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Mutational Analysis of the Regulatory Mechanism of PKN: The Regulatory Region of PKN Contains an Arachidonic Acid-Sensitive Autoinhibitory Domain[1]

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PKN is a fatty acid- and Rho GTPase-activated protein kinase whose catalytic domain in the carboxyl terminus is homologous to those of protein kinase C (PKC) family members. The amino terminal region of PKN is suggested to function as a regulatory domain, since tryptic cleavage or the binding of Rho GTPase to this region results in protein kinase activation of PKN. The structural basis for the regulation of PKN was investigated by analyzing the activity of a series of deletion/site-directed mutants expressed in insect cells. The amino-terminally truncated form of PKN (residue 455-942) showed low basal activity similar to that of the wild-type enzyme, and was arachidonic acid-dependent. However, further deletion (residue 511-942) resulted in a marked increase in the basal activity and a decrease in the arachidonic acid dependency. A $(His)_6$-tagged protein comprising residues 455-511 of PKN (designated His-Iα) inhibited the kinase activity of the catalytic fragment of PKN in a concentration-dependent manner in competition with substrate ($K_i = 0.6 \pm 0.2$ μM). His-Iα also inhibited the activity of the catalytic fragment of PRK2, an isoform of PKN, but had no inhibitory effect on protein kinase A or protein kinase Cδ. The IC_{50} value obtained in the presence of 40 μM arachidonic acid was two orders of magnitude greater than that in the absence of the modifier. These results indicate that this protein fragment functions as a specific inhibitor of PKN and PRK2, and that arachidonic acid relieves the catalytic activity of wild-type PKN from autoinhibition by residues 455-511 of PKN. Autophosphorylation of wild-type PKN increased the protein kinase activity, however, substitution of Thr64, Ser374, or Thr531 in the regulatory region of PKN with alanine, abolished this effect. Substitution of Thr774 in the activation loop of the catalytic domain of PKN with alanine completely abolished the protein kinase activity. These results suggest that these phosphorylation sites are also important in the regulation of the PKN kinase activity. Potential differences in the mechanism of activation between the catalytic regions of PKN and PRK2 are also discussed.

Key words: arachidonic acid, I alpha, PKN, PRK2, Rho.

PKN is a serine/threonine protein kinase with a catalytic domain homologous to those of PKC family members (*1*); its kinase activity is enhanced by fatty acids such as arachidonic acid (*2, 3*) and small GTP-binding protein Rho in a GTP-dependent manner (*4-6*). The N-terminal region of PKN is assumed to restrict the kinase activity of the catalytic domain in the absence of activators for the following reasons: (i) Limited proteolysis of PKN results in the formation of a catalytically active fragment lacking the N-terminal region (*3, 7, 8*); (ii) Rho binds to residues 33-111 (*5*), a region containing the first leucine zipper-like sequence; and (iii) the yeast two-hybrid system and *in vitro* binding analysis indicate that the N-terminal regulatory region binds directly to the C-terminal catalytic domain of PKN (*9*). However, the precise mechanism by which the N-terminal region regulates the catalytic domain of PKN has not been elucidated.

There have been a number of reports concerning the regulation of enzyme activity by the phosphorylation of co-factor-dependent protein kinases themselves. Regulation by phosphorylation in the regulatory domain is important in several protein kinases. For example, CaM-kinase II

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Abbreviations: CaM-kinase, Ca^{2+}/calmodulin-dependent protein kinase; CIAP, calf intestine alkaline phosphatase; C-terminal, carboxyl-terminal; N-terminal, amino-terminal; GST, glutathione S-transferase; PAK, protease-activated protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PRK2, protein kinase C-related kinase 2.

autophosphorylates its Thr286 in the autoinhibitory domain and obtains Ca^{2+}/calmodulin-independent kinase activity (*10-13*). The importance of phosphorylation in the kinase domain has also been reported. For example, CaM-kinases I and IV are activated by phosphorylation at the threonine residue in the "activation loop" of subdomain VIII of the protein kinase domain (*14-16*) by CaM-kinase kinase (*17-21*). Protein kinase B/c-Akt, which is activated by phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate as a second messenger, has been shown to be regulated by PDK1 through the phosphorylation of Thr308 in the activation loop (*22, 23*). We previously reported that the autophosphorylation activity of PKN purified from rat testis is partially inhibited by pretreatment with alkaline phosphatase, suggesting that the phosphorylation of PKN itself also plays an important role in the activation of the enzyme (*2*). Recently, several phosphorylation sites in rat liver PKN/PAK-1 have been reported (7). Among them, Thr64, Ser377, Thr534, and Thr778 of rat PKN are conserved in the human PKN homology (*24*) and in an isoform of PKN, PRK2/PAK2 (*25*). Thr64, Ser377, and Thr534 are located in the N-terminal regulatory region and Thr778 is located in the activation loop of the protein kinase domain. However, the roles of these phosphorylation events and the possible regulatory mechanism have not been clarified.

In this study, we demonstrated that the peptide fragment comprising residues 455-511 of PKN selectively inhibit the catalytic activity of PKN and PRK2, and that arachidonic acid abrogates the inhibitory effect of this fragment, suggesting an activation mechanism of PKN by unsaturated fatty acids. We also showed the potential importance of the phosphorylation sites described above in PKN activation. In addition, catalytic regions of PKN and PRK2 expressed in bacteria were used to show the potential difference in the mechanism of activation between these two kinases.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—A scheme for the fusion constructs of the various deletion mutants and point mutants used in this study is presented in Fig. 1. Transfer vectors to generate the recombinant viruses were constructed as follows. For GST/PKNf, GST-tagged wild-type human PKN, a cDNA fragment for the coding region of GST prepared by PCR amplification from pGEX-4T vector (Pharmacia Biotech), and the *Sal*I fragment of phPKN-H4 (*1*) were subcloned into the *Xho*I and *Sal*I sites of pGFPN3 (Clontech Laboratories). The insert fragment for GST/PKNf was obtained by digesting the vector with *Kpn*I, blunting with T4 DNA polymerase, ligating with *Nco*I linker, and digesting with *Bgl*II/*Nco*I. pBlueBacHis/GST/PKNf for the expression of GST-tagged full length of PKN in Sf9 cells was constructed by subcloning the above insert fragment (*Bgl*II/*Nco*I ~3.7 kbp) into the *Bgl*II/*Nco*I site of pBlueBacHis-C (Invitrogen). pBlueBacHis/GST for the expression of the GST-tagged protein in Sf9 cells was made by digesting pBlueBacHis/GST/PKNf with *Bgl*II/*Hin*dIII, and recovering the DNA fragment containing only the GST region. This fragment was ligated into the *Bam*HI/*Hin*dIII site of pBlueBacHis-B. For GST/PKN-(109-942), a cDNA fragment encoding PKN-(109-207) was amplified by PCR from phPKN-H4, and subcloned into pT7Blue-T (Novagen). Then the resulting plasmid was digested with *Hin*dIII/*Sph*I, phPKN-H4 was digested with *Sph*I/*Xho*I, and the two insert fragments were simultaneously subcloned into the *Hin*dIII site of pBlueBacHis/GST. A cDNA fragment encoding PKN-(367-942) was made by digesting phPKN-H4 with *Sma*I, ligating with *Bgl*II linker, and digesting with *Bgl*II/*Hin*dIII. For GST/PKN-(367-942), this cDNA fragment was subcloned into the *Bam*HI/*Hin*dIII site of pBlueBacHis/GST. For GST/PKN-(455-942), a cDNA fragment encoding human PKN-(455-511) prepared by PCR amplification from phPKN-H4, and a cDNA fragment encoding PKN-(512-942) made by digesting phPKN-H4 with *Cla*I/*Hin*dIII were simultaneously ligated into the *Bgl*II/*Hin*dIII site of pBlueBacHis/GST. For GST/PKN-(511-942), the *Not*I fragment from pVP/PKNC1 (*26*) was subcloned into the *Not*I site of pAcGHLT-C (Pharmingen). pAcGHLTC/PKN-(543-634) was made by subcloning the cDNA fragment encoding PKN-(543-634) amplified by PCR from phPKN-H4 to the *Xho*I/*Eco*RI site of pAcGHLT-C. pAcGHLTC/PKN-(543-942) was made by subcloning the 1.0 kbp *Eco*RI fragment of phPKN-H4 into the *Eco*RI site of pAcGHLTC/PKN-(543-634). pAc/PKN-(543-942) for the expression of GST/PKN-(543-942) in Sf9 cells was made by digesting pAcGHLTC/PKN-(543-942) with *Xho*I, filling in the ends with T4 polymerase, ligating with *Bgl*II linker, digesting with *Bgl*II/*Pst*I, and ligating the resultant fragment into the *Bam*HI/*Pst*I site of pAcGHLT-C to eliminate the PKA phosphorylation site of pAcGHLT-C. pRSET/GST/PKNf was made by digesting pBlueBacHis/GST/PKNf with *Nco*I/*Bgl*II, and ligating to the *Nco*I/*Bgl*II site of pRSET-A (Invitrogen). A cDNA fragment encoding PKN-(561-942) was amplified by PCR from pMhPKN3 (*1*). pRSET/GST/PKN-(561-942) was made by substituting the above cDNA fragment for the *Sal*I insert of pRSET/GST/PKNf. pBlueBacHis/GST/PKN-(561-942) was made by digesting pRSET/GST/PKN-(561-942) with *Nco*I/*Bgl*II, and ligating into the *Nco*I/*Bgl*II site of pBlueBacHis-C. pGEX/PKN-(601-942) was made by subcloning the cDNA fragment encoding PKN-(601-942) amplified by PCR from phPKN-H4 into the *Eco*RI site of pGEX-4T. pAc/PKN-(601-942) was made by digesting pGEX/PKN-(601-942) with *Nco*I/*Not*I, and ligating into the *Nco*I/*Not*I site of pAcGHLT-A. For GST/PRK2-(550-984), the cDNA fragment encoding PRK2-(550-984) was amplified by PCR from phPRK2 (human PRK2 cDNA in pUC118, isolated from a human lung carcinoma cDNA library) and subcloned into pT7Blue-T. pBlueBacHis/GST/PRK2-(550-984) was made by digesting the plasmid with *Sma*I/*Hin*dIII, and ligating into the *Sma*I/*Hin*dIII site of pBlueBacHis/GST. pBlueBacHis/PKCδ-(328-673) for the expression of the constitutively active catalytic region of rat PKCδ (*27, 28*) was made by subcloning a cDNA fragment encoding PKCδ-(328-673) amplified by PCR from pTB801 (*28*) into the *Bam*HI site of pBlueBacHis/GST. A QuickChange site-directed mutagenesis kit (Stratagene) was used to incorporate mutations into the cDNA encoding PKN or PRK2 according to the manufacturer's instructions. For mutations involving Thr64, Ser374, Thr531, and Thr774, the original residues were changed to alanine; for the Lys644 mutation of PKN and the Lys684 mutation of PRK2, the original residues were changed to glutamate (Fig. 1). All constructs were verified by automated DNA sequencing.

Vectors to express recombinant proteins in *Escherichia coli* were constructed as follows. pGEX/PKN-(543-942) for the expression of GST-tagged PKN-(543-942) the same length as that expressed in Sf9 cells was made by digesting pAcGHLTC/PKN-(543-942) with *Xho*I, filling in the end with T4 DNA polymerase, ligating with *Bgl*II linker, digesting with *Bgl*II/*Not*I, and ligating into the *Bam*HI/*Not*I site of a modified pGEX-4T vector, in which the *Bam*HI site was created at position −16 from an original *Bam*HI site of pGEX-4T by QuickChange site-directed mutagenesis. pGEX/PRK2-(550-984) was made by digesting pBlueBacHis/GST/PRK2-(550-984) with *Sma*I/*Hin*dIII, filling in the end with T4 DNA polymerase, and ligating into the *Sma*I site of pGEX-4T. pRSET/PKN-(455-511) for His/PKN-(455-511) was constructed by subcloning a cDNA fragment encoding human PKN-(455-511) into the *Bam*HI site of pRSET. pRSET/GST was made by subcloning a cDNA fragment encoding GST amplified by PCR into the *Xho*I site of pRSET.

Expression and Purification of Recombinant PKN—To generate the recombinant baculovirus, each transfer vector described above was cotransfected with BacVector-2000 Triple Cut Virus DNA into Sf9 cells. The isolation and amplification of the baculovirus, and the expression of GST-tagged proteins in Sf9 cells were performed according to the manufacturer's instructions (Novagen). Sf9 cells expressing recombinant proteins were lysed for 15 min in buffer A (50 mM Tris/HCl at pH 8.0, 1 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride with rotation at 4°C. The lysate was then centrifuged at 30,000 × *g* for 10 min at 4°C and the resulting supernatant was collected. An appropriate volume of glutathione Sepharose 4B was added to the supernatant, and the mixture was rotated for 1 h at 4°C. After washing with buffer A, the bound proteins were eluted with elution buffer (50 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin, 10 mM reduced glutathione). Purified recombinant enzymes were stored in 50% glycerol at −80°C. The expression and purification of GST-tagged proteins in *E. coli* were performed using glutathione Sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech). The expression and purification of $(His)_6$-tagged proteins in *E. coli* were performed using Ni-NTA agarose according to the manufacturer's instructions (Qiagen). His-tagged fusion proteins were dialyzed in 50 mM Tris/HCl at pH 7.5 containing 1 mM DTT.

Protein Kinase Assay—To assess the protein kinase activity of PKN or PKCδ, purified enzyme (10 ng) from Sf9 cells was incubated for 5 min at 30°C in a reaction mixture (final volume 25 μl) containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 40 μM ATP, 18.5 kBq of [γ-^{32}P]ATP, 40 μM δPKC peptide (*3*) as phosphate acceptor, 0.1 mg/ml recombinant GST as the stabilizer, with or without 40 μM arachidonic acid. The kinase activity of PKA (kindly provided by Dr. Kuno, Kobe University) was measured by incubating the catalytic subunit of the enzyme (10 ng) in a reaction mixture containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 40 μM ATP, 18.5 kBq of [γ-^{32}P]ATP, 0.1 mg/ml recombinant GST as the stabilizer, and 40 μM Kemptide (*29*) as the phosphate acceptor for 5 min at 30°C. Reactions were terminated by spotting them onto Whatman P81 phosphocellulose papers, submersing them in 75 mM phosphate, and then washing three times for 10 min. The incorporation of ^{32}P phosphate into the δPKC peptide was assessed by liquid scintillation counting.

Dephosphorylation of Recombinant PKN—PP1 was purchased from New England Biolabs. PP2A was purchased from Upstate Biotechnology. CIAP was purchased from Takara Shuzo. Calcineurin was purified from bovine brain as described (*30*). Calmodulin was purified from bovine brain using a phenyl-Sepharose column, as described (*31*). GST/PKN-(543-942) was incubated for 1 h at 30°C with the indicated phosphatases, which could dephosphorylate the same amount of phosphocasein phosphorylated by PKA, in a reaction mixture containing 50 mM Tris/HCl at pH 7.5, 0.1 mM EDTA, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 8 mM $MgCl_2$, 5 mM DTT, and 0.01% Brij 35 (1 μM calmodulin for calcineurin) with or without phosphatase inhibitor (1 μM okadaic acid for PP1 and PP2A; 2 mM EGTA for calcineurin; 10 mM phosphate buffer at pH 7.5 for CIAP). Dephosphorylation reactions were terminated by adding phosphatase inhibitors to the reaction mixtures, and then the kinase assay toward the δPKC peptide was performed.

Autophosphorylation of Recombinant PKN—The recombinant enzyme (50 ng) was pre-incubated at 30°C in a reaction mixture containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 100 μM ATP, 10 mM phosphate buffer at pH 7.5, and 0.1 mg/ml recombinant GST as the stabilizer for the indicated times, 20 μl aliquots were removed, and the protein kinase assay toward the δPKC peptide was performed.

RESULTS

Expression of Various PKN Mutants in Insect Cells—We used the baculovirus expression system to investigate the regulatory mechanism of PKN by mutational analysis. Figure 1 summarizes the constructs used to generate various PKN mutants in this study. Recombinant enzymes fused to GST were affinity-purified from Sf9 cells (Fig. 2A). Full-length PKN fused to GST appeared as a concentrated single band, while GST/PKN-(543-942) consisted of multiple bands. As shown in Fig. 2B, dephosphorylation of these catalytic fragments of PKN by CIAP resulted in the migration of the bands to a faster position. These results suggest that there are multiple phosphorylated sites in the catalytic domain which cause electrophoretic mobility shifts on SDS-PAGE.

Determination of the Essential Regulatory Region Restricting the Kinase Activity of PKN—The wild-type enzyme expressed as a fusion protein with GST in Sf9 cells had the same properties as native PKN purified from rat testis (*2*) with regard to substrate specificity and response to effectors (data not shown). As shown in Fig. 3, the wild-type enzyme underwent ~7-fold activation in the presence of 40 μM arachidonic acid. Since the peptide kinase activity of PKN was competitively inhibited by a synthetic peptide comprising residues 39-53 of PKN itself (*9*), and the RhoA-binding region of PKN was identified as residues 33-111 (*5*), we first truncated residues 1-108 and examined the kinase activity [Asp108 was identified as a cleavage site for a caspase-3-like protease during apoptosis induced by treatment with δFas, staurosporine, or etoposide in Jurkat cells (*8*)]. As shown in Fig. 3, GST/PKN-

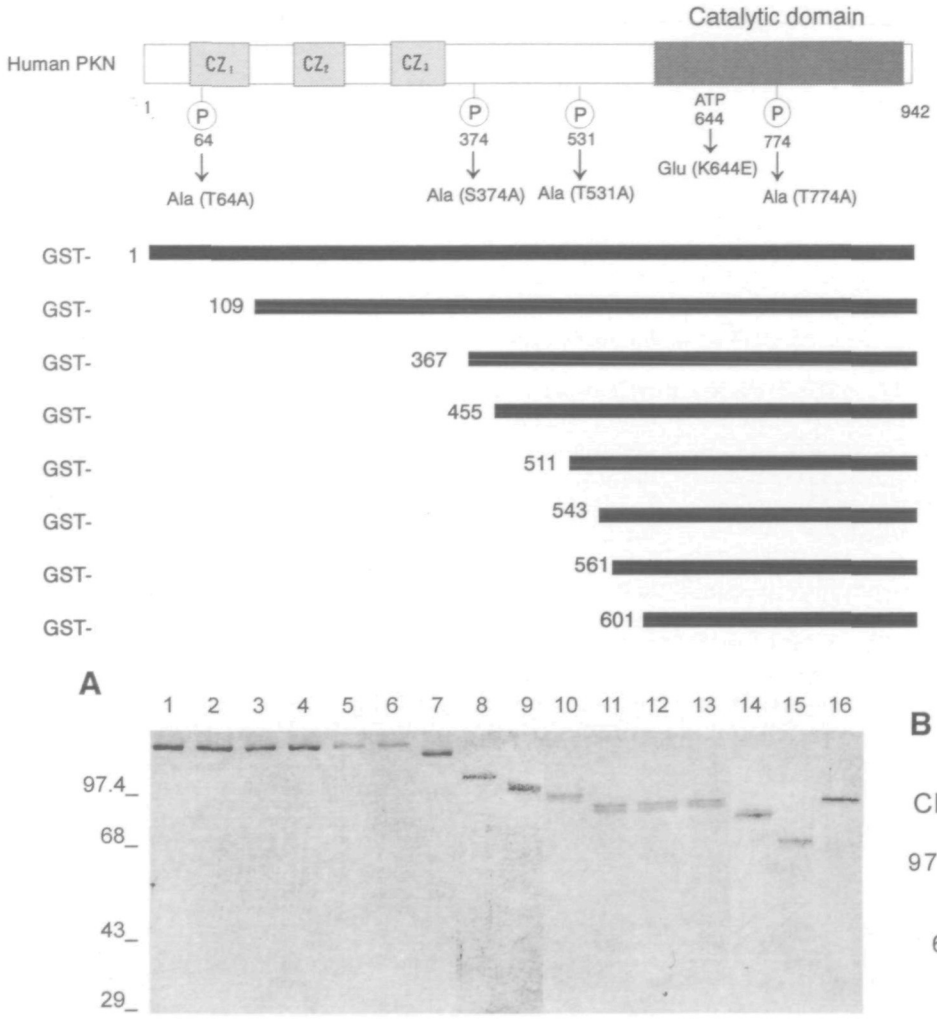

Fig. 1. **Schematic representation of the various constructs for expression in insect cells used in this study.** The schematic structure of human PKN is represented at the top of the figure, the deletion mutants of PKN are aligned below as solid boxes. *CZ* indicates regions rich in charged amino acids with a leucine zipper-like sequence, and are conserved among various vertebrates [*CZ1*, *CZ2*, and *CZ3* correspond to the previously reported regions A, B, and C in (*24*), respectively]. *P* indicates the putative phosphorylation sites corresponding to those in rat PKN reported by Peng *et al.* (*7*). ATP indicates the ATP binding site in the catalytic domain.

Fig. 2. **Various mutants of PKN expressed in Sf9 cells.** Molecular mass markers are indicated in kDa on the left. A, expression of various mutants of PKN in Sf9 cells. Ten microliter aliquots of purified enzymes were subjected to 8% SDS-PAGE, and stained with Coomassie Brilliant Blue. Lane 1, GST/PKNf; lane 2, GST/PKNf-T64A; lane 3, GST/PKNf-S374A; lane 4, GST/PKNf-T531A; lane 5, GST/PKNf-T774A; lane 6, GST/PKNf-K644E; lane 7, GST/PKN-(109-942); lane 8, GST/PKN-(367-942); lane 9, GST/PKN-(455-942); lane 10, GST/PKN-(511-942); lane 11, GST/PKN-(543-942); lane 12, GST/PKN-(543-942)-T774A; lane 13, GST/PKN-(543-942)-K644E; lane 14, GST/PKN-(561-942); lane 15, GST/PKN-(601-942); lane 16, GST/PRK2-(550-984). B, effect of dephosphorylation on the electrophoretic mobility of GST/PKN-(543-942). GST/PKN-(543-942) (*WT*), GST/PKN-(543-942)-K644E (*K644E*), or GST/PKN-(543-942)-T774A (*T774A*) was incubated at 37°C for 2 h with (+) or without (−) CIAP. Proteins were subjected to 7% SDS-PAGE with Coomassie Brilliant Blue staining.

(109-942) showed low basal activity similar to that of the wild-type enzyme, suggesting that the N-terminal region necessary for RhoA-binding is not essential for the restriction of the kinase activity of the catalytic domain. Since the major site of proteolytic activation of the rat enzyme by trypsin is Arg546 (*7*), we next tested the protein kinase activity of the corresponding fragment of the human enzyme GST/PKN-(543-942). The basal protein kinase activity of GST/PKN-(543-942) was markedly increased to a level almost as high as that of the full-length enzyme in the presence of 40 μM arachidonic acid. GST/PKN-(561-942), which corresponds to the major fragment generated by cleavage with the caspase-3-like protease during apoptosis in Jurkat cells (*8*), had a high basal kinase activity completely independent of arachidonic acid (Fig. 3). GST/PKN-(511-942) had a basal activity as high as GST/PKN-(543-942) with a slight dependence on arachidonic acid. However, GST/PKN-(455-942), which is a little longer than GST/PKN-(543-942), had a low basal activity similar to the wild-type enzyme, and was highly dependent on arachidonic acid. These results suggest that an essential autoinhibitory domain exists in the region of residues 455-511 of PKN. A $(His)_6$-tagged protein fragment corresponding to residues 455-511 of PKN (designated as "His-Iα") expressed in bacteria potently inhibited the kinase activity of GST/PKN-(543-942) in a dose-dependent manner (Fig. 4, A and B). The IC_{50} value for His-Iα obtained in the absence of modifiers was ~50 nM. GST/PKN-(543-942) was inhibited by neither $(His)_6$-tagged GST fragment (Fig. 4A) nor $(His)_6$-tagged fragment corresponding to residues 540-560 of PKN at concentrations up to 20 μM (data not shown). His-Iα also inhibited the activity of GST/PKN-(561-942) as well as GST/PKN-(543-942) (data not shown). Kinetic analyses were carried out to characterize

further the inhibition of GST/PKN-(543-942) by His-Iα. The enzyme was inhibited in a manner competitive with respect to the δPKC peptide with a K_i of 0.6±0.2 μM (Fig. 4C). These results suggest that His-Iα associates with the substrate-binding domain of the catalytic domain of PKN, blocking access of the enzyme to the substrate peptide, and that the region comprising residue 455 to 511 of PKN functions as an autoinhibitory domain. To assess whether the inhibition of the protein kinase activity by His-Iα is specific to PKN, we examined the ability of the fragment to inhibit the activities of PKC. As shown in Fig. 4B, His-Iα also potently inhibited the activity of the catalytic fragment of PRK2, GST/PRK2 (550-984), whereas it did not inhibit the basal activities of the catalytic regions of PKA or PKCδ (Fig. 4B). These observations indicate that His-Iα is a selective and competitive inhibitor of PKN activity, but that it does not exhibit a PKN isoform selectivity when assayed *in vitro* in the absence of other modifiers. In the presence of 40 μM arachidonic acid, His-Iα also inhibited GST/PKN-(543-942) but with an IC_{50} two orders of magnitude greater than in the absence of the modifier, as illustrated by the rightward shift in the IC_{50} for the inhibitory fragment (Fig. 4A). These results suggest that arachidonic acid directly interferes with the interaction between residues 455-511 and the catalytic region of PKN, and that arachidonic acid can recover the catalytic activity from autoinhibition by residues 455-511 of the wild-type PKN itself.

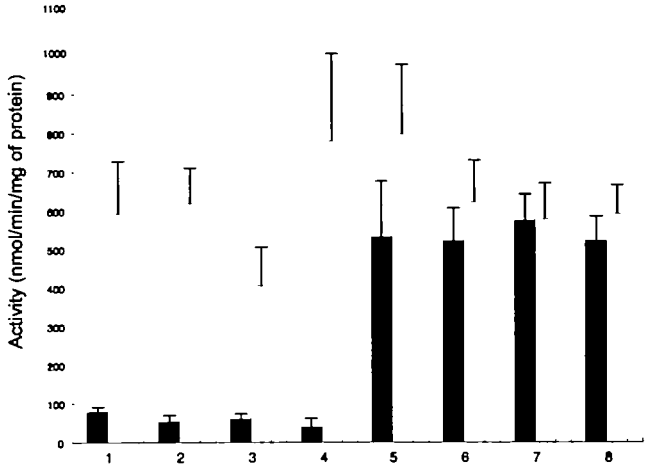

Fig. 3. **Protein kinase activity of various mutants with N-terminal deletions.** Enzyme samples (10 ng) were assayed for δPKC peptide in the absence (solid bar) or presence (open bar) of 40 μM arachidonic acid. The mean values and standard deviations were calculated from five independent experiments. 1, GST/PKNf; 2, GST/PKN-(109-942); 3, GST/PKN-(367-942); 4, GST/PKN-(455-942); 5, GST/PKN-(511-942); 6, GST/PKN-(543-942); 7, GST/PKN-(561-942); 8, GST/PKN-(601-942).

Role of Autophosphorylation of PKN in the Amino-Terminal Region—Recently, Peng *et al.* identified several

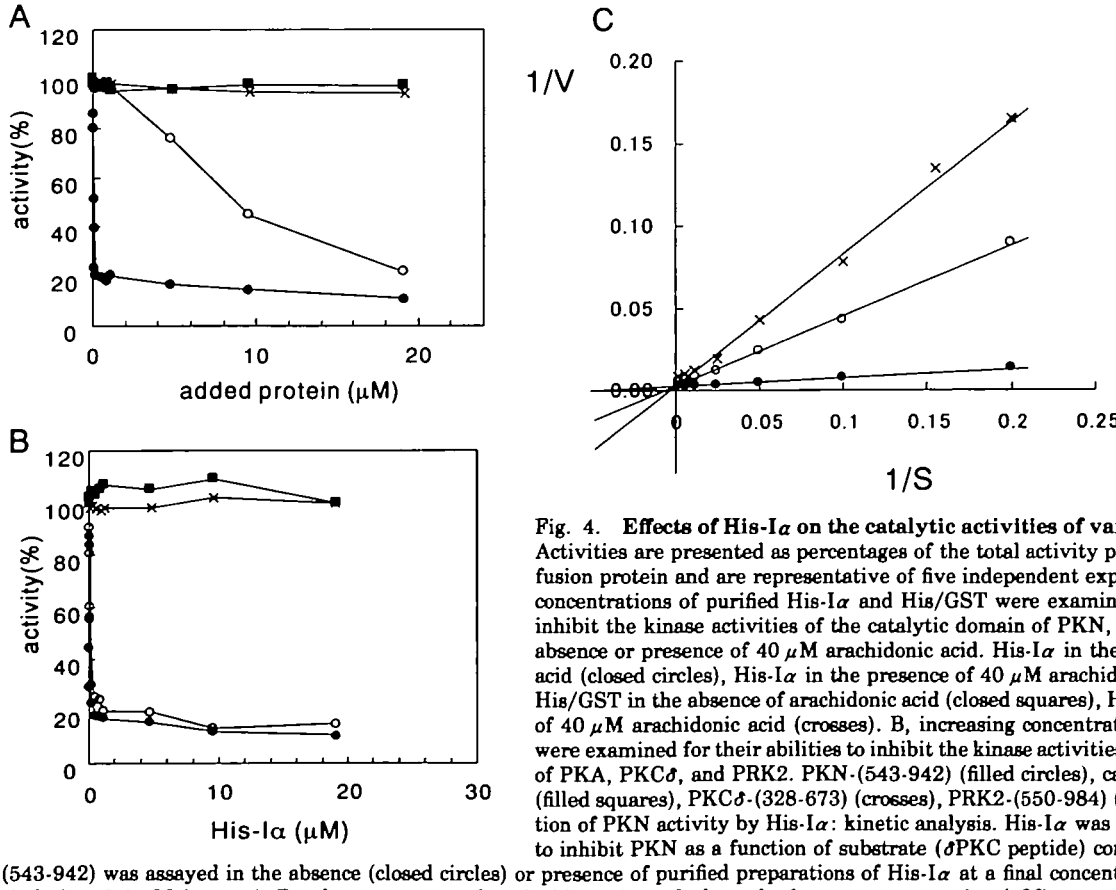

Fig. 4. **Effects of His-Iα on the catalytic activities of various protein kinases.** Activities are presented as percentages of the total activity present in the absence of fusion protein and are representative of five independent experiments. A, increasing concentrations of purified His-Iα and His/GST were examined for their abilities to inhibit the kinase activities of the catalytic domain of PKN, PKN-(543-942), in the absence or presence of 40 μM arachidonic acid. His-Iα in the absence of arachidonic acid (closed circles), His-Iα in the presence of 40 μM arachidonic acid (open circles), His/GST in the absence of arachidonic acid (closed squares), His/GST in the presence of 40 μM arachidonic acid (crosses). B, increasing concentrations of purified His-Iα were examined for their abilities to inhibit the kinase activities of the catalytic domain of PKA, PKCδ, and PRK2. PKN-(543-942) (filled circles), catalytic subunit of PKA (filled squares), PKCδ-(328-673) (crosses), PRK2-(550-984) (open circles). C, inhibition of PKN activity by His-Iα: kinetic analysis. His-Iα was evaluated for its ability to inhibit PKN as a function of substrate (δPKC peptide) concentration. GST/PKN-(543-942) was assayed in the absence (closed circles) or presence of purified preparations of His-Iα at a final concentration of 4.8 μM (open circles) or 9.6 μM (crosses). Results are expressed as double-reciprocal plots of substrate concentration (μM) *versus* enzymatic activity expressed as nanomoles of ^{32}P/min/mg of protein.

phosphorylation sites in rat liver PKN/PAK-1 (*7*). Among them, Thr63, Ser377, Ser534, and Thr778 are conserved in human PKN and in its isoform PRK2 (*24, 25*). Thr63, Ser377, and Thr534 are located in the N-terminal regulatory region, and have been reported to be autophosphorylated (*7*). These sites correspond to Thr64, Ser374, and Thr531 of human PKN, respectively (Fig. 1). To investigate the role of autophosphorylation at these sites, we introduced point mutations at each site. As shown in Fig. 5, changing each residue to alanine did not affect the basal kinase activity of PKN, whereas the arachidonic acid-dependent activity of PKN was decreased by changing Ser374 to alanine. Peng *et al.* reported that the kinase activity of PKN was slightly stimulated after pre-incubation with ATP (*7*). We prepared the autophosphorylated form of wild-type PKN by incubation with ATP. As shown in Fig. 6, the protein kinase activity of wild-type PKN in the absence of arachidonic acid was increased up to ~3-fold by 120 min pre-incubation with ATP. However, the protein kinase activity of GST/PKN-(543-942) was not activated under the same conditions (data not shown). To define the residue whose phosphorylation is essential for the autoactivation of PKN, the effects of pre-incubation on each mutant were analyzed. As shown in Fig. 6, replacing Thr64, Ser374, or Thr531 with alanine resulted in the repression of autoactivation by autophosphorylation of the enzyme. When assayed in the presence of arachidonic acid, neither wild-type nor any of these mutants was activated by preincubation with ATP. Therefore, Thr64, Ser374, and Thr531 may all be involved in the autoactivation of PKN in the initial phase.

The Critical Role of Phosphorylation of Thr774 in the Protein Kinase Activity of PKN—Thr778 in rat PKN has been reported to be stoichiometrically phosphorylated in the mature enzyme *in vivo* (*7*). This residue corresponds to Thr774 of human PKN, and is located in the activation loop of the catalytic domain (*14-16*). The threonine residue in the activation loop corresponding to Thr774 of human PKN is conserved in several serine/threonine kinases such as PKA and PKC, and the importance of the phosphorylation of this threonine residue has been reported in these kinases (*20, 32-35*). As shown in Fig. 5, the replacement of Thr774 of PKN by alanine resulted in a complete loss of the kinase activity as observed for the mutation of Lys644, the ATP-binding site, to glutamate [lane 5 for GST/PKNf; lane 8 for GST/PKN-(543-942)]. This observation suggests the importance of the phosphorylation of Thr774 in the protein kinase activity of PKN. We tested the effects of various phosphatases on the protein kinase activity of the catalytic fragment of PKN. GST/PKN-(543-942) expressed in Sf9 cells was preincubated for 1 h with various phosphatases at levels that could dephosphorylate the same amounts of PKA-phosphorylated phosphocasein, then the remaining protein kinase activity was assayed. As shown in Fig. 7A, GST/PKN-(543-942) pretreated with phosphatases, especially PP2A and CIAP, had lower δPKC peptide kinase activity than the non-pretreated enzyme. PKN might be down-regulated by the dephosphorylation of residues in the catalytic domain, such as Thr774.

GST/PKN-(543-942) expressed in *E. coli* was a nonfunctional enzyme, although the same fragment expressed in Sf9 cells showed constitutive kinase activity (Figs. 7B and 3). It has been reported that PKCα and βII expressed in *E. coli* have no protein kinase activity, and that this is due to the absence of the putative PKC-kinase in *E. coli* required for the phosphorylation of residues in the kinase activation loop (*36, 37*). GST/PKN-(543-942) expressed in *E. coli* migrated to almost the same position on SDS-PAGE as the protein expressed in Sf9 cells and treated with CIAP (data not shown). Thus, phosphorylation by another protein kinase may have been necessary for the activation of PKN,

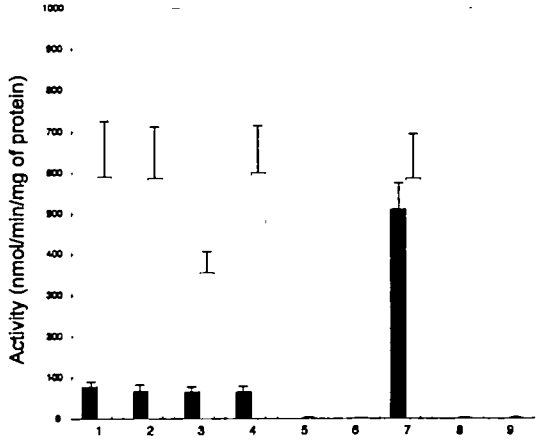

Fig. 5. **Protein kinase activity of enzyme with mutations in the putative phosphorylation site or the ATP-binding site.** Enzyme (10 ng) was assayed for δPKC peptide in the absence (solid bars) or presence (open bars) of 40 μM arachidonic acid. The mean values and standard deviations were calculated from five independent experiments. 1, GST/PKNf; 2, GST/PKNf-T64A; 3, GST/PKNf-S374A; 4, GST/PKNf-T531A; 5, GST/PKNf-T774A; 6, GST/PKNf-K644E; 7, GST/PKN-(543-942); 8, GST/PKN-(543-942)-T774A; 9, GST/PKN-(543-942)-K644E.

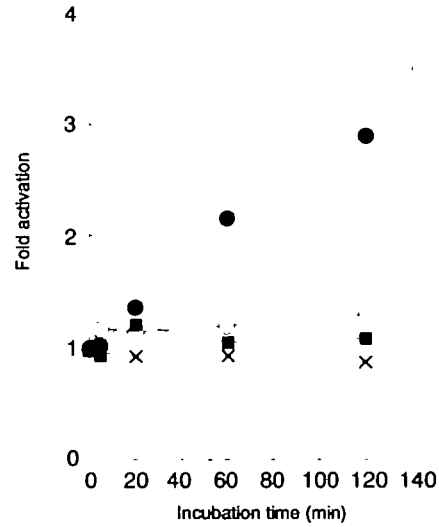

Fig. 6. **Effect of autophosphorylation on the protein kinase activity of PKN.** GST/PKNf (filled circles), GST/PKNf-T64A (open circles), GST/PKNf-S374A (crosses), and GST/PKNf-T531A (filled squares) were preincubated with ATP at 30°C. At the indicated time points, aliquots were removed and the protein kinase activity was assayed at 30°C for 2 min in the absence of arachidonic acid. Values are expressed as the fold activation relative to the activity observed without preincubation with ATP in the absence of arachidonic acid. The results shown are of four independent experiments.

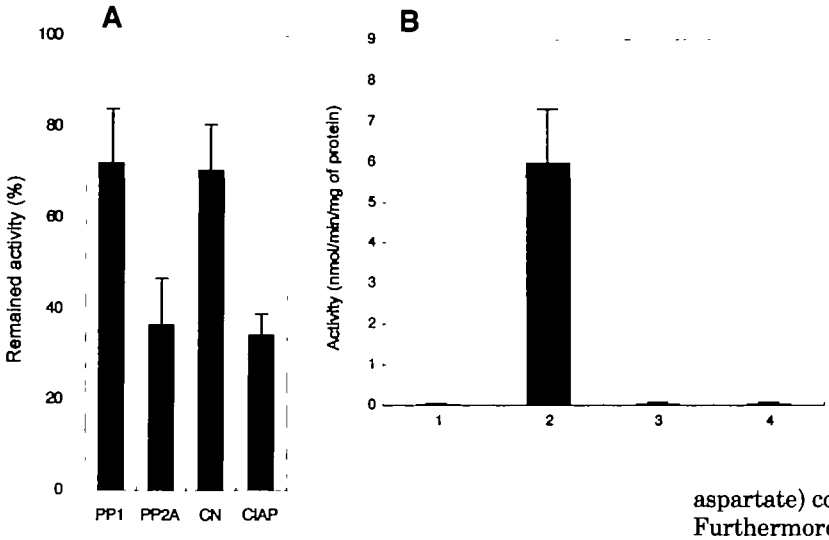

Fig. 7. **Important role of Thr774 in the kinase activity of PKN.** The mean values and standard deviations were calculated from four independent experiments. A, effect of various phosphatases on the protein kinase activity of PKN. GST/PKN-(543-942) was incubated at 30°C for 1 h with the indicated phosphatases, and the remained kinase activity was assessed. The protein kinase activity of GST/PKN-(543-942) incubated without phosphatases was taken as 100% activity. CN indicates calcineurin. B, protein kinase activity of bacterially-expressed catalytic domain of PKN or PRK2. Affinity-purified enzymes were assayed for δPKC peptide. 1, GST/PKN-(543-942); 2, GST/PRK2-(550-984); 3, GST/PRK2-(550-984)-K686E; 4, GST/PRK2-(550-984)-T816A.

and *E. coli* appears to lack this putative PKN kinase. However, the catalytic fragment of PRK2, GST/PRK2-(550-984), expressed in *E. coli* showed a relatively low (~6 nmol/min/mg protein) but significant protein kinase activity (Fig. 7B). Mutational substitution of alanine for Thr816 in the activation loop of PRK2, corresponding to Thr774 of PKN, resulted in the complete loss of protein kinase activity (Fig. 7B). These results suggest that phosphorylation of threonine in the activation loop underlies the post-translational activation of the wild-type catalytic fragment of PRK2 in *E. coli*. It has been proposed that the phosphorylation of the recombinant protein on this residue is catalyzed by an endogenous kinase in *E. coli* or by the activity of the catalytic fragment of PRK2 itself. If PRK2 can phosphorylate itself at Thr816 in the activation loop, the enzyme would be able to phosphorylate Thr774 in PKN, since sequence comparison of the catalytic regions of PKN and PRK2 revealed extensive identity, and especially the activation loop of PKN has the same sequence as that of PRK2. However, preincubation of the catalytic fragment of PKN expressed in *E. coli* with active PRK2 expressed in Sf9 cells and ATP did not induce the protein kinase activation of PKN, and the co-expression of the catalytic fragments of PRK2 and PKN in *E. coli* also did not activate PKN (data not shown). These results support the hypothesis that the phosphorylation of Thr774 in the activation loop also occurs to some extent in *E. coli*, whereas post-translational modifications of PKN other than the phosphorylation of Thr774 are lacking in this bacterium. The relatively low catalytic activity of PRK2 expressed in *E. coli* as compared to that expressed in Sf9 cells might be the result of the low stoichiometric phosphorylation of residues in the activation loop in *E. coli*.

Several kinases that are activated by phosphorylation on the activation loop can be activated by mutagenesis of the phosphorylated residue to glutamate or aspartate (review in Ref. *33*). In fact, replacement of Thr500 in the PKCβII or Thr495 in PKCα (each residue is in the activation loop) with glutamate restores protein kinase activity (*36, 37*). However, mutations PKN expressed in *E. coli* (replacement of Thr772 with glutamate, Thr774 to glutamate, Ser773 to aspartate/Thr774 to glutamate, or Thr774 to aspartate) could restore the activity at all (data not shown). Furthermore, the substitution of Thr774 in PKN with glutamate completely abolished the kinase activity of the enzyme even when expressed in Sf9 cells (data not shown). These results suggest that only the addition of a negative charge to the region in the activation loop of PKN does not lead to the expression of an active PKN, and it remains possible that the mutation of Thr774 to another amino acid simply disrupts the structure of the enzyme preventing its activation.

DISCUSSION

Recently, expression vectors encoding the regulatory domain or inactivated catalytic domain of enzymes have been used in transfection studies to gain dominant negative effects on signal transduction. Since several proteins, such as the GTP-bound form of Rho (*4, 6*), α-actinin (*38*), and PCD17 (*39*) bind to regions in PKN containing N-terminal leucine zipper-like sequences (~amino acid residues 33-306, roughly corresponding to "CZ1" and "CZ2" in Fig. 1), overexpression of the entire regulatory region of PKN is presumed to exert its effect in part through the sequestration of these PKN binding proteins. In fact, overexpression of the N-terminal region of PKN in 293 cells has been reported to cause abnormalities in cell division that do not directly reflect the loss of specific PKN functions, since the simple sequestration of Rho alone may block other Rho-mediated signaling involved in the regulation of cytoskeletal function (*40*). On the other hand, the primary structure of the catalytic region of PKN is similar to that of PKC family members, a finding consistent with their similar substrate specificities (*2, 41*). If relevant substrates of PKN overlap with those of PKC family members, overexpression of the kinase-negative mutant of the catalytic domain of PKN may induce interference with substrates. Furthermore, in the case of PKN, it is not certain that the kinase-negative mutant can interact with its target protein even if it has a point mutation, since K644E or T774A mutants in the catalytic domain do not bind to the N-terminal region of PKN in the two-hybrid system [data not shown; the wild-type catalytic domain of PKN binds directly to the N-terminal region of PKN (*9*)]. Thus, in assessing the specific roles of PKN in signal transduction, it will be helpful to obtain peptide fragments that act as specific dominant inhibitors of PKN by interfering with

PKN substrate utilization and not by interfering with the interaction of PKN with the binding proteins described above. As demonstrated here, His-Iα inhibits substrate phosphorylation with marked specificity through direct effects on the catalytic region of the enzyme, and does not inhibit the activity of the catalytic domain of PKCδ. Although His-Iα exhibits only modest PKN/PRK family isoform selectivity *in vitro*, if members of the PKN/PRK family are functionally redundant, non-selectivity within these members would be useful to elucidate the functions of the family. Residues 455-511 of PKN (designated as "Iα") are relatively well-conserved between PKN and PRK2, and the N-terminal part of this region shows some homology to the E4 region/C-terminal part of V0 of nPKC (*7, 25, 42*); the carboxyl-terminal part of this fragment shows slight homology to the pseudosubstrate region of nPKC. Dekker *et al.* reported that deletion of the V0 domain does not affect the cofactor dependence, but deletion of the pseudosubstrate region generates cofactor-independent activity of PKCη (*43*). Thus, the C-terminal part of Iα might function as a pseudosubstrate region for PKN and PRK2. Recently, the E region has been reported to play an important role in the regulation of phorbol ester interaction with the C1 domain of PKC (*44*), and to mediate protein-protein interactions (*45, 46*). Although the region of PKC that interacts with *cis*-unsaturated fatty acids such as arachidonic acid has not been identified, our results suggest the possibility that the E4-pseudosubstrate region of nPKC is also involved in the binding to unsaturated fatty acids such as arachidonic acid.

In this paper, we present a possible mechanism for the protein kinase activation of PKN by phosphorylation, which is evoked by the autophosphorylation of several sites including Thr64, Ser374, and Thr531 in the regulatory region and by phosphorylation of Thr774 in the catalytic region. By analogy to CaM-kinase II, the phosphorylation of residues located in or near the autoinhibitory region, such as Thr531 of PKN, may release the restriction of the protein kinase activity as well. However, the phosphorylation of Thr64 and Ser374 were also required for autoactivation by autophosphorylation. Peng *et al.* reported that the phosphorylation of Ser377 in rat PKN, corresponding to Ser374 in human PKN, occurs earlier than the autophosphorylation of other sites (*7*). We confirmed by immunoblotting using an antibody that reacts specifically with a peptide containing phosphorylated Ser374 that the wild-type PKN purified from Sf9 cells contains some phosphate on Ser374, and the addition of ATP to the enzyme results in a rapid increase in the phosphorylation of this residue (data not shown). Thus, the phosphorylation of Ser374 or Thr64 may proceed early in the preincubation with ATP followed by the autophosphorylation of Thr531, and the mutation of Thr64 or Ser374 to alanine may prevent this ordered phosphorylation, resulting in the suppression of the autophosphorylation of Thr531. In our experiments, the extent of activation by autophosphorylation *in vitro* was smaller than the activation induced by proteolysis. Autoactivation of PKN may be a precursor for the full activation of the activator-induced protein kinase activity rather than a secondary event that stabilizes an active conformation generated by the activator-enzyme interaction as observed in the case of CaM-kinase II.

Recently, there have been several reports of differences in the structural regulation of PKN and PRK2. Quilliam *et al.* reported that the NCK adaptor protein binds to the N-terminal unique proline-rich sequence of PRK2 that is absent from PKN, suggesting differences in subcellular localization and/or signaling pathway (*47*). Vincent and Settleman reported that PKN and PRK2 exhibit different nucleotide requirements for Rho binding, suggesting distinct binding mechanisms and possibly distinct biological roles for these two related kinases (*48*). Studies of the differential regulation between these protein kinases has focused on the structure of the N-terminal regulatory sequence as mentioned above, since the primary structures of the catalytic regions of the two kinases are very similar to one another. In this study, we demonstrated that the catalytic region of PRK2 expressed in *E. coli* has low but significant peptide kinase activity. However, we were unable to express the enzymatically active catalytic region of PKN in *E. coli* using the same expression vector and the same conditions used for the expression of PRK2. In the case of PKC family members, it has been reported that PKCδ, in constrast to PKCα and βII, can be expressed in *E. coli* in a functional form (*36, 37, 49*). The phosphorylation of Thr505 in the activation loop of PKCδ, which corresponds to the critical phosphorylation site for the putative PKC kinase, was not shown to be required for the formation of functional PKCδ by site-directed mutagenesis (*49*). According to our results, however, the phosphorylation of threonine in the activation loop of PRK2, which corresponds to Thr505 in PKCδ, appears to be essential for a kinase activity similar to that of PKN. As discussed in "RESULTS," certain post-translational modifications, such as the phosphorylation of other sites in the catalytic region, might play critical roles in the activation of PKN, and be responsible for the differences in the activation mechanism between PKN and PRK2. The extent to which the post-translational phosphorylation of PKN is subjected to acute regulation remains to be established, but the identification of other modifications will permit an assessment of this issue and will also aid in identifying the relevant PKN-specific modifiers.

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