicular bodies, Golgi bodies, secretory vesicles, and cell nuclei (*25*). PKNα localized mainly in the cytosolic fraction in various cultured cells such as NIH 3T3, rat-1, and HeLa cells under normal growing conditions. However, stresses such as heat shock or arsenite treatment, and serum deprivation induced nuclear translocation of PKNα (*26*). On the other hand, PKNβ was detected in the cell nucleus and the perinuclear region, presumably corresponding to the Golgi apparatus, but not in the cytoplasmic region (*12*).

Gene maps

The PKNα gene *PRKCL1*/*Prkcl1* maps to human chromosome 19p12-p13.1, and to mouse chromosome 8 with close linkage to the Myodystrophy (*myd*) mutation (*27*). The mouse PKNα gene is situated at the mouse EP1 prostanoid receptor gene locus, the two genes overlapping in a tail-to-tail manner (*28*) and being transcribed in opposite directions. Overlapping, bidirectionally transcribed genes are uncommon, and only a few cases have been reported in mammalian genomes. Examples of this phenomenon include a member of the rat thyroid/steroid hormone receptor family encoded by the opposite strand of the rat *c-erb*Aα gene (*29*), and an exon of the *c-myb* proto-oncogene that is encoded on the antisense strand of the SC35 splicing factor gene (*30*). Antisense transcripts often have a regulatory function (*29*, *31*). The transcription, stability, and translation of PKNα mRNA may be regulated by a naturally occurring antisense transcript. It is also possible, although highly speculative, that PKNα and the EP1 prostanoid receptor are functionally coupled.

Regulation of activity

As speculated from the structural resemblance between the catalytic domain of PKN and PKC, PKN efficiently phosphorylates peptide substrates based on the pseudosubstrate sequence of PKC *in vitro,* and PKNα phosphorylates basically the same sites of MARCKS (*32*) and vimentin (*33*) as PKC *in vitro*. In some cases, however, PKNα phosphorylates different sites of the same protein from those phosphorylated by PKC *in vitro* (*34*). While possible physiological substrates of PKN are now beginning to be presented, the kinase activity of PKN has hitherto basically been discussed with respect to common substrates such as MBP, a peptide based on the pseudosubstrate site of PKC, or PKN itself.

Unsaturated fatty acids such as arachidonic and linoleic acids were identified as potential activators of PKN immediately after the enzyme was isolated in 1994 (*35*). Around the same time, major cardiolipin-activated protein kinase activity was reported in cytoplasmic extracts of rat liver using ribosomal protein S6- (aa 229–239) peptide substrate (*36*), and later the enzyme responsible for this activity was revealed to be PKNα (*5*). Besides cardiolipin, phospholipids such as phosphatidylinositol 4,5-bisphosphate (PI4,5-P2) and phosphatidylinositol 3,4,5-

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Table 1. **List of clones of PKN.**

Species	cDNAs		
Human	PKN(1)	PKNβ(12)	
Human	PRK1(7)		PRK2(7)
Rat	PKN(1)		
Rat	PAK-1(5)		PAK-2(18)
Frog	PKN(10)		
Starfish			PRK2(24)
Fly		PKN(11)[a]	
Nomenclature used here	**PKNα**	**PKNβ**	**PRK2/PKNγ**

[a]It is difficult to classify this clone into the corresponding isoform. Numbers in parentheses indicate the reference numbers listed below.

trisphosphate (PI3,4,5-P3), and lysophospholipids such as lysophosphatidic acid (LPA) and lysophosphatidylinositol can also stimulate the protein kinase activity of PKNα. On the contrary, calcium/phosphatidylserine/diolein, which is a combination of potent activators of PKCs, does not enhance the PKNα activity (*2*, *37*). The peptide kinase activities of PKNβ and PRK2/PKNγ are significantly less sensitive to arachidonic acid than that of PKNα (*12*, *18*). In 1996, it was found that the small GTPase RhoA binds to PKNα in a GTP-dependent manner, and that PKNα purified from a bovine brain membrane fraction is stimulated by the addition of the GTPγS-bound form of RhoA (*38–41*). The addition of LPA to Swiss 3T3 fibroblasts induces activation of RhoA, and phosphorylation of PKNα was detected by metabolic labeling experiments with ^{32}P-orthophosphate. PKNα can interact with RhoB and RhoC as well as with RhoA, but it has been reported to interact very weakly with and to be activated by Rac since the early stages of the study (*19*, *38*, *39*, *41*). However, controversial results have been reported regarding the binding of mammalian PRK2/PKNγ with Rho family GTPases. Quilliam et al. reported that PRK2/PKNγ specifically binds to RhoA in a GTP-dependent manner (*14*), whereas Vincent and Settleman reported that the interaction of PRK2/PKNγ with RhoA *in vitro* was nucleotide-independent, but that the interaction with Rac was strictly GTP-dependent (*19*). *Drosophila* Pkn protein exhibited approximately equivalent GTP-dependent binding to each of *Drosophila* Rho1, Rac1, and Rac2 GTPases, and each of these interactions resulted in an increase in the catalytic activity of Pkn (*11*). Additional Pkn-related family members have not been found in *Drosophila* (*11*). Thus, PKN may originally have acted as an effector target of both Rho and Rac GTPases, and in its subsequent evolution it may have differentiated into various isoforms in mammals, accepting each input relatively selectively to perform distinct cellular functions.

The N-terminal region of PKN is thought to restrict the kinase activity of the catalytic domain in the absence of activators (*5*, *35*, *42*, *43*), but the precise regulation mechanism of the PKN activity has not been elucidated. In the early stages of the studies on PKN, Kitagawa *et al.* reported that the synthetic peptide corresponding to aa 39 to 53 of PKNα but with Ser substituted Ile 46 served as an efficient substrate for PKNα and suggested that this region functioned as a pseudosubstrate (*42*). Since the first ACC finger domain overlaps this potential pseudosubstrate site, the activation model was presented that RhoA binding to the ACC finger domain could produce an unmasked and active catalytic domain of PKNα (*6*). However, the N-terminally truncated form of PKNα (aa 455–942, lacking all of the three ACC fingers) still showed a low basal activity similar to that of the wild-type enzyme. Further analysis revealed that the C-terminal part of the C2-like region (aa 455–511) functions as an arachidonic acid-sensitive auto-inhibitory region (*9*). The mechanism by which activated RhoA induces conformational changes in PKN, however, remains unknown.

PKN belongs to the AGC subfamily of protein kinases (*44*). Several members of the AGC protein kinase family, such as protein kinase B (PKB/Akt) (*45–48*), PKC (*49–52*), cAMP-dependent protein kinase (PKA) (*51*, *53*), p70 ribosome S6 kinase (S6K) (*52*, *54*, *55*), and serum and glucocorticoid-induced kinase (SGK) (*56*, *57*), are reported to be phosphorylated in the activation loop and activated by phosphoinositide-dependent protein kinase 1 (PDK1). PKNα and PRK2/PKNγ were also reported to bind to PDK1 (*58–61*) and to be activated by PDK1 (*59*, *60*). Recently, Parekh *et al.* (*62*) presented a model of Rho-dependent PKN activation *via* PDK1 based on the data reported in (*60*). Activated Rho binds to PKN and induces a conformational change that is permissive for binding to PDK1. PDK1 phosphorylates PKN in the activation loop and stimulates the protein kinase activity of PKN. This model is also supported by an *in vivo* study showing that overexpression of RhoB recruits PDK1 to an endosomal compartment only when PKNα is coexpressed, suggesting that a heterotrimeric complex is formed between these molecules *in vivo* (*60*). Both of the activation loop site mutants (T774A for PKNα and T816A for PRK2/PKNγ) result in a complete loss of kinase activity. However, at least for PRK2/PKNγ, activation loop phosphorylation by PDK1 may not be a critical point of acute regulation, for the following reasons: (i) co-expression of PDK1 with PRK2/PKNγ only very mildly activates the enzyme compared to the increase in the activation loop site phosphorylation (*60*); (ii) the catalytic domain of PRK2/PKNγ expressed in *E. coli* has mild but significant kinase activity, far more than PKNα (*9*), although *E .coli* lacks a PDK1 homolog (*63*); (iii) Flynn *et al.* reported that PKNα and PRK2/PKNγ show very low basal activation loop phosphorylation *in vivo* (*60*). However, Balendran et al. reported that the activation loop site was already phosphorylated to a high stoichiometry when PRK2/PKNγ was overexpressed in unstimulated 293 cells (*64*). In addition, purified enzyme from rat tissue or unstimulated insect cells also shows relatively high levels of peptide kinase activity (*2*, *9*, *18*); (iv) Balendran *et al.* reported that enhanced association with PDK1 and increased phosphorylation of the activation loop site (Thr 816) were not observed in PRK2/PKNγ when it was co-expressed with the activated form of Rho (*64*). Further study is necessary to determine the importance of activation loop phosphorylation by PDK1 in the signal-induced activation of PKN.

It was recently reported that PRK2/PKNγ associates with MEK kinase 2 (MEKK2) but not MEKK3, and that MEKK2 dramatically activates PRK2/PKNγ activity *in vitro* and *in vivo* (*65*). The MEKK2 binding site maps to the region between the Pro-rich region and the catalytic

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domain of PRK2/PKNγ, which is divergent among PKN family members. Interestingly, kinase-negative MEKK2 can also activate the PRK2/PKNγ activity (albeit to a lesser degree). Scaffolding and possibly oligomerization of MEKK2 and PRK2/PKNγ may regulate the kinase activity of PRK2/PKNγ, by analogy to the case in which PKCα interacts with and activates PLD independently of PKCα kinase activity (*66*).

Potential activators of PKN have been discussed above. On the other hand, evidence has been accumulated that the function of these potential PKN activators can be affected by interaction with PKN. The endogenous GTPase activity and the p122 Rho GAP-stimulated GTPase activity of RhoA are inhibited by the interaction with PKNα, suggesting the presence of a regulatory mechanism that sustains the GTP-bound active form of RhoA (*40*). Sun *et al.* reported that co-incubation of PRK2/PKNγ and MEKK2 leads to an increased phosphorylation level of MEKK2, suggesting that PRK2/PKNγ phosphorylates or increases the autophosphorylation activity of MEKK2 (*65*). With regard to PDK1, much evidence for its regulation by PKN has been reported, although the situation is complex. Balendran *et al.* isolated the C-terminal 77 aa peptide of PRK2/PKNγ (corresponding to aa 908–984 of PRK2/PKNγ, termed PDK1-interacting fragment, PIF) as a specific binding partner of PDK1 and reported that PIF converts PDK1 from a kinase with activation loop specificity (Thr 308, termed the "PDK1 site") to a kinase capable of phosphorylating both the "PDK1 site" and the C-terminal hydrophobic phosphorylation site (Ser 473, termed the "PDK2 site") of PKB (*58*). Since phosphorylation of the "PDK2 site" is necessary for the maximal activation of PKB, PRK2/PKNγ could regulate PKB activity through this mechanism, although whether phosphorylation of the "PDK2 site" of PKB is mainly controlled by PIF remains hightly controversial (*67*). The potential regulation of PDK1 by PKN in a different way has been presented as described below after the report by Balendran *et al*. (*58*). Most of the PDK1 substrates identified to date, apart from PKA, possess a "PDK2 site" about 160 aa C-terminal to the "PDK1 site" (*68*), and phosphorylation of both the "PDK1 site" and "PDK2 site" are known to be required for maximal activation of not only PKB but also S6K and/or for stability of isoforms of PKC (*69*). Then the regulation of PDK1 by PKN may extend to the regulation of AGC kinases other than PKB. Koh et al. reported that the C-terminal fragments (both aa 700–984 and aa 862–984), but not full-length PRK2/PKNγ, bind to PKB and down-modulate the PKB activity by completely inhibiting the "PDK2 site" (Ser 473) and "PDK1 site" (Thr 308) phosphorylations (*70*). Wick *et al.* reported that co-expression of full-length PKNα, PRK2/PKNγ, or the catalytic domain of PKNα (aa 560–942) with PDK1 inhibits PKB phosphorylation at both the "PDK1 site" and the "PDK2 site", although co-expression of PIF with PDK1 leads to an increase of PKB phosphorylation at the "PDK2 site" (*71*). Recently, Hodgkinson and Sale reported that the C-terminal region of PRK2/PKNγ (aa 695–985) inhibits PDK1 autophosphorylation by >90%, and inhibits the ability of PDK1 to phosphorylate PKC-ζ and δ (*61*). Koh *et al.* claimed that the C-terminal fragments of PRK2/PKNγ directly inhibit PKB activity (*70*). However, together with the above reports by Wick *at al.* and by Hodgkinson and Sale, the C-terminal fragment of PKN (except for PIF) probably down-regulates PKB activity by at least partly affecting PDK1. Furthermore, Biondi *et al.* reported that PIF and PIFtide (24 aa fragment of PIF that encompasses the hydrophobic motif of PRK2/PKNγ; aa 957–980 of PRK2/PKNγ) also bind tightly to PDK1 and inhibit S6K and SGK (*72*). [Remarkably, PIFtide interacts with the PDK1 with >1,000-fold higher affinity than the S6K1, SGK1, and PKB hydrophobic motif peptides (*72*).] The C-terminal fragment can be produced by the proteolysis of PKN physiologically as described below (in the "Role in the apoptotic model" section). PKN may regulate various AGC kinases by affecting their interaction with PDK1. These results also indicate that the overexpression of PKN, especially the C-terminal fragment of PKN, and the induction of the proteolysis of PKN in cells, potentially prevent PDK1 from interacting with various AGC kinases. Thus, great caution should be taken in interpreting the results of such experiments. Using the fragment based on the auto-inhibitory domain of PKN ("Iα" *etc.*), which can inhibit PKN kinase activity selectively (*9*), may help to solve the problem.

Biological function

Recent reports suggest that PKN is involved in a variety of processes, including the following.

Cytoskeletal regulation. Rho family GTPases have been identified as important regulators of the actin cytoskeleton. A number of target molecules have been shown to be involved in the effector pathways that link these GTPases to their effects on the cytoskeleton (*73*). The precise roles of PKN family members in regulating the reorganization of the actin cytoskeleton have remained elusive, in part because of the seemingly different results between closely-related isoforms of PKN that have emerged from some studies. Vincent and Settleman reported that the expression of a kinase-negative form of human PRK2/PKNγ in microinjected NIH 3T3 fibroblasts results in the disruption of actin stress fibers, suggesting a normal role for PRK2/PKNγ in regulating actin reorganization (*19*). Dong *et al.* reported that the ectopic expression of PKNα stimulates actin stress fiber depolymerization and membrane ruffling in 3T3 L1 and Rat1-IR fibroblasts (*59*). The kinase-negative forms of PKNα (T774A or K644D) prevent insulin-induced actin stress fiber breakdown and membrane ruffling, suggesting that PKNα is involved in insulin-induced actin cytoskeletal reorganization. One of the cytoskeletal functions of PKN may have been elucidated from studies of a loss of function mutant of *Drosophila* Pkn. In the late stages of *Drosophila* development, the dorsal trunk region of the embryo remains without ectoderm and is instead covered by a transient epithelial structure, the amnioserosa (*74*). During the process of dorsal closure, the dorsal-most, or leading edge (LE) cells of the lateral epidermis elongate along the dorsal-ventral axis, which is associated with the accumulation of actin and nonmuscle myosin beneath the dorsal-most cell membranes. It has been proposed that the accumulation of F-actin and myosin at the leading edge forms the actomyosin contractile apparatus driving the elongation of the LE cells, and finally the

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leading edges of both sides meet at the dorsal midline and fuse in general. However, Pkn loss of function mutants display a dorsal closure defect (*11*). This dorsal closure defect closely resembles those observed with loss of function of the Rho1 gene and several components of the Rac-mediated JNK cascade. Pkn may regulate the actin-cytoskeleton for cell shape changes downstream of Rho family GTPases during the dorsal closure process (*11*).

What are the target molecules of PKN in regulating cytoskeletal rearrangements? One potential target is a molecule involved in the regulation of movements between actin and myosin filaments. In mammalian cells, nonmuscle myosins are regulated by phosphorylation of their light chain (MLC), and this phosphorylation represents an important regulatory mechanism for actomyosin-based contraction such as stress fiber formation (*75*). Myosin light chain kinase (MLCK) and Rho kinase/ROKα/ROCKII are thought to be involved in the regulation of the phosphorylation level of myosin directly or indirectly through inhibition of myosin phosphatase (*76*). It was demonstrated that PKNα *in vitro* neither phosphorylates myosin phosphatase nor inhibits its activity (*77*). PKN can directly phosphorylate myosin *in vitro,* though further studies are necessary to demonstrate if this is relevant to the actin-cytoskeletal regulatory function of PKN. In smooth muscle cells, Ca^{2+} sensitization often occurs following agonist stimulation and is thought to involve inhibition of myosin phosphatase via a G-protein (*78*) and arachidonic acid (*79*) . RhoA and Rho kinase are also known to play a pivotal role in this process (*76*). However, another potential pathway to increase Ca^{2+} sensitization exists independently of Rho/Rho-kinase links (*80*) and can be mediated by phosphorylation of CPI-17 (*81*), a phosphorylation-dependent inhibitory protein for myosin phosphatase. PKNα can phosphorylate CPI-17 efficiently *in vitro*, and phosphorylation of PKNα increases the inhibitory effect of CPI-17 on myosin phosphatase activity (*82*). PKNα might be involved in the Ca^{2+} sensitization of smooth muscle cells as a downstream effector of Rho and arachidonic acid. However, it was recently reported that in vascular smooth muscle cells PKN contributes little to histamine-induced CPI-17 phosphorylation based on sensitivity to different inhibitors (*83*). PKNα binds to the third spectrin-like repeats of actin cross linking protein α-actinin (*84*). PKN also binds to the region containing EF-hand-like motifs of non-skeletal muscle type α-actinin in a Ca^{2+}-sensitive manner, and to that of skeletal muscle type α-actinin in a Ca^{2+}-insensitive manner. PI4,5P2 regulates the F-actin–gelating activity of α-actinin *in vitro*, and also activates the protein kinase activity of PKNα *in vitro* (*37*). Since actin-cytoskeletal proteins such as caldesmon and G-actin are relatively preferred substrates for PKNα *in vitro* (*84*), PKNα might participate in the regulation of actin-cytoskeleton through phosphorylating these proteins by anchoring to α-actinin. Recently it was reported that protein Tyr phosphatase-basophil like (PTP-BL), a large non-transmembrane protein Tyr phosphatase implicated in the modulation of the cytoskeleton, binds to PRK2/PKNγ (*22*). This interaction is mediated by the PSD-95, *Drosophila* discs large, zonula occludens (PDZ) 3 domain of PTP-BL and the extreme C-terminus of PRK2/PKNγ. Gross et al. described the strong colocalization of endogenous PTP-BL and PRK2/PKNγ in lamellipodia-like structures, regions of large actin turnover, suggesting the close correlation of these proteins in the regulation of the actin-cytoskeleton.

PKN also directly binds to and efficiently phosphorylates the head-rod region of the intermediate filament proteins, another major cytoskeletal component, and inhibits the polymerization of the following proteins *in vitro*: the subunits of neurofilament (NF) (*85*), vimentin, and glial fibrillary acidic protein (GFAP) (*86*). Thus PKN may be widely implicated in the regulation of organization of intermediate filaments (IF). The sites in these proteins phosphorylated by PKN are almost identical to those phosphorylated by PKC (*33*). NF is known as one of the constituents of neurofibrillary tangles (NFTs). PKN can efficiently phosphorylate microtubule-associated protein (MAP) tau, another major constituent of NFTs, both *in vitro* and *in vivo* (*34*, *87*). Sensitive immunocytochemical examination of postmortem human brain tissues revealed that PKN is closely associated with NFTs and abnormally modified tau in Alzheimer disease-affected neurons (*25*). PKN is also found in degenerative neurites within senile plaques. PKN may be involved in NFT formation and neurodegeneration in Alzheimer disease-damaged neurons.

Cell adhesion. Rho-type GTPases play an important role as regulators of cell-cell adhesion in a manner which varies substantially depending on cell type and cellular context (*88*). In keratinocytes, both Rac and Rho activities are required for cell–cell junction formation (*89*). The activated Rho induces Tyr phosphorylation of adherens junction components such as β and γ-catenins and $p120^{ctn}$ by Fyn kinase, independently of actin-cytoskeleton (*20*). A Rho V14-Y42C mutant which is selectively defective in PKN binding fails to promote recruitment of E-cadherin to cell–cell junctions in keratinocytes triggered by calcium treatment to induce keratinocyte differentiation (*20*). Autophosphorylation activities of immunoprecipitated endogenous PKNα and PRK2/PKNγ were elevated 1 h after exposure of cells to calcium. In particular, PRK2/PKNγ activity remained at elevated levels for at least 24 h. Overexpression of wild-type PRK2 enhanced the cell–cell adhesion in keratinocytes triggered by calcium treatment. Tyr phosphorylation of β- and γ-catenin and $p120^{ctn}$, and Fyn kinase activity are induced by overexpression of PRK2/PKNγ in keratinocytes, and also the active catalytic domain of PKN increases the kinase activity of Fyn immunoprecipitated from keratinocytes. The link between Rho and Fyn activation in control of cell–cell adhesion in keratinocytes may be provided at least by PRK2/PKNγ.

Vesicle transport. Electron microscopic analysis revealed that PKNα is enriched in a subset of endoplasmic reticulum (ER) and ER-derived vesicles localized to the apical compartment of the juxtanuclear cytoplasm, as well as to late endosomes, multivesicular bodies, Golgi bodies, and secretory vesicles in neurons in human brain, as described above (*25*) , suggesting that the enzyme is implicated in the regulation of vesicle movement. Several Rho family members are localized to vesicular compartments, and increasing evidence suggests that they play important roles in the trafficking of vesicles on both

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endocytic and exocytic pathways (*90*). In particular, RhoA, RhoB, RhoD, Rac, and Cdc42 have been shown to affect various steps of membrane trafficking. RhoB was reported to localize predominantly to late endosome/lysosomes (*91*). Gampel *et al.* reported that overexpression of RhoB retards EGF receptor movement from early to late endosomal compartments after stimulation by EGF, and that a kinase-negative form of PKN mutant completely blocks this effect (*92*). Thus PKNα may regulate the kinetics of EGF receptor trafficking.

Glucose transport. In 3T3/L1 cells, stable expression of wild-type Rho and PKNα activates glucose transport, and a dominant negative-form of Rho and kinase-negative PKNα inhibits insulin-stimulated glucose transport (*93*). Insulin-stimulated glucose uptake is primarily mediated by the facilitative transporter Glut4, a member of a family of related transporters that are highly expressed in adipose tissue and skeletal and cardiac muscle (*94*). Insulin stimulates the translocation of a pool of Glut4 from vesicular compartments within the cell to the plasma membrane through a process of targeted exocytosis. In rat adipocytes, transient expression of wild-type PKNα provokes an increase in the translocation of HA-Glut4 to the plasma membrane. However, kinase-negative PKNα inhibits the effects of both insulin and GTPγS on HA-Glut4 translocation (*93*), suggesting that PKNα contributes to Glut4 translocation during insulin and GTPγS action. The precise intracellular location and traffic pathways of specialized vesicles containing Glut4 remains unclear, however. One potential site for tethering Glut4 vesicles in resting cells is the actin cytoskeleton. PKNα is reported to be involved in insulin-induced actin stress fiber breakdown and membrane ruffling, as described above (*59*). PKNα might regulate Glut4 translocation by regulating actin cytoskeletal reorganization. Phospholipase D1 (PLD1) has been shown to localize with Glut4 vesicles and to potentiate the effects of insulin on Glut4 translocation (*95*). Recently PKNα has been shown to bind to PLD1 and activate PLD1 in the presence of PIP2 (*96*). PKN might regulate PLD1 activity under insulin stimulation.

Role in the apoptotic model. PKNα is cleaved at specific sites in apoptotic Jurkat and U937 cells on Fas ligation and treatment with staurosporin or etoposide, respectively (*43*). This cleavage seems to be catalyzed by caspase-3 or related proteases. The major proteolysis takes place between the N-terminal regulatory domain and the C-terminal catalytic domain, and it generates a constitutively active kinase fragment. The cleavage of PKNα may contribute to signal transduction, eventually leading to apoptosis. In addition, PRK2/PKNγ cleaved in early stages of apoptosis binds to and prevents phosphorylation of PKB *in vivo* at Ser 473 and Thr 308 (*70*). Since phosphorylation of PKB is necessary for its full activation, PRK2/PKNγ may inhibit PKB downstream signaling and abrogate its anti-apoptotic effects.

Many laboratories have provided evidence for cell death by an apoptotic mechanism in several models of individual cerebral ischemia (*97–99*). Ueyama et al. analyzed the fragmentation of PKNα at various regions of the rat brain after middle cerebral artery occlusion (MCAO) modeled in rats (*100*). Interestingly, an intense 55-kDa band which corresponds to the active catalytic domain of PKNα appeared 5 days after MCAO, peaking at 21 days and was sustained at an increased level until at least day 28. Since the apoptosis after MCAO is known to peak at 24–72 h, and DNA laddering and TUNEL-positive cells were barely detected 14 days after MCAO, the active catalytic domain of PKN seems to participate in the recovery from ischemia rather than to work as an execution phase of apoptosis in this model. Since PKN increases in microglia after MCAO, constitutive activity of PKN might be necessary for regenerative processes regulated by microglia, such as scavenging dead cells and the production/secretion of cytotoxic/neurotrophic molecules.

Regulation of meiotic maturation and embryonic cell cycles. During early development, oocytes arrest late in G2 of the first meiotic cell cycle. Hormonal stimulation results in the resumption of meiosis, known as meiotic maturation (*101*). In the case of starfish oocytes, meiotic maturation is characterized by the activation of Cdc2/CyclinB, breakdown of the germinal vesicle (GVBD), and the subsequent completion of meiosis I and II. Stapleton *et al.* reported that "maturation-inducing hormone" 1-methyladenine induces *in vivo* phosphorylation of the closely related homolog of PRK2/PKNγ prior to Cdc2/cyclin B activation and translation initiation of stored mRNA, and induces translocation of PRK2/PKNγ from the cytoplasm into the germinal vesicle (*24*). PRK2/PKNγ may regulate the early events during meiotic maturation, and the potential targets of PRK2/PKNγ are the activation of Cdc2/cyclinB, translation initiation, and actin cytoskeletal changes. The increase of translation is due in part to the activation of eIF4F, and which requires phosphorylation of eIF4E. Since eIF4E is phosphorylated after 1-MA stimulation at the same site (Ser 209) *in vivo* as is phosphorylated *in vitro* by PRK2/PKNγ, it is plausible that PRK2/PKNγ is the eIF4E kinase functioning during meiotic maturation in starfish oocytes (*23*). PI3K inhibitors and the reagents which can increase intracellular elevation of cAMP block PRK2/PKNγ activation and eIF4E phosphorylation. PI3K and cAMP may be upstream regulators of PRK2/PKNγ and eIF4E.

In the case of *Xenopus* embryos, microinjection of the active catalytic domain of PKNα into the two-cell stage results in cell cleavage arrest in the injected blastomeres (*102*). On the other hand, microinjection of the kinase-negative form of PKNα or active catalytic domain of PKCε, whose primary structure is very similar to that of PKNα, does not prevent normal cell division. Exogenous addition of the active catalytic domain of PKN delays mitotic timing in *Xenopus* egg cycling extracts, as judged by morphology of sperm nuclei and histone H1 kinase activity, which correlates with the phosphorylation level of Tyr-15 in Cdc2. PKNα efficiently phosphorylates Cdc25C *in vitro* and the Cdc25C activity for the dephosphorylation of Tyr-15 in Cdc2 is suppressed by pretreatment with the active form of PKNα. PKNα may participate in G2/M transition control in the cell cycle.

Signaling to the cell nucleus. PKNα translocates from the cytosol to the nucleus in response to various stresses such as heat shock, and PRK2/PKNγ translocates from the cytoplasm to germinal vesicles during meiotic maturation in starfish oocytes, as described above.

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However, the relevant nuclear targets of PKN have not been clearly identified in these cases.

Exposure of cells to heat shock leads to the heat shock response and the induction of heat shock proteins (HSPs) (*103*). In mammalian cells, HSP genes are thought to be primarily regulated at the transcriptional level by heat shock transcription factor (HSF), a sequence-specific transcription factor that binds to the heat shock element (HSE) at their promoters (*104*). At least 3 HSFs have been isolated from mammalian cells, and among them HSF1 functions as the acute stress-responsive activator (*105*). After heat shock, HSF1 monomers oligomerize to a trimeric state, translocate to the nucleus, where they become inducibly phosphorylated, and bind to HSE, resulting in stress-induced transcription. Whereas inducible phosphorylation appears to be essential for transcriptional phosphorylation, the kinase(s) involved are still unknown (*105*). Co-expression of HSF1 and the active catalytic domain of PKNα in HeLa S3 cells and C6 glioma cells shows accumulation of small HSP αB-crystallin but not closely related HSP27 and HSP70, whereas individual expression of HSF1 or PKNα separately does not show accumulation of αB-cyrstallin (*106*). αB-crystallin expression is dependent on the HSE of its promoter. Since expression of recombinant HSF1 in cells forms nucleic, DNA-binding, homotrimeric proteins, PKNα might induce phosphorylation of these homotrimeric HSF1 proteins for subsequent expression of αB-crystallin. Interestingly, inhibitors of cyclooxygenases and activators of phospholipase A2 stimulate the induction of αB-crystallin during heat and arsenite stress, suggesting that arachidonic acid stimulates the production of αB-crystallin (*107*). αB-crystallin is suggested to confer increased stress resistance, especially by associating with cytoskeletal elements to protect cellular integrity.

Activated Rho stimulates c-fos gene expression in NIH 3T3 cells (*108*), which is mediated through the c-fos serum response element (SRE) in a ternary complex factor (TCF)–independent manner. Each expression of the active form of PKNα, PRK2/PKNγ, and Rho kinase induces transcriptional activation of c-fos SRE in NIH 3T3 cells (*14*, *109*), but the relevant signaling pathways linking RhoA to SRE remain a controversy. Geneste et al. recently reported that the relative importance of different RhoA effector pathways for SRE activation differs between cell types (*110*). For example, mDia function is required both in NIH 3T3 cells (*111*) and PC12 cells (*110*), Rho kinase and LIMK function plays a significant role in PC12 cells (*110*), and Rho kinase is necessary in rat aortic smooth muscle cells (*112*). Also, in cardiomyocytes, RhoA was shown to stimulate c-fos SRE activation in a TCF-independent manner (*113*). RhoA also mediates hypertrophic growth and atrial natriuretic factor (ANF) gene expression in neonatal rat ventricular cardiomyocytes (*114*). PKNα and PRK2/PKNγ, but not Rho kinase, generate a robust stimulation of a luciferase reporter gene driven by the ANF promoter containing an SRE-like sequence which lacks flanking Ets motifs responsible for TCF binding. The ability of PKN to stimulate the ANF reporter gene was dependent on this SRE-like sequence. PKN may be implicated as a downstream effector of Rho in transcriptional responses in cardiomyocytes, which is associated with cardiac hypertrophy.

Activated Rho can also stimulate c-jun expression and the activity of the c-jun promoter. PKNα, MKK3/MKK6, and ERK6 (p38γ) were reported to be involved in this signaling pathway, with consequent stimulation of transcription factors ATF2 and MEF2A, which act on the c-jun promoter through the jAP1 and MEF2 responsive elements (*115*). It was also reported that activation of this pathway is required for the ability of Rho to induce cellular transformation (*115*). PKNα may act as MAPKKKK and mediate Rho and lipid signals to the p38γ MAP kinase cascade, by analogy to the Rac/PAK/p38 MAP kinase pathway.

In addition to these reports, neuron-specific Helix-LooP-Helix (bHLH) transcription factor, NDRF/NeuroD2 (*116*), and potential transcription factor PCD17/CDR62 (*117*) were isolated as binding partners of PKNα. Transient transfection assays revealed that co-expression of a catalytically active form of PKNα significantly enhances NDRF/NeuroD2-mediated transactivation activity (*116*). PKN might be involved in the mechanism of neuronal differentiation.

Tumorigenesis. The human papillomaviruses (HPVs) are associated with epithelial tumors or benign lesions, especially those of anogenital origin (*118*). HPVs are categorized into low risk and high risk HPVs. Low risk HPVs are usually associated with benign warts, whereas high risk HPVs are associated with carcinomas. Only E6 and E7 genes are necessary for the immortalization activity of HPVs (*119*, *120*). E6 oncoproteins of high risk HPVs (HPV16 and HPV18) preferentially interact with the C-terminal region of PKNα as compared with the E6 protein of low risk HPVs (HPV6 and HPV11) (*121*). All of the E6 mutants that can immortalize epithelial cells retain the ability to interact with PKNα. These results suggest the possible role of PKNα in E6-mediated cellular transformation. However, the binding ability to PKNα was also retained in certain E6 mutants that do not immortalize epithelial cells, suggesting that binding to PKNα by itself is not sufficient for E6-induced cellular transformation. PKNα induces phosphorylation of E6 when both are co-expressed *in vivo*. Functional regulation of E6 and PKNα between the two awaits further studies.

Concluding remarks

A large number of proteins that are capable of interacting with PKN have been identified, suggesting that PKN is a versatile signaling molecule that regulates a diverse set of cellular functions. Currently, the significance of many of these interactions remains enigmatic, and it will be a challenge in the future to establish their physiological relevance. The multiple PKN family members do not appear to be conserved in genetically tractable systems such as *Drosophila* and *C. elegans*. In this regard, a promising approach will be the generation of a "pure" inhibitor of each isoform of PKN that can prevent unwanted effects on other Rho effectors or AGC kinases in mammalian cells or a conditional targeting of each isoform of PKN genetically or using an RNAi technique. Recent studies have revealed that multiple Rho effectors are involved in stress fiber formation or SRE activation (*122*, *123*). As this review has described, PKN also may

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be implicated in multiple functions of Rho signals concomitantly with other Rho target molecules.

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