PKN Associates and Phosphorylates the Head-Rod Domain of Neurofilament Protein*

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PKN is a fatty acid-activated serine/threonine kinase that has a catalytic domain highly homologous to that of protein kinase C in the carboxyl terminus and a unique regulatory region in the amino terminus. Recently, we reported that the small GTP-binding protein Rho binds to the amino-terminal region of PKN and activates PKN in a GTP-dependent manner, and we suggested that PKN is located on the downstream of Rho in the signal transduction pathway (Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Science 271, 648-650; Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y. Kakizuka, A., and Narumiya, S. (1996) Science 271, 645-648). To identify other components of the PKN pathway such as substrates and regulatory proteins of PKN, the yeast two-hybrid strategy was employed. By this screening, a clone encoding the neurofilament L protein, a subunit of neuron-specific intermediate filament, was isolated. The amino-terminal regulatory region of PKN was shown to associate with the head-rod domains of other subunits of neurofilament (neurofilament proteins M and H) as well as neurofilament L protein in yeast cells. The direct binding between PKN and each subunit of neurofilament was confirmed by using the in vitro translated amino-terminal region of PKN and glutathione S-transferase fusion protein containing the head-rod domain of each subunit of neurofilament. PKN purified from rat testis phosphorylated each subunit of the native neurofilament purified from bovine spinal cord and the bacterially synthesized head-rod domain of each subunit of neurofilament. Polymerization of neurofilament L protein in vitro was inhibited by phosphorylation of neurofilament L protein by PKN. The identification and characterization of the novel interaction with PKN may contribute toward the elucidation of mechanisms regulating the function of neurofilament.

We have reported a novel serinethreonine protein kinase, designated PKN, having a catalytic domain homologous to the PKC1 family and unique amino-terminal sequences (1, 2). The amino-terminal region of PKN contains repeats of a leucine zipper-like motif, suggesting promotion of protein-protein association through hydrophobic interactions (3), and the basic region adjacent to the first leucine zipper-like motif, which is conserved through evolution in vertebrates (4) and among the various isoforms of PKN (5). In the previous study, we demonstrated that truncation of the amino-terminal region of PKN by limited proteolysis results in the generation of the catalytically active form in vitro, and unsaturated fatty acid and some detergent can remove restriction of the catalytic activity by its amino-terminal region at relatively low concentrations in vitro (1, 6). Thus, regions contained in the amino-terminal portion of PKN are presumed to be critical for the regulation of the biological activity of this enzyme.

Recently, we demonstrated that Rho, a small GTP-binding protein, binds to PKN in a GTP-dependent fashion and that this binding leads to the activation of PKN (7, 8), suggesting that PKN is one of the targets of Rho. Several reports have been accumulated showing that multiple protein-protein interactions within the regulatory region of serine/threonine protein kinases are important for the regulation of the enzyme activities in vivo, such as Raf-1 and p21-activated protein kinase (9–19). For example, current models of Raf-1 activation suggest that this kinase is bound in a native complex with 14-3-3 proteins (10, 11) and p50 and hsp90 proteins (12, 13). Raf-1 also binds to Ras in a GTP-dependent fashion (14–17), and active Ras is thought to recruit Raf-1 to the plasma membrane, where it interacts with other modulators of its activity and relevant substrates (18, 19). The binding site on Raf-1 for 14-3-3 proteins is distinct from the Ras binding domain, and Raf-1 molecules bound to 14-3-3 proteins may simultaneously bind to Ras (10, 11). In our preliminary experiment, PKN immunoreactivity in the crude extract from rat brain was eluted broadly on gel filtration, suggesting that PKN formed complexes. Thus, in the analogy with Raf-1, we attempted to identify other proteins that interact with PKN and potentially regulate its activity by using a yeast two-hybrid system, with the amino-terminal regulatory region of PKN as a target protein. By this screening, a cDNA clone encoding the head-rod domain of NFL, a subunit of neuron-specific intermediate filament protein, was isolated from a human brain cDNA library. In this report we characterize the interaction between PKN and each subunit of NF and vimentin and raise the possibility that PKN can play a role in the regulation of assembly of NF and provide a potential kinase A; NF, neurofilament; NFL, neurofilament L; NFH, neurofilament H; NFM, neurofilament M; DTT, dithiothreitol; GST, glutathione S-transferase; Gal4ad, transcription activation domain of Gal4; Gal4bd, DNA binding domain of Gal4; VP16ad, transcription activation domain of VP16; LexAbd, DNA binding domain of LexA; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); NAK, neurofilament-associated kinase.

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1The abbreviations used are: PKC, protein kinase C; PKA, protein kinase A; NF, neurofilament; NFL, neurofilament L; NFH, neurofilament H; NFM, neurofilament M; DTT, dithiothreitol; GST, glutathione S-transferase; Gal4ad, transcription activation domain of Gal4; Gal4bd, DNA binding domain of Gal4; VP16ad, transcription activation domain of VP16; LexAbd, DNA binding domain of LexA; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); NAK, neurofilament-associated kinase.
junction from signals generated by growth factor stimulation to intermediate filaments.

**EXPERIMENTAL PROCEDURES**

Two-hybrid Screens and Constructs for the Two-hybrid System—A scheme of the fusion constructs for each subunit of NF used in this study is represented in Fig. 1. An EcoRI/BamHI fragment encoding amino acids 1–540 of human PKN, designated as PKNN1, was inserted into the vector pGBT9 (20). This plasmid, which contains a LexA DNA binding domains (hatched boxes), and glutathione S-transferase (solid boxes) were fused to the various deletion mutants of NF (open boxes). The dashed box indicates the deleted sequence. The original clone isolated from the library pGAD10-NFL #21 is shown at the bottom of the figure. Restriction sites: T, Thrl111; X, XhoI; P, PstI; B, BglII; K, KpnI.

**In Vitro Transcription and Translation**—For in vitro transcription, truncated human PKN was made as follows. pPKNC2 for the amino-terminal region of PKN (amino acids 1–474, this region was designated as PKNC2) was made by digesting pPKNC4 (human PKN cDNA in pBluescript II SK; see Ref. 2) with XbaI, and cRNAs were transcribed using T7 RNA polymerase. For NFL, pBL(22) (kindly provided by Dr. N. Hirokawa) was linearized by cutting with XbaI, and cRNAs were transcribed using T7 RNA polymerase. For NFL, pH6 (23) (kindly provided by Dr. N. Hirokawa) was linearized by cutting with HindIII, and cRNA was transcribed using T3 RNA polymerase. For In vitro transcription, these cRNAs were translated in the rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine.

Preparation of GST Fusion Proteins—pGST/NFL#21 for GST fused to NFL#21 (amino acids 1–349 of human NFL) was made by subcloning an EcoRI insert of plasmid 21 into the pGEX4T vector. pGST/NFLdelA for GST fused to NFLdelA (amino acids 1–175 and 335–349) was made by digesting pGST/NFL#21 with PstI, removing the 480-base pair insert, filling in the ends with T4 DNA polymerase, and self-ligating. This removed the original amino acid sequences carboxyl-terminal of the TthII site (1425 nucleotides from the initiating ATG), and a stop codon was created in the plasmid sequence. The fragment encoding amino acids 614–942 (this region was designated as PKNC2) containing the conserved catalytic domain of human PKN was made by polymerase chain reaction amplification, and pGST/NFL delB for GST fused to NFLdelB (amino acids 1–175 and 335–349) was made by subcloning this fragment into pGEX4T. pGST/NFL for GST fused to the head-tail domain of NFL was made by digesting pGST/NFL6 with EcoRI, filling in the ends with T4 DNA polymerase, and self-ligating. This removed the original amino acid sequences carboxyl-terminal of the BglII site (1425 nucleotides from the initiating ATG), and a stop codon was created in the plasmid sequence. The fragment encoding amino acids 614–942 (this region was designated as PKNC2) containing the conserved catalytic domain of human PKN was made by polymerase chain reaction amplification, and pGST/NFLdelA for GST fused to NFLdelA (amino acids 1–175 and 335–349) was made by digesting pGST/NFL#21 with PstI, removing the 480-base pair insert, filling in the ends with T4 DNA polymerase, and self-ligating. This removed the original amino acid sequences carboxyl-terminal of the BglII site (1425 nucleotides from the initiating ATG), and a stop codon was created in the plasmid sequence. The fragment encoding amino acids 614–942 (this region was designated as PKNC2) containing the conserved catalytic domain of human PKN was made by polymerase chain reaction amplification, and pGST/NFL delB for GST fused to NFLdelB (amino acids 1–175 and 335–349) was made by subcloning this fragment into pGEX4T. pGST/NFL for GST fused to the head-tail domain of NFL was made by subcloning the EcoRI/BamHI fragment of human PKN into pBTM116 and pVP16, respectively. pBTM/PKNC1 for GST fused to PKNC1 was constructed by subcloning the Clal/EcoRI fragment of human PKN into pBTM116 and pVP16, respectively.

**Preparation of GST Fusion Proteins**—pGST/NFL#21 for GST fused to NFL#21 (amino acids 1–349 of human NFL) was made by subcloning an EcoRI insert of plasmid 21 into the pGEX4T vector. pGST/NFLdelA for GST fused to NFLdelA (amino acids 1–175 and 335–349) was made by digesting pGST/NFL#21 with PstI, removing the 480-base pair fragment, and self-ligating with T4 DNA ligase. pGST/NFLdelB for GST fused to NFLdelB (amino acids 245–349) was made by subcloning the BglII/EcoRI fragment of pGST/NFL#21 into the pGEX4T vector. pGST/NFL for GST fused to the head-tail domain of NFL was made by subcloning the ~700-base pair KpnI/EcoRI fragment of pBL into the pGEX4T vector. pGST/NFLdelA for GST fused to the tail-tail domain of NFL was made by subcloning the ~700-base pair KpnI/EcoRI fragment of pBL into the pGEX4T vector. pGST/NFLdelB for GST fused to the head-tail domain of NFL was made by subcloning the ~700-base pair KpnI/EcoRI fragment of pBL into the pGEX4T vector.
Expression and purification of GST or GST fusion proteins were performed according to the manufacturer’s instruction (Pharmacia Bio-Tech Inc.) The eluate from glutathione-Sepharose 4B (Pharmacia Bio-Tech Inc.) was dialyzed overnight against 10 mM Tris/HCl at pH 8.8 containing 1 mM EDTA, 1 mM DTT, and 0.1 μg/ml leupeptin.

In vitro Binding Assay—For the in vitro NF binding experiment, 2.5 μl of in vitro translated PKNN2 or PKNC2 was mixed with 5 μg of each GST-NF fusion protein or with 25 μg of GST alone in 100 μl of GST binding buffer (20 mM Tris/HCl at pH 7.5, 0.5 mM DTT, 150 mM NaCl, 0.05% Triton X-100, 1 mM EDTA, 1 μg/ml leupeptin) and incubated at 1 h at 4°C. After the addition of 25 μl of glutathione-Sepharose 4B pretreated with 10 ng/ml Escherichia coli extract to block nonspecific binding, the binding reaction was continued for an additional 30 min at 4°C. The glutathione-Sepharose 4B was then washed three times in GST wash buffer (20 mM Tris/HCl at pH 7.5, 0.5 mM DTT, 1 mM EDTA, 1 μg/ml leupeptin) containing 0.5 mM NaCl and 0.05% Triton X-100 and washed further with GST wash buffer. Bound proteins were eluted with GST elution buffer (100 mM Tris at pH 8.0, 20 mM glutathione, 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin) and subjected to 10% SDS-PAGE. The binding was visualized and quantitated by an imaging analyzer (Fuji BAS1000).

Preparation of NF Proteins from Native Bovine Tissues—NF proteins were prepared from bovine spinal cords as reported (23).

Kinase Assay—Soluble cytosolic extract of spinal cord or purified NF proteins were boiled for 5 min to destroy endogenous protein kinase activity, and the extract was used as phosphate acceptors. The phosphorylation of NF preparations was carried out at 30°C in an assay mixture containing 20 μg Tris/HCl at pH 7.5, 8 mM MgCl₂, 100 μM ATP, 185 kBq of [γ-³²P]ATP, phosphate acceptors, 20 ng/ml PKN purified from rat testis (6), with/without 40 μM arachidonic acid as indicated in each experiment. After incubation for various times, the reaction was terminated by the addition of an equal volume of Laemmli’s sample buffer and separated on 7% SDS-polyacrylamide gels. The gels were dried under vacuum, and the phosphorylation was visualized by an imaging analyzer (Fuji BAS1000). Dephosphorylation of NF proteins was conducted with calf intestine alkaline phosphatase as described (24), and NF proteins were boiled for 5 min to destroy phosphatase activity.

Effects of Phosphorylation on the Polymerization of NF—For the in vitro NF polymerization experiment, phosphorylated forms of bacterial-synthesized GST fused to the full length of NFL and GST fused to NFL#21 were prepared by incubation of 5 μg of these proteins with 1 mM MgCl₂, 60 ng PKN, and 100 μM ATP for 2 h at 30°C. Bacterially synthesized proteins incubated with PKN in the absence of ATP were employed as a nonphosphorylated control. 2.5 μl of in vitro translated NFL was added to the above mixtures in depolymerization buffer (20 μg Tris/HCl at pH 8.5, 1 mM DTT, 1 μg/ml leupeptin, 1 mM MgCl₂), then the pH of the reaction mixture was shifted to 7.2 by the addition of appropriate volumes of 1 M PIPES at pH 6.8 and incubated for 1 h at 35°C. After adding 25 μl of glutathione-Sepharose 4B pretreated with 10 mg/ml E. coli extract to block nonspecific binding, the binding reactions were continued for an additional 30 min at 4°C. The glutathione-Sepharose 4B was then washed twice in depolymerization buffer containing 0.5% Triton X-100 and washed further with depolymerization buffer. Bound proteins were eluted with GST elution buffer and subjected to 10% SDS-PAGE. Quantitation of the binding reactions was carried out by an imaging analyzer (Fuji BAS1000).

RESULTS

Isolation of PKN-binding Proteins Using the Yeast Two-hybrid System—To identify proteins that interact with the amino-terminal region of human PKN, we used the yeast two-hybrid system. The chimeric protein construct contained the DNA binding domain of the GAL4 protein fused to the unique amino-terminal region of human PKN. The 82 plasmids were isolated representing 16 different cDNAs as judged by DNA sequencing. One of these cDNAs encoded the head-rod domain of NFL (411 amino acids of the amino-terminal region) of NFM was made by polymerase chain reaction amplification from a human hippocampus cDNA library and subcloning into the pGEX4T vector. pGST/NFL for GST fused to the full length of NFL was constructed by subcloning the BamHI/EcoRI insert of pGL into the pGEX4T vector, pGST/NFM for GST fused to the tail domain of NFM was made by subcloning the ~1-kilobasepair XhoI/NotI fragment of pBM (22) (kindly provided by Dr. N. Hirokawa) into the pGEX4T vector, pGST/NFH for GST fused to the full length of NFH was constructed by digesting pBH with BglII, and the cDNA insert was subcloned into the BamHI restriction site of the pGEX4T vector. pGST/NFM for GST fused to the head-rod domain of NFH was made by digesting pGST/NFH with Thul1111/EcoRI, filling in the ends with T4 DNA polymerase, and ligating them together with T4 DNA ligase. This removed the DNA sequences 3’-terminal to the Thul1111 site, which encodes the tail domain of NFH. pGST/NFH for GST fused to the tail domain of NFH was made by digesting pBH with Thul1111/BglII, filling in the ends, and subcloning the ~2-kilobasepair fragment into the pGEX4T vector.

For the in vitro PKN binding experiment, 2.5 μl of in vitro translated PKNN2 or PKNC2 was mixed with 5 μg of each GST-NF fusion protein or with 25 μg of GST alone in 100 μl of GST binding buffer (20 μg Tris/HCl at pH 7.5, 0.5 mM DTT, 150 mM NaCl, 0.05% Triton X-100, 1 mM EDTA, 1 μg/ml leupeptin) and incubated at 1 h at 4°C. After the addition of 25 μl of glutathione-Sepharose 4B pretreated with 10 ng/ml Escherichia coli extract to block nonspecific binding, the binding reaction was continued for an additional 30 min at 4°C. The glutathione-Sepharose 4B was then washed three times in GST wash buffer (20 μg Tris/HCl at pH 7.5, 0.5 mM DTT, 1 mM EDTA, 1 μg/ml leupeptin) containing 0.5 mM NaCl and 0.05% Triton X-100 and washed further with GST wash buffer. Bound proteins were eluted with GST elution buffer (100 mM Tris at pH 8.0, 20 mM glutathione, 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin) and subjected to 10% SDS-PAGE. The binding was visualized and quantitated by an imaging analyzer (Fuji BAS1000).
protein. Specificity of this interaction was tested further by measuring the ability of other combinations of the two-hybrid constructs, LexAbd (instead of Gal4bd)-PKN and VP16ad (instead of Gal4ad)-NFL, or LexAbd-NFL and VP16ad-PKN, to support lacZ expression in L40 cells. Fig. 2 summarizes these data and demonstrates a specific interaction between the amino-terminal region of PKN and the head-rod domain of NFL. To characterize further the interaction between PKN and NFL, we tested the ability of in vitro translated PKN to bind to GST-NFL in an in vitro binding assay. As shown in Fig. 3A, the amino-terminal region of PKN bound to the head-rod domain of NFL, and little binding of the carboxyl-terminal catalytic domain of PKN to the head-rod domain of NFL was observed in this assay.

We reported previously that RhoA binds to the amino-terminal region of PKN. Given the interaction of NFL with the amino-terminal regulatory region of PKN, it was possible that this protein interacts with the Rho binding domain of PKN and might compete with Rho for binding to PKN. In an in vitro binding assay, bacterially synthesized NFL did not compete with the bacterially synthesized RhoA for the binding to the amino-terminal region of PKN (data not shown).

The Amino-terminal Region of PKN Binds to the Head-Rod Domain of Each Subunit of NF—To test the ability of PKN to phosphorylate each subunit of NF, NF purified from spinal cord was subjected to in vitro phosphorylation by PKN. PKN phosphorylated efficiently all three NF subunits (Fig. 4). Initial velocities of phosphorylation of each subunit by PKN in the presence of arachidonic acid were ~5–10 times higher than those in the absence of modifier (Fig. 4A). In the presence of arachidonic acid, phosphate incorporation into each subunit of NF reached a maximum at ~60 min and then continued to plateau up to 120 min (Fig. 4, B and C). Although purified PKN was labile in its diluted condition, it was unlikely that the decline in phosphorylation speed was mainly the result of the inactivation of PKN because the phosphorylation level did not decrease appreciably from 60 to 120 min in the absence of arachidonic acid (data not shown). The maximal phosphorylation by PKN per mol of protein subunit was estimated by image quantitation to be ~2 mol/mol of NFH, ~6 mol/mol of NFM, and ~1 mol/mol of NFL, respectively. The NFH, which was reported to be the most intensely radiolabeled subunit in vivo (28), was a relatively poor substrate for PKN in vitro compared with NFL. It seemed possible that potential phosphorylation sites of NFH and NFM for PKN had already been masked since these proteins were purified from bovine tissue. Therefore, we tested whether we could reveal additional phosphorylation sites using enzymatically dephosphorylated NF. As shown in Fig. 4D, the change of electrophoretic mobility was accompanied by the dephosphorylation of NFH and NFM (24). Dephosphorylation of bovine NF with alkaline phosphatase did not result in any significant difference in phosphorylation of the NFH and NFM subunits by PKN, suggesting that native NF contains sites that are accessible to phosphorylation (Fig. 4, B and C).

To examine the phosphorylation site of NF by PKN, we prepared bacterially synthesized GST fused to the head-rod domain and GST fused to the tail domain of each subunit of NF and subjected them to an in vitro phosphorylation assay. As shown in Fig. 5, 32P was incorporated to the head-rod domain of each NF in the ratio of 3:10:2 for NFH:NFM:NFL. The GST fused to the tail domain of each subunit was not labeled at all. PKN also phosphorylated the head-rod domain of vimentin (data not shown). This clearly indicated that phosphorylation...
sites were located exclusively in the head-rod domain of these intermediate filaments.

The Effect of Phosphorylation on Filamentous Structure of NFL in Vitro—

It is widely accepted that the NFL subunit forms the "core" of the NF, suggesting that NFL may assemble first and may then provide the signal as well as the scaffold for coassembly or polymerization of NFM and NFH. It has been shown in vitro that phosphorylation of NFL by PKA and PKC inhibited its polymerization and also depolymerized the filaments (29–31).

We investigated whether NFL polymerizes in an in vitro binding analysis. The bacterially produced GST fused to the head-rod domain of NFL or GST fused to the full length of NFL was mixed with in vitro translated NFL in buffer at pH 8.5 with 1 mM MgCl₂. Then the pH of the reaction mixture was shifted to 7.2 and incubated for 1 h at 35°C. After extensive washing, sites were located exclusively in the head-rod domain of these intermediate filaments.

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has a high specificity for NFH and is insensitive to 5 mM Ca^2+.

Toru-Delbauffe and Pierre (47) and Toru-Delbauffe characteristics are unknown. One of these, partially purified by laboratories, although their molecular weights and structural protein kinase by molecular weight, by their marked preference for phosphorylating NF. PKN is clearly distinguished from this protein kinase of 67 kDa from bovine spinal cord which phosphorylates NF. Wible mainly bound to vertebrate NF through purification procedures directed their attention to phosphorylating activities that resembled the intact tissue are yet unclear. Several laboratories have reported the isolation of a NF-associated kinase (32–35). Recently, increasing evidence has accumulated to suggest mechanisms of the growth-dependent regulation of NF turnover at discrete sites on the filaments (41). One of the possible proteins into preexisting intermediate filament networks (32–35) and the disruption of endogenous intermediate filament networks by the integration of assembly-incompetent mutant proteins (38–40). NF, a neuronal intermediate filament, was also reported to have a dynamic structure, which turns over within a small region of the axoplasm by exchanging subunits at discrete sites on the filaments (41). One of the possible mechanisms of the growth-dependent regulation of NF turnover is the phosphorylation of NF triplet proteins. Protein kinases responsible for the phosphorylation of NF subunits in the intact tissue are yet unclear. Several laboratories have directed their attention to phosphorylating activities that remain bound to vertebrate NFs through purification procedures (42–45). Wible et al. (46) reported the isolation of a NF-associated protein kinase of 67 kDa from bovine spinal cord which phosphorylates NF. PKN is clearly distinguished from this protein kinase by molecular weight, by their marked preference for the tail domain of NFH, and by their lack of autophosphorylation activity. Protein kinases that phosphorylate all three subunits of NF were partially purified by several other laboratories, although their molecular weights and structural characteristics are unknown. One of these, partially purified by Toru-Delbauffe and Pierre (47) and Toru-Delbauffe et al. (48), has a high specificity for NFH and is insensitive to 5 mM Ca^2+.

Another one, purified by Dosemeci et al. (49), has marked preference for casein rather than histone or NF as substrate. Proteins bound to the beads were analyzed by autoradiography. As shown in Fig. 6, phosphorylation of NF was clearly detected. Next, we determined whether phosphorylation of NF by PKN inhibits the polymerization of NF in this assay system. The GST fused to the head-rod domain of NF and GST fused to the full length of NF were phosphorylated by PKN, transferred to the reaction mixture, and mixed with in vitro translated NF. As shown in Fig. 6, the binding of the head-rod domain and the full length of NF to in vitro translated NF was very weakly detected, indicating that phosphorylation of NF by PKN inhibited the polymerization of NF.

DISCUSSION

Recently, increasing evidence has accumulated to suggest that continuous turnover of intermediate filaments is involved in maintaining the intermediate filament structure and function in living cells. Transfection of native and mutated intermediate filament genes or microinjection of biotin-labeled intermediate filaments has revealed the successive incorporation of newly synthesized or microinjected intermediate filament proteins into preexisting intermediate filament networks (32–37) and the disruption of endogenous intermediate filament networks by the integration of assembly-incompetent mutant proteins (38–40). NF, a neuronal intermediate filament, was also reported to have a dynamic structure, which turns over within a small region of the axoplasm by exchanging subunits at discrete sites on the filaments (41). One of the possible mechanisms of the growth-dependent regulation of NF turnover is the phosphorylation of NF triplet proteins. Protein kinases responsible for the phosphorylation of NF subunits in the intact tissue are yet unclear. Several laboratories have directed their attention to phosphorylating activities that remain bound to vertebrate NFs through purification procedures (42–45). Wible et al. (46) reported the isolation of a NF-associated protein kinase of 67 kDa from bovine spinal cord which phosphorylates NF. PKN is clearly distinguished from this protein kinase by molecular weight, by their marked preference for the tail domain of NFH, and by their lack of autophosphorylation activity. Protein kinases that phosphorylate all three subunits of NF were partially purified by several other laboratories, although their molecular weights and structural characteristics are unknown. One of these, partially purified by Toru-Delbauffe and Pierre (47) and Toru-Delbauffe et al. (48), has a high specificity for NFH and is insensitive to 5 mM Ca^2+.

Another one, purified by Dosemeci et al. (49), has marked preference for casein rather than histone or NF as substrate. Another, purified by Hollander and Bennett (50), phosphorylates efficiently the carboxyl-terminal tail domain of NFM. Judging from these characteristics, PKN seems to be different from these kinases. Recently, Xiao and Monteiro (51) detected four prominent kinases (NAKs) with molecular masses of 115, 95, 89, 84 kDa by in situ gel kinase assay in an affinity-purified fraction by using recombinant NFH from mouse brain extract (51). The 115-kDa molecular mass of NAK is close to that of human PKC, which is 120 kDa (2). These investigators reported that NAKs phosphorylate all three NF subunits and that partial dephosphorylation of NF with alkaline phosphatase does not affect the phosphorylation of the NFH and NFM by NAKs. Thus PKN is similar in this respect. However, PKN bound to the head-rod domain of NFH, and NAKs were found on the basis of their retention by the tail domain of the NFH affinity column. Purification and information about molecular aspects of NAKs are expected. Dosemeci co-workers (49, 52) reported that bovine NF preparation contains known Ca^2+-dependent kinase, PKC, and PKA as well as activator-independent kinase activities. In vitro phosphorylation of NF at the amino-terminal domain by PKA or PKC inhibits the assembly process of NF and induces disassembly of preexisting filaments (53). In the present study, we revealed that PKN phosphorylated NF at the amino-terminal domain, was able to inhibit the assembly of NF in vitro, and that PKN was another candidate for being a regulator of NF assembly.

We reported here that the regulatory domain of PKN binds to the head-rod domain of NF and vimentin. What is the meaning of this association? Although additional evidence is required for determining whether PKN phosphorylates NF in vivo, one possible explanation for the interaction between the regulatory domain of PKN and NF is that an association interface outside the catalytic domain is required for substrate recognition and could serve to target PKN to physiologically important substrates. Precedence for protein association outside the conserved catalytic domain of protein kinase as an important targeting mechanism during signal transduction is provided by studies of Src homology domains (54–57) and the regulatory region of PKC (58, 59). In our preliminary experiment, some clones that were isolated by this two-hybrid screening were indicated to be phosphorylated in vitro by PKN. It is possible that PKN uses its regulatory domain as an association domain for all physiologically important substrates. An alternative possibility is that the binding of PKN to one substrate serves to promote the phosphorylation of other proteins in the same complex or that NF acts only as an adaptor that promotes the activity of PKN toward adjacent specific cytoskeletal targets.

We recently found that PKN directly binds to the GTP-bound form of Ras-related small GTPase Rho and is activated by this binding both in a cell-free system and in cultured cells (7, 8). Rho works as a molecular switch in diverse cellular processes such as stimulus-evoked cell adhesion (60–62), cell motility (63), regulation of smooth muscle contraction (64), and cytokinesis (65, 66). Rho was presented to exert these actions by inducing certain types of cytoskeletal structures (60, 65, 66). Paterson et al. (67) reported the collapse of the intermediate filament into irregular thick bundles after microinjection of constitutive activated Rho (Val^14Rho) into subconfluent Swiss 3T3 cells; however, the biochemical basis of the Rho effect is not known. From the data presented in this paper, PKN, positioned in the downstream of Rho signal, might regulate the assembly-disassembly of intermediate filaments.

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