



Interacting proteins for the serine/threonine protein kinase PKN family

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博士論文

Interacting proteins for the serine/threonine
protein kinase PKN family

(セリン/スレオニン蛋白質リン酸化酵素 PKN ファミリーの相互作用蛋白質)

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Abbreviations

bHLH, basic Helix-Loop-Helix
CG-NAP, centrosome- and Golgi-localized PKN-associated protein
CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CZ, charged amino acids with a leucine zipper-like sequence
DAG, diacylglycerol
DTT, dithiothreitol
GAP, GTPase-activating protein
GEF, guanine nucleotide exchange factor
GFAP, glial fibrillary acidic protein
GST, glutathione-S transferase
HA, hemagglutinin
IF, intermediate filament
MBP, maltose-binding protein
PAGE, polyacrylamide gel electrophoresis
PCR, polymerase chain reaction
PDGF, platelet-derived growth factor
PH, pleckstrin homology
PI4,5P2, phosphatidylinositol 4,5-bisphosphate
PKA, protein kinase A
PKC, protein kinase C
PMA, phorbol 12-myristate 13-acetate
PP1, protein phosphatase 1
PP2A, protein phosphatase 2A
PS, phosphatidylserine
RACE, rapid amplification of cDNA ends
RIPE3, rat insulin promoter element 3
SDS, sodium dodecyl sulfate
SH3, src homology 3
X- α -gal, 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside

Summary

The PKN family protein kinase comprising PKN α /PRK1/PAK1, PKN β , and PKN γ /PRK2/PAK2, constitutes a subclass of lipid and proteolysis activated serine/threonine protein kinase with a catalytic domain highly homologous to those of PKC family members in the carboxyl-terminal region. The amino-terminal region of the PKN family contains three repeats of the CZ region (charged amino acids and leucine-zipper like sequence, CZ1-CZ3) and the D region, and is assumed to restrict the protein kinase activity of the catalytic domain in the absence of activators. The small GTPase Rho binds to the amino-terminal region of PKN α and activates the protein kinase activity in a GTP-dependent manner. In the first step of this study, the yeast two-hybrid system and *in vitro* binding assay were employed to characterize the interaction between PKN α and Rho. It was revealed that the region corresponding to the amino acid residues from 33 to 111 in the amino-terminal region of PKN α was sufficient to confer the ability to associate with Rho. Each of synthetic peptide fragment corresponding to the amino acid residues from 74 to 93 and from 94 to 113 of PKN α inhibited the interaction between PKN α and Rho in *in vitro* binding assay, suggesting that this region is important in the association with Rho. The endogenous and the GTPase-activating protein stimulated GTPase activities of Rho were inhibited by the interaction with PKN α , suggesting the presence of a regulatory mechanism that sustains the GTP-bound active form of Rho.

Next, the yeast two-hybrid screening was carried out to identify the other compartments of the PKN family pathways such as substrates and regulatory proteins of the PKN family. Using the amino-terminal region corresponding to the amino acid residues from 1 to 540 of PKN α as bait, 15 different clones were isolated from human brain cDNA library. The responsible region for the interaction between PKN α and candidate proteins were determined by the yeast two-hybrid system and *in vitro* binding assay using the various truncated mutants of these proteins. The amino-terminal region of PKN α outside the Rho-binding domain was sufficiently shown to associate with actin cross-linking protein α -actinin, a novel 450-kDa coiled-coil protein CG-NAP

(centrosome and Golgi localized PKN-associated protein) and a paraneoplastic cerebellar degeneration-associated antigen PCD17. On the other hands, the whole of the amino-terminal region of PKN α required for the interaction with a neuron specific intermediate filament protein neurofilament-L and a basic Helix-Loop-Helix transcription factor NDRF/NeuroD2.

In vitro binding studies using the deletion mutants of NDRF/NeuroD2 indicated that the internal region containing the basic Helix-Loop-Helix domain of NDRF/NeuroD2 was necessary and sufficient for the interaction with PKN α . In addition, recombinant NDRF/NeuroD2 purified from *Escherichia coli* could bind PKN α , suggesting the direct interaction between NDRF/NeuroD2 and PKN α . Transient transfection assays using P19 cells revealed that expression of NDRF/NeuroD2 increased the transactivation of the rat insulin promoter element 3 (RIPE3) enhancer up to approximately 12-fold and that co-expression of catalytically active form of PKN α , but not kinase-deficient derivative, resulted in a further 3-fold increase of NDRF/NeuroD2-mediated transcription. These findings suggest that PKN α may contribute to transcriptional responses through the post-translational modification of the NDRF/NeuroD2-dependent transcriptional machinery.

Using the PKN β linker region containing proline-rich motifs as bait, the clones encoding Graf (GAP for Rho associated with focal adhesion kinase) and a novel Graf-related protein, termed Graf2, were isolated from human embryonic kidney 293 cell cDNA library. Full length of Graf2 contains putative PH domain, a RhoGAP domain, and a SH3 domain as well as Graf. Northern and Western blot analyses demonstrated that Graf2 is expressed in several tissues, with the highest expression in skeletal muscle. Recombinant Graf2 exhibited GTPase activating activity toward the small GTPase RhoA and Cdc42Hs but not toward Rac1 *in vitro*. The SH3 domains of Graf and Graf2 purified from *E. coli* bound directly to PKN β . Graf or Graf2 were co-immunoprecipitated with PKN β in COS-7 cells transiently transfected with Graf or Graf2 and PKN β expression constructs. The catalytic active form of PKN β phosphorylated Graf and Graf2 *in vitro*. The interplay of PKN β and GTPase activating protein, Graf and Graf2, may

offer a novel mechanism regulating the Rho-mediated signaling.

Introduction

Protein phosphorylation is one of the most important mechanisms for regulation in the eukaryotic cell. The protein kinases encoded for by the human genome alone are predicted to number over 500. The first protein kinase to be purified was phosphorylase kinase in 1959 (Krebs et al., 1959), followed in 1968 by another protein-serine kinase, the cAMP-dependent protein kinase (Walsh et al., 1968). In 1977, Protein kinase C (PKC) was defined as a histone kinase activity from rat brain which could be activated by limited proteolysis (Inoue et al., 1977). This was followed by the discovery that PKC could also be activated by phosphatidylserine (PS) and diacylglycerol (DAG) in a Ca^{2+} -dependent manner and also by tumour-promoting phorbol esters such as PMA (Nishizuka, 1984). PKC has been recognized as an important regulator of a variety of physiological processes. Some of these, such as adhesion, migration, morphology and cell-cell communication, primarily involve cytoskeletal remodeling whereas others, such as regulation of growth and gene expression, involve nuclear events.

Extensive molecular cloning analysis has defined a superfamily of mammalian PKC isotypes that comprises some eleven distinct genes (Fig. 1) (Nishizuka, 1992). The first PKCs to be identified and cloned were the α , β , and γ isotypes, which were isolated from brain cDNA libraries (Ono et al., 1986a; Ono et al., 1986b; Parker et al., 1986; Coussens et al., 1986). This tissue has proven to be a rich source of PKC isotypes, and low-stringency screening of brain cDNA libraries with probes derived from the α , β , and γ isotypes yielded three additional PKCs, the δ , ϵ , and ζ isotypes (Ono et al., 1987). Further low-stringency screens of other tissue cDNA libraries have delivered PKC η (Osada et al., 1990), PKC θ (Osada et al., 1992; Baier et al., 1993), and PKC ι/λ (Selbie et al., 1993; Akimoto et al., 1994). The known members of the PKC family all depend on PS, but show different lipid requirement for their activation (Nishizuka, 1995). As mentioned above, the conventional or classical PKCs (cPKC), which comprise the α , β I, β II, and γ isotypes (the PKC β gene is alternatively spliced to produce two gene products which differ only in their extreme carboxyl-terminal ends), are activated by PS in a Ca^{2+} dependent manner.

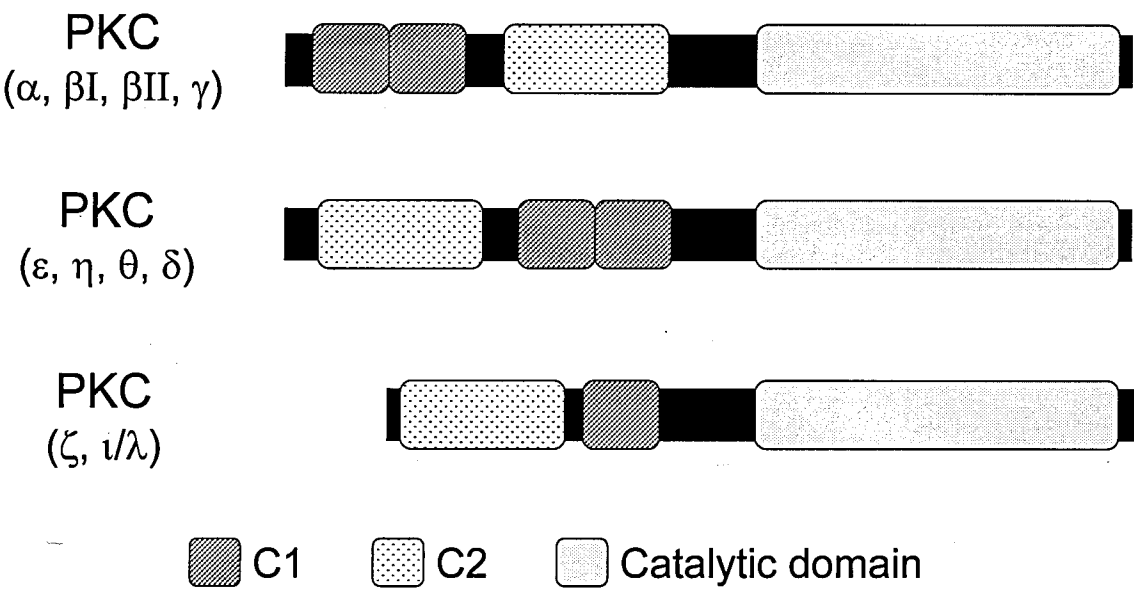


Figure 1 Domain structure of the PKC family

The Figure shows a comparison of the protein architecture of the various subgroups of the PKC family.

These cPKCs also bind DAG, which both increases the specificity of the enzyme for PS and also shifts the affinity for Ca^{2+} into the micromolar range (Takai et al., 1979). On the other hand, the novel PKCs (nPKC), which consist of δ , ϵ , η , θ , and μ isotypes, are Ca^{2+} -insensitive, but respond to DAG or phorbol esters in the presence of PS (Ono et al., 1988; Baier et al., 1993; Osada et al., 1992; Johannes et al., 1994). The atypical PKCs (aPKC), which consist of ζ and ι/λ isotypes, are Ca^{2+} -insensitive nor do they respond to PMA/DAG (Ono et al., 1989). The categorization of PKC isotypes based on their enzymic properties is identical with the relatedness of these kinases at the protein-sequence level. The protein-sequence alignments between PKC isotypes reveal the presence of blocks of homology between family members (Nishizuka, 1988; Nishizuka, 1995; Mellor and Parker, 1998). The amino-terminal half of the regulatory domain of cPKC isotypes contains two conserved regions, C1 and C2, which play a critical role in the regulation of enzymatic activity. The nPKC and aPKC isoforms lack the C2 region. The carboxyl terminal half of the enzyme contains two additional highly conserved regions, C3 and C4. The C3 region contains the ATP-binding consensus sequence, and the C4 region is responsible for protein substrate binding.

In 1994, Mukai and Ono isolated a cDNA for a novel serine/threonine protein kinase, designated PKN, from a human hippocampus cDNA library by utilizing the catalytic domain of PKC β II as a probe (Mukai and Ono, 1994). PKN is widely distributed in tissues and organs of mammals and other organisms (Kitagawa et al., 1995; Mukai et al., 1995). The catalytic half of PKN is highly similar to the catalytic domain of PKC family members and PKN efficiently phosphorylates PKC δ peptide (corresponding to amino acids 137-153 of PKC δ , substituting Ser for Ala147) *in vitro* (Mukai et al., 1994; Kitagawa et al., 1995). The amino-terminal region of PKN contains repeats of charged amino acids and leucine-zipper like sequence (designated CZ region) (Yoshinaga et al., 1999), suggesting protein-protein interaction. PKN is cleaved at specific sites in apoptotic Jurkat and U937 cells on Fas ligation and treatment with staurosporin or etoposide, respectively (Takahashi et al., 1998). The cleavage is indicated to be catalyzed by

caspase-3 or related proteases. The major proteolysis took place between the amino-terminal region and the carboxyl-terminal catalytic domain, and it generated a constitutively active kinase fragment, indicated that the amino-terminal region restricts the protein kinase activity. The catalytic activity of PKN is also stimulated by lipid and fatty acid such as arachidonic acid (Mukai et al., 1994; Kitagawa et al., 1995). In 1996, Amano et al. and Watanabe et al. reported that PKN is one of the targets for small GTPase Rho. GTP-bound form of Rho specifically binds the amino-terminal region of PKN and activates the protein kinase activity (Amano et al., 1996; Watanabe et al., 1996).

Like PKC, PKN makes a family that comprises at least three gene products (Figure 2). The cDNA encoding PKN β and PKN γ /PRK2 are isolated by PCR with redundant primers designed to the amino acid sequences of the catalytic domain of PKN and PKC, respectively (Originally isolated PKN was designated as PKN α) (Oishi et al., 1999; Palmer et al., 1995b). The comparison of the primary sequences of PKN α from various organisms (Kitagawa et al., 1995) and the three members of PKN family revealed that three CZ regions, a D region that located at carboxyl adjacent of third CZ region, and carboxyl-terminal catalytic domain are highly conserved (Mukai and Ono, 1994; Oishi et al., 1999; Palmer et al., 1995b). This has allowed us to refine our understanding of the domain structure of the PKN family and has given valuable insight into the many regulatory elements present in these proteins. Yoshinaga et al. reported that the carboxyl-terminal part of the D region of PKN α (designated I α fragment) selectively inhibits the catalytic activity of PKN α and PKN γ /PRK2, and that arachidonic acid abrogates the inhibitory effect of I α (Yoshinaga et al., 1999). These results indicate that this region functions as fatty acid-sensitive autoinhibitory domain of the PKN family. Since the conserved CZ regions contain leucine-zipper like sequences, it is considerable that the interacting partners of these regions of the PKN family regulate the protein kinase activities and/or subcellular localization of these enzymes or are phosphorylated by PKNs as physiological substrates. Further, the sequences of the linker region of the PKN family between the D region and the

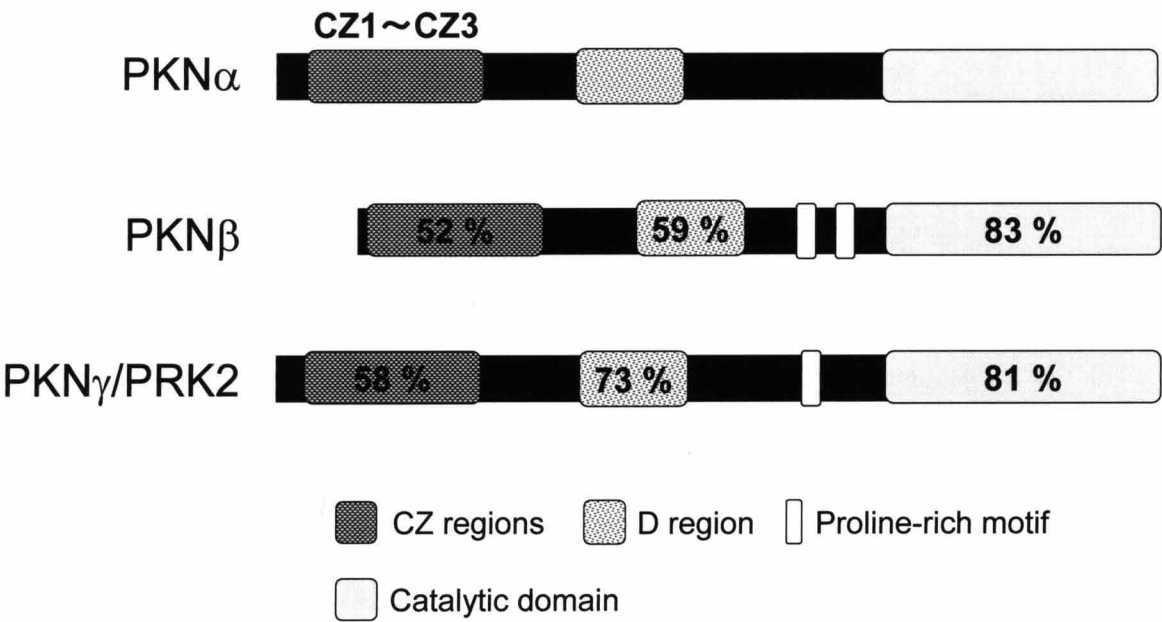


Figure 2 Domain structure of the PKN family

The Figure shows a comparison of the protein architecture of the various subtypes of the PKN family. All subtypes of the PKN family contain three copies of the CZ region. The percentage identities of the respective regions to their counterparts in PKN α are shown underneath.

carboxyl-terminal catalytic domain are structurally divergent, in which PKN β and PKN γ /PRK2 possesses proline-rich motifs (Oishi et al., 1999; Quilliam et al., 1996). It is possible that the linker regions of the PKN family function as the interaction interfaces of the specific partners and these interactions promote differential regulation of the PKN family.

The present studies focuses on the interacting proteins for the PKN family that has been obtained from the yeast two-hybrid system using the various regions of the PKN family as bait, and discusses the possibilities that the PKN family protein kinases are involved in the various cellular signaling pathways.

Materials and Methods

Materials and chemicals

[γ -³²P]GTP (1.11 TBq/mmol) was purchased from DuPont-New England Nuclear. Nitrocellulose filters (BA-85, 0.45 μ m pore size) were obtained from Schleicher & Schuell. Synthetic peptide fragments for potential RhoA binding region of PKN α were synthesized with an automated peptide synthesizer (Applied Biosystems, model 431). All other chemicals were from commercial sources.

Yeast Strain and Cell Culture

For the two-hybrid system, the yeast strain YGH1 (*a*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *Can^r*, *gal4-542*, *gal80-538*, *LYS2::gal1_{uas}-gal1_{tata}-HIS3*, *URA3::gal1-lacZ*), L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*), and AH109 (*MATa*, *ura3-52*, *his3-200*, *trp1-901*, *leu2-3*, *112*, *gal4 Δ* , *gal80 Δ* , *Lys::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*) were used. COS-7 and P19 cells were grown in DMEM containing 10 % heat-inactivated fetal bovine serum, 50 units/ml penicillin and 50 μ g/ml streptomycin at 37 °C in a humidified 5 % CO₂ atmosphere.

Plasmid Constructs

The following plasmids were constructed for use in the yeast two-hybrid system. pGBT9-p53 (amino acid residues 72-390, pVA3) and pGAD3F-largeT (amino acid residues 84-708, pTD1) were purchased from Clontech. The cDNA fragments encoding various length of human PKN α were inserted into the vector pBTM116 in frame with the upstream LexA DNA-binding domain sequences, the vector pGBT9 and pGBKT7 in frame with the upstream Gal4 DNA-binding domain sequences, or the vector pVP16 in frame with the upstream VP16 transcription activation domain sequences. The cDNA fragments encoding human RhoA, RhoA^{Val14}, and RhoA^{Ala37} were obtained by digesting pGEX-RhoA, pGEX-RhoA^{Val14}, and

pGEX-RhoA^{Ala37} (Amano et al., 1996), respectively, then cloned into pBTM116 and pVP16. The cDNA fragment for human RhoA lacking the carboxyl-terminal lipid modification site (amino acid residues 190-193), which was designated "RhoA Δ C", was prepared by PCR and ligated into pBTM116 and pVP16. The 5' end of human NDRF/NeuroD2 was isolated by polymerase chain reaction (PCR) with the human brain cDNA Matchmaker library in the pGAD10 vector (Clontech) as the template and the primers hND2FP2 (5'-TTGAATTCATGCTGACCCGCCTGTT-3') and hND2RP1 (5'-GCGAATTCCGAGAGCGCCCAGATATA-3'). The PCR product digested with *Eco*RI was ligated into the *Eco*RI site of pBluescript II SK(+) to generate pBSIISK(+)-NDRF/NeuroD2 (amino acid residues 1-175). The fidelity of the constructs was confirmed by sequencing. The cDNA fragment encoding the full-length NDRF/NeuroD2 was inserted into the vector pBTM116 or pGAD10. The cDNA fragments encoding the PKN α linker region (amino acid residues 511-632) and the PKN β linker region (amino acid residues 465-557) were fused in-frame to the carboxyl-terminus of the Gal4 DNA-binding domain in vector pGBKT7.

The following plasmids were constructed for use in mammalian cell transfections. pMhPKN3, for expression of the full-length of human PKN α , was described previously (Mukai and Ono, 1994). pRc/CMV-AF3 and pRc/CMV-AF3 K644E, for expression of FLAG-tagged catalytically active form of PKN α (amino acid residues 561-942) and kinase-deficient mutant of PKN, respectively, were constructed as described previously (Takahashi et al., 1998). pRc/CMV/hPKN β /FLAG, for FLAG-tagged full-length of PKN β was constructed as described previously (Oishi et al., 1999). The cDNA fragment encoding the full-length NDRF/NeuroD2 was inserted into the vector pTB701FLAG (Kuroda et al., 1996). To detect the NDRF/NeuroD2-dependent transcriptional activation, the following plasmids were constructed. pGL3-ova contains the minimal promoter sequence of the chicken ovalbumin gene from -50 to +43, fused to the luciferase reporter gene of pGL3-Basic vector (Promega). pGL3-4xRIPE3-ova was constructed by inserting four tandem repeats of the oligonucleotide

covering the RIPE3 (-126 to -86) in the upstream of the chicken ovalbumin minimal promoter of pGL3-ova (Hwung et al., 1990). The mouse NDRF/NeuroD2 and NeuroD cDNAs (Yasunami et al., 1996) was subcloned into pCI-neo mammalian expression vector (Promega). pCI-neo-T7mNDRF/NeuroD2 contains an epitope-tagged derivative of NDRF/NeuroD2 sequence, which codes for a 12-amino acid peptide of the bacteriophage T7 gene 10 protein fused to the amino-terminus (Leu2) of the mouse NDRF/NeuroD2 (Yasunami et al., 1995).

The full-length cDNA of human Graf2 was obtained by 5'-RACE (5' rapid amplification of cDNA ends) using Marathon-Ready HeLa cell cDNA according to the manufacturer's instruction (Clontech). The full-length cDNA of human Graf was isolated from HeLa cell cDNA library by PCR.

The following plasmids were constructed for use in the expression of maltose binding protein (MBP) fusion proteins in *E. coli*. The cDNA fragments encoding human PKN α (amino acid residues 7-155), human NDRF/NeuroD2 (amino acid residues 120-381), and human PKN β (amino acid residues 301-610) were inserted into the vector pMAL-c2 (New England Biolabs).

The following plasmids were constructed for use in the expression of glutathione-S transferase (GST) fusion proteins in *E. coli*. The cDNA fragments encoding the amino-terminal region of human PKN α (amino acid residues 1-540), the carboxyl-terminal region containing the SH3 domain of human Graf (amino acid residues 596-759), the SH3 domain of human Graf2 (amino acid residues 734-786), the RhoGAP domain of human Graf (amino acid residues 375-583) and the RhoGAP domain of human Graf2 (amino acid residues 381-587) were inserted into the vector pGEX4T (Amersham Pharmacia Biotech). The plasmids containing the GST-RhoA, GST-Rac1, and GST-Cdc42Hs constructs were kindly provided from Dr. Kozo Kaibuchi (Nagoya University).

The following plasmids were constructed for use in the expression of 6xHis-tagged protein in *E. coli*. The cDNA fragment encoding the SH3 domain of human Graf2 (amino acid residues 734-786) was cloned into pRSET (Invitrogen).

The following plasmids were constructed for use in *in vitro* translation. The cDNA fragments encoding the various length of human NDRF/NeuroD2 (Fig. 9) were inserted into the vector pGBKT7 (Clontech).

The following plasmids were constructed for use in the expression in Sf9 cells. The cDNA fragment encoding the full-length of human Graf was cloned into the *Bam*HI/*Not*I site of pAcGHLT-C (PharMingen). The PKA phosphorylation site of pAcGHLT-C was eliminated in this construct. The cDNA fragment encoding the full-length of human Graf2 was cloned into pBlueBacHis/GST (Yoshinaga et al., 1999). The cDNA fragment encoding the catalytic domain of human PKN β (amino acid residues 520-889) was cloned into pAcGHLT-C.

Two-hybrid system

For the interaction assay, different combinations of the baits and the preys were co-transformed into L40 cells and plated on selection medium lacking leucine and tryptophan. The filter lift assay for the β -galactosidase activity was performed according to the manufacturers' protocols (Clontech).

Screening of the associated proteins with the amino-terminus of human PKN α (amino acid residues 1-540) was performed as described by Hannon et al. (Hannon et al., 1993). Primary positive clones were recovered and retransfected into the original yeast host strain YGH1 in combination with DNA binding domain of Gal4 (Gal4bd)-PKN α (amino acid residues 1-540) or Gal4bd-p53 tumor suppresser (amino acid residues 72-390). Library plasmids that activated marker expression only in the presence of PKN α were sequenced and the obtained sequences were analyzed using the BLAST program on the World Wide Web.

Screening of the associated proteins with the linker region of human PKN β (amino acid residues 465-667) was performed as follows: Gal4bd-linkPKN β was used to transform the yeast reporter strain AH109 together with human kidney embryonic 293 cell library cDNA cloned carboxyl-terminal to the activation domain of Gal4 in vector pACT2 (Clontech). A total of 1.5

$\times 10^6$ transformants were plated on synthetic complete plates lacking tryptophan, leucine, histidine and adenine (SD/-Trp/-Leu/-His/-Ade), but supplemented with 15 mM 3-amino-1,2,4-triazole (3-AT, Sigma) and X- α -gal (Wako). After 5 days of incubation at 30 °C, 6 of growing clones were picked and streaked on SD/-Trp/-Leu/-His/-Ade plate containing 5 mM 3-AT and X- α -gal. The pACT2 library plasmids were isolated from *His*⁺, *Ade*⁺, *Mell*⁺ clones and retransformed into the original yeast host strain in combination with the Gal4bd-linkPKN β , Gal4bd-linkPKN α , or Gal4bd-p53 tumor suppresser (pGBKT7-53, Clontech). At least 5 independent yeast colonies on SD/-Trp/-Leu plate were streaked onto SD/-Trp/-Leu/-His/-Ade plate containing 5 mM 3-AT and X- α -gal. The plates were incubated at 30 °C and examined for growth (histidine and adenine prototrophy) and blue color development (*Mell* gene activation). Library plasmids that activated marker expression only in the presence of PKN β were sequenced and the obtained sequences were analyzed using the BLAST program on the World Wide Web.

Preparation of recombinant proteins

His-tagged proteins, GST fusion proteins, or MBP fusion proteins were expressed in *E. coli* and purified by nickel-NTA agarose (Qiagen), glutathione-Sepharose-4B (Amersham Pharmacia Biotech) or amylose resin (New England Biolabs), respectively, according to the manufacturers' protocols. The expression and purification of the recombinant proteins in Sf9 cells was performed as follows: Each transfer vector was co-transfected with BacVector-2000 Triple Cut Virus DNA into Sf9 cells. The isolation and amplification of the baculovirus, and the expression and purification of GST fusion proteins in Sf9 cells were performed as described previously (Yoshinaga et al., 1999). The purified protein was dialyzed against dialysis buffer (50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂). *In vitro* translation was performed in the presence of [³⁵S]methionine (DuPont/NEN) with the T7-coupled reticulocyte lysate system according to the manufacturer's instruction (TNT; Promega).

Antibodies

Mouse anti-HA 12CA5 (Roche Molecular Biochemicals), anti-FLAG M2 (Eastman Kodak), and peroxidase-conjugated secondary antibodies (Santa Cruz) were purchased. The anti-PKN α monoclonal antibody, PRK1/PKN, was purchased from Transduction Laboratories. The polyclonal antisera against PKN α , α N2 and α C6, were previously described (Mukai et al., 1994; Mukai et al., 1996a)

The polyclonal antibody, α G2SH3, was raised against the SH3 domain of Graf2. Purified His-tagged protein (His-Graf2SH3) from *E. coli* was resolved by SDS-PAGE. The corresponding band was visualized by staining with Coomassie Brilliant Blue, excised from the gel, and used to immunize rabbits. His-Graf2SH3 coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech) were used to affinity-purify antibody from the whole serum.

In vitro binding assay

The analysis of the interaction between PKN α and RhoA was performed as follows: fifty nM of the guanine nucleotide bound form of RhoA fused to GST was incubated with 1.5 μ l of *in vitro* translated PKN α in a total volume of 200 μ l of RhoA-binding buffer (20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 0.5 mM DTT, 5 mM MgCl₂ and 1 μ g/ml leupeptin) for 60 min at 4°C. Then 40 μ l of glutathione-Sepharose beads preincubated in binding buffer containing 10 mg/ml *E. coli* extracts to block non specific binding (Lee et al., 1995), were added and rotated for 30 min at 4°C. Unbound proteins were removed by four washes with RhoA-binding buffer containing 0.2 % Nonidet P-40 and 50 mM NaCl, and by two washes with RhoA-binding buffer. Specifically bound proteins were eluted with binding buffer containing 10 mM reduced glutathione, subjected to SDS-PAGE, and to autoradiography. Experiments with synthetic peptides were performed using the various concentrations of peptide in 200 μ l of the binding mixture.

The analysis of the interaction between PKN α and NDRF/NeuroD2 was performed as

follows: two μg of GST-PKN α or GST mixed with 3 to 7 μl of the respective *in vitro* translation reaction mixture in 200 μl of NDRF-binding buffer (20 mM Tris/HCl at pH 7.5, 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM DTT) and incubated for 3 h at 4 °C. After addition of 30 μl of glutathione-Sepharose 4B pretreated with 10 mg/ml of *E. coli* extracts to block nonspecific binding, the binding reaction was continued for additional 30 min at 4 °C. The glutathione-Sepharose 4B was washed three times with NDRF-binding buffer. Then bound proteins were eluted with GST elution buffer (50 mM Tris/HCl at pH 8, 10 mM glutathione, 1 mM EDTA, 1 mM DTT) and subjected to SDS-PAGE. The radioactive bands were visualized using an imaging analyzer FUJI BAS1000. For direct binding studies, 2 μg of GST-PKN α mixed with 2 μg of MBP-NDRF/NeuroD2 (amino acid residues 120-381) or MBP in 200 μl of NDRF-binding buffer and incubated for 3 h at 4 °C. After addition of 30 μl of amylose resin pretreated with 10 mg/ml of *E. coli* extracts to block nonspecific binding, the binding reaction was continued for additional 30 min at 4 °C. The amylose resin was washed three times with NDRF-binding buffer. Then bound proteins were eluted with MBP elution buffer (20 mM Tris/HCl at pH 7.5, 10 mM maltose, 1 mM EDTA, 1 mM DTT), subjected to SDS-PAGE and then immunoblotted with anti-PKN α antisera, αC6 . The immunoreactive bands were visualized with Enhanced Chemiluminescence immunoblotting detection kit.

The analysis of the interaction between PKN β and Graf or Graf2 was performed as follows: GST fusion and MBP fusion protein was mixed in Graf-binding buffer (20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5 % Triton X-100, 1 $\mu\text{g}/\text{ml}$ leupeptin) and incubated for 2 hours at 4 °C. Glutathione-Sepharose 4B or amylose resin was subsequently added, followed by further incubation for 1 hour at 4 °C. After extensively washing the resin with Graf-binding buffer, proteins were eluted with SDS sample buffer. The eluates were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

Co-immunoprecipitation assays

COS-7 cells were transfected with the expression plasmids by electroporation using GenePulser II (Bio-Rad). After 2 days, cells were lysed in lysis buffer (20 mM Tris/HCl at pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 % Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 µg/ml leupeptin). Cleared lysate were incubated with appropriate antibody at 4 °C for 2 hour, then protein G-Sepharose (Amersham Pharmacia Biotech) was added and the reaction was continued for a further 1 hour. After extensively washing the resin with the same buffer, the bound proteins were subjected to SDS-PAGE and then immunoblotted with appropriate antibody. The immunoreactive bands were visualized with Enhanced Chemiluminescence immunoblotting detection kit (Amersham Pharmacia Biotech).

GTPase assay

Assay for the inhibitory effects of the amino-terminal region of PKN α on the GTPase activity of RhoA was carried out as follows: GST-RhoA (2 µM) was incubated with 5 µM of [γ -³²P]GTP (1.11 TBq/mmol) in 50 mM Tris/HCl at pH 7.5, 10 mM EDTA, 1 mM MgCl₂, 50 mM NaCl, 1 mM DTT, 0.3 % CHAPS for 10 min at 30°C. The exchange reaction was stopped by placing the reaction mixture on ice, followed by addition of MgCl₂ to a final concentration of 10 mM. The [γ -³²P]GTP-loaded RhoA fused to GST or RhoA^{Val14} fused to GST (0.2 µM) was incubated at 30°C with either the presence or the absence of PKN α (amino acid residues 1-540) fused to GST in 60 µl of hydrolysis buffer (50 mM Tris/HCl at pH 7.5, 5 mM MgCl₂, 1 mM DTT, 1 mM GTP and 0.1 mg/ml bovine serum albumin). The reaction was stopped by the addition of about 2 ml of ice-cold 20 mM Tris/HCl at pH 7.5 containing 100 mM NaCl and 25 mM MgCl₂, followed by rapid filtration on a nitrocellulose filter. The filter was washed three times with the same ice-cold buffer. The radioactivity collected on the filter was determined. The p122 RhoGAP-stimulated GTPase activity of RhoA was also assayed by measuring the decreased radioactivity of [γ -³²P] GTP bound form of RhoA fused to GST for 2 min at 25°C in the presence

of 50 nM of p122 RhoGAP fused to GST (Homma and Emori, 1995) and the various concentration of PKN α (amino acid residues 7-155) fused to MBP (Amano et al., 1996) in 100 μ l of reaction mixture (20 mM Tris/HCl at pH 7.5, 10 mM EDTA, 1 mM DTT, 10 mM MgCl₂, 1 mM GTP, 0.075% CHAPS, 0.25 mM L- α -dimyristoylphosphatidylcholine, 100 nM [γ -³²P] GTP bound form of RhoA fused to GST) (Homma and Emori, 1995).

Assay for the GAP activity of Graf and Graf2 was carried out as follows: GST-RhoA, GST-Rac1, and GST-Cdc42Hs (400 nM) were incubated at 30 °C with [γ -³²P] GTP (1.11 TBq/mmol) in 20 μ l of GTP binding buffer (20 mM Tris-HCl at pH 7.5, 10 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 0.3 % CHAPS, 1 μ M GTP), respectively, and the nucleotide exchange was stopped after 10 min by the addition of MgCl₂ (final concentration of 10 mM). Preloaded GTPases (final concentration, 100 nM) were diluted with 60 μ l of GAP buffer (20 mM Tris-HCl at pH 7.5, 10 mM EDTA, 1 mM DTT, 10 mM MgCl₂, 1 mM GTP) and each GAP protein was added to the reaction mixture. Following incubation at 25 °C, the reaction was terminated by the addition of 2 ml of ice-cold stop buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 25 mM MgCl₂) and the mixture was filtered through a nitrocellulose membrane (Schleicher & Schuell). The membrane was washed three times with ice-cold stop buffer and the radioactivity collected on the membrane was determined.

Luciferase Assays

For the transient transfection assays, the plasmid DNAs were transfected using a cationic liposome-mediated method. Approximately 1×10^5 P19 cells were seeded in 35 mm dishes and grown for 24 hour. Cells were washed with serum free medium and then overlaid with 1 ml of transfection mixture containing 4.5 μ l of Tfx-50 reagent (Promega), 1.0 μ g of pGL3 reporter plasmid and 1.0 μ g of pCI-neo expression vector plasmid for the experiments in Fig. 10A. For the experiments including PKN α expression plasmids in Fig. 10B, 0.7 μ g each of pGL3 reporter, pCI-neo expression vector and PKN α expression vector plasmids were used. After a 1 hour of

incubating period, 1 ml of complete medium was added to the dishes, which were then grown for a further 23 hour. The cells were lysed in 200 µl of PicaGene reporter lysis buffer and luciferase assay was performed by mixing 20 µl of cell lysate with 100 µl of PicaGene assay solution according to the manufacturer's instruction (Toyo ink). Luciferase activity was measured by using a Berthold luminometer. Transfection experiments were repeated independently at least three times.

Analysis of Graf2 mRNA expression

Multiple tissue Northern blot (Human MTN blot, Clontech) was hybridized with the ³²P-labeled *EcoRV/EcoRI* fragment of human Graf cDNA (1318-2388) or *EcoRI/BglII* fragment of human Graf2 cDNA (1336-2361), followed by extensive washing. Blot was exposed on an imaging plate, which was then examined using a BAS1000 imaging analyzer (Fuji Film).

Analysis of Graf2 protein expression

Rat tissues were removed quickly after decapitation, and homogenized in the 10 volume of RIPA buffer (50 mM HEPES-NaOH at pH 7.2, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EDTA, 2 µg/ml leupeptin, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS) with 10 strokes of a Teflon/glass homogenizer. After centrifugation at 100,000 x g for 1 hour at 4 °C, the supernatant was precleared on protein A-Sepharose 4B (Amersham Pharmacia Biotech). Cleared lysate was incubated for 2 hour at 4 °C in the presence of αG2SH3 antibody. Protein A-Sepharose 4B was subsequently added and followed by further incubation for 1 hour at 4 °C. After washing three times with RIPA buffer, proteins eluted with SDS sample buffer were resolved by SDS-PAGE and electrotransferred onto Immobilon-P membrane (Millipore). The membrane was incubated with αG2SH3 antibody in RIPA buffer for 1 hour at room temperature. Immunoreactive bands were visualized with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech).

Protein kinase assays

The phosphorylation of Graf or Graf2 was carried out at 30°C in a kinase assay mixture (20 µl) containing 20 mM Tris-HCl at pH 7.5, 8 mM MgCl₂, 100 µM ATP, 185 kBq of [γ -³²P]ATP, 5 µg/ml of phosphoacceptors, and 0.5 µg/ml of the constitutive active form of PKN β fused to GST (GST-PKN β cat). After incubation for the indicated time, the reaction was terminated by the addition of an equal volume of SDS sample buffer and separated on SDS-PAGE. The gel was dried under vacuum and phosphorylation was visualized by a BAS1000 imaging analyzer (Fuji Film).

Results

CZ1 region of PKN α interacts with GTP-bound form of Rho GTPase.

Amano et al. and Watanabe et al. have shown that PKN α forms a complex with the GTP-bound form of RhoA but not with the GDP-bound form (Amano et al., 1996; Watanabe et al., 1996). To characterize the association between PKN α and RhoA further, the yeast two-hybrid system was carried out. The amino-terminal regulatory region of human PKN α fused to the LexA DNA binding domain (LexAbd) was co-expressed with human RhoA or RhoA mutants fused to the VP16 activation domain (VP16ad) in yeast strain L40. As shown in Fig. 3A, the β -galactosidase activity was obviously induced in the transformants expressing PKN α and RhoA^{Val14}, a GTPase-defective mutant (Ridley and Hall, 1992), whereas it was detected in neither the transformants expressing PKN α and wild type RhoA nor the transformants expressing PKN α and RhoA^{Ala37}, an effector domain mutant (Amano et al., 1996; Paterson et al., 1990). The Rho family members have a CAAX motif (C, cysteine; A, aliphatic acid; X, any amino acid) in their carboxyl-terminal region, and undergo post-translational modifications including geranylgeranylation, proteolysis, and carboxyl-methylation (Adamson et al., 1992; Katayama et al., 1991). To prevent association of RhoA fusion proteins with membranes, RhoA mutants designated “ Δ C”, which lack the carboxyl-terminal lipid modification site, were tested by the yeast two-hybrid system. PKN α interacted with RhoA Δ C and with RhoA^{Val14} Δ C, though did not interact with RhoA^{Ala37} Δ C. Similar results were obtained from the combination of RhoA fused to LexAbd and PKN α fused to VP16ad (Fig. 3A). Thus, the specific interaction between activated RhoA and PKN α was supported by the two-hybrid system, and these results suggest that the CAAX motif of RhoA is not required for the interaction with PKN α .

To identify the binding region of PKN α for RhoA, the various expression plasmids for the truncated PKN α were transfected with RhoA^{Val14} Δ C into yeast cells. As shown in Fig. 3B, RhoA^{Val14} Δ C interacted with the region corresponding to the amino acid residues from 33 to 111 of PKN α in the yeast two-hybrid system. Since this region of PKN α contains the CZ1 region, this

motif may have some roles in the interaction between PKN α and RhoA.

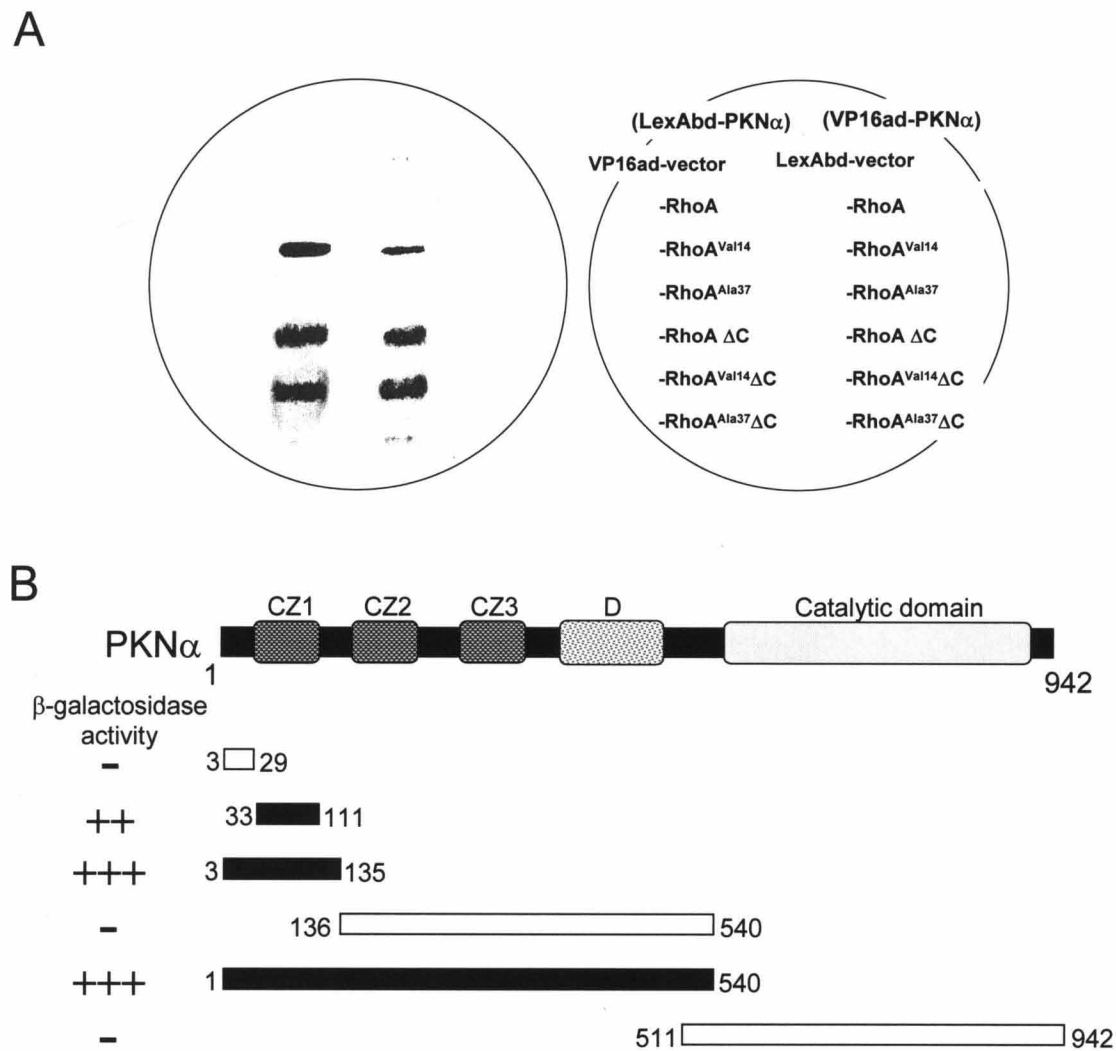


Figure 3 Interaction of PKN α with RhoA in the yeast two-hybrid system
 Yeast L40 cells were cotransfected with expression vectors encoding various LexA DNA-binding domain (LexAbd) and VP16 transcription activation domain (VP16ad) fusion protein. Interaction was examined using a filter lift assay for the β -galactosidase activity. **(A)** Specificity of the interaction of the amino-terminal regulatory region of PKN α with wild type or mutants of RhoA. “PKN α ” indicates the amino-terminal regulatory region of PKN α (amino acid residues 1-540). “ Δ C” indicates the deletion mutant which lacks the carboxyl-terminal lipid modification site. **(B)** Identification of the RhoA binding region of PKN α . Schematic whole structure of PKN α is represented at the top of the figure, and the deletion mutants are aligned below. “CZ” indicates charged amino acid-rich region with leucine-zipper-like sequences. “+++” and “++” indicate the development of strong color within 20 and 60 min from initiation of the assay, respectively. “-” indicates no development of color within 24 hours. The results shown are representative of three independent experiments.

Next, we measured the binding between *in vitro* translated PKN α and the GTP γ S-bound form of RhoA fused to GST in the presence of synthetic peptides corresponding to the various amino-terminal regions of PKN α . As shown in Fig. 4A, in the absence of peptide, *in vitro* translated PKN α bound specifically to the GTP γ S-bound form of RhoA fused to GST. Each of synthetic peptide fragments corresponding to the amino acid residues from 74 to 93 and from 94 to 113 of PKN α inhibited the binding of PKN α to the GTP γ S-bound form of RhoA in a concentration-dependent manner (Fig. 4B). These results suggest that the region corresponding to the amino acid residues from 74 to 113 of PKN α is critical for this interaction.

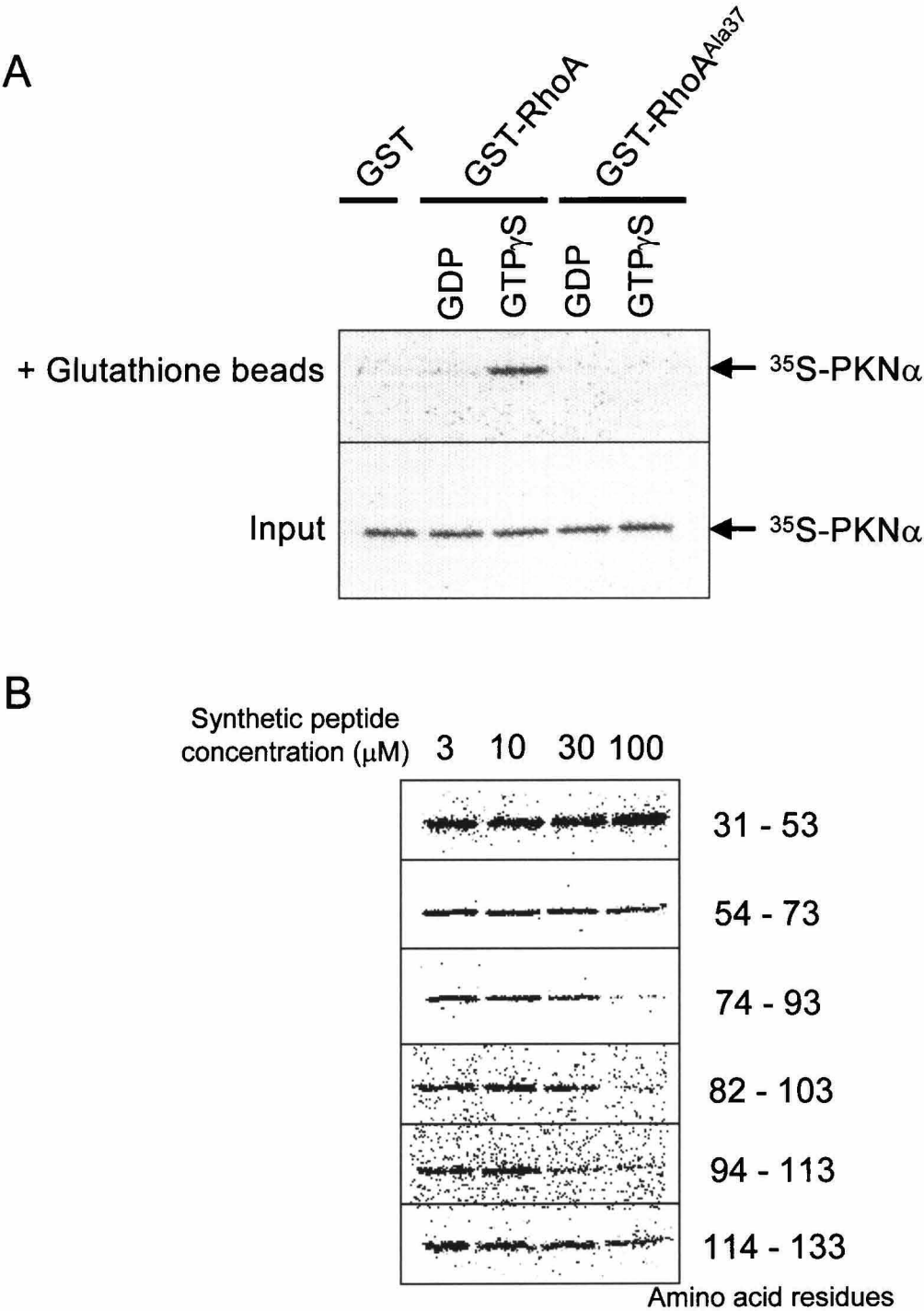


Figure 4 *In vitro* interaction of the amino-terminal regulatory region of PKN α with RhoA. *In vitro* translated PKN α (amino acid residues 1-474) and GST fusion proteins were incubated with glutathione-Sepharose beads, washed, and subjected to SDS-PAGE and to autoradiography, as described in the “**Materials and methods**”. (A) Specific interaction between the amino-terminal regulatory region of PKN α and the GTP γ S-bound form of RhoA. (B) Effects of the various synthetic peptide fragments on the interaction between PKN α and RhoA. The GTP γ S-bound form of RhoA fused to GST was incubated with *in vitro* translated PKN α in the presence of the indicated concentration of the peptides for potential RhoA binding region of PKN α . The results shown are representative of three independent experiments.

The amino-terminal region of PKN α inhibits the intrinsic and GAP-stimulated GTPase activity of RhoA.

Several protein kinases, such as the activated Cdc42Hs-associated kinase p120^{ACK} and the p21-(Cdc42/Rac) activated kinase p65^{PAK}, which bind to the active form of small GTPases, have been found to inhibit the endogenous and the GAP-stimulated GTPase activity of these small GTP binding proteins (Manser et al., 1993; Manser et al., 1994). These effects of target protein kinases on the GTPase activity are important for signal duration. To determine whether the GTPase activity of RhoA could be effected by the binding to PKN α , the residual bound radioactivity of RhoA fused to GST or RhoA^{Val14} fused to GST preloaded with [γ -³²P]GTP was measured in the presence or the absence of PKN α . As shown in Fig. 5A, the half-life of GTP bound to RhoA fused to GST was 12 min, while that of RhoA^{Val14} fused to GST was increased to greater than 100 min. The addition of the amino-terminal region of PKN α (amino acid residues 1-538) fused to GST increased the half-life of GTP bound to RhoA fused to GST from 12 to more than 20 min at 30°C, but the addition of GST alone did not affect the GTP hydrolysis rate. These results indicate that the amino-terminal region of PKN α has an activity to inhibit the endogenous GTPase activity of RhoA. This inhibition was dependent on the concentration of PKN α (Fig. 5B). As shown in Fig. 5C, the amino-terminal region of PKN α fused to MBP (amino acid residues 7-155) inhibited p122 RhoGAP-stimulated GTPase activity of RhoA fused to GST in a concentration-dependent manner, suggesting that the amino-terminal region of PKN α inhibit the interaction of GAP with RhoA. Rhotekin, a putative target for Rho, bearing a homologous sequence to the amino-terminal region of PKN α , inhibits both the endogenous and the GAP-stimulated GTPase activity of RhoA (Reid et al., 1996), suggesting this conserved Rho binding motif is implicated in the regulation of the GTPase activity.

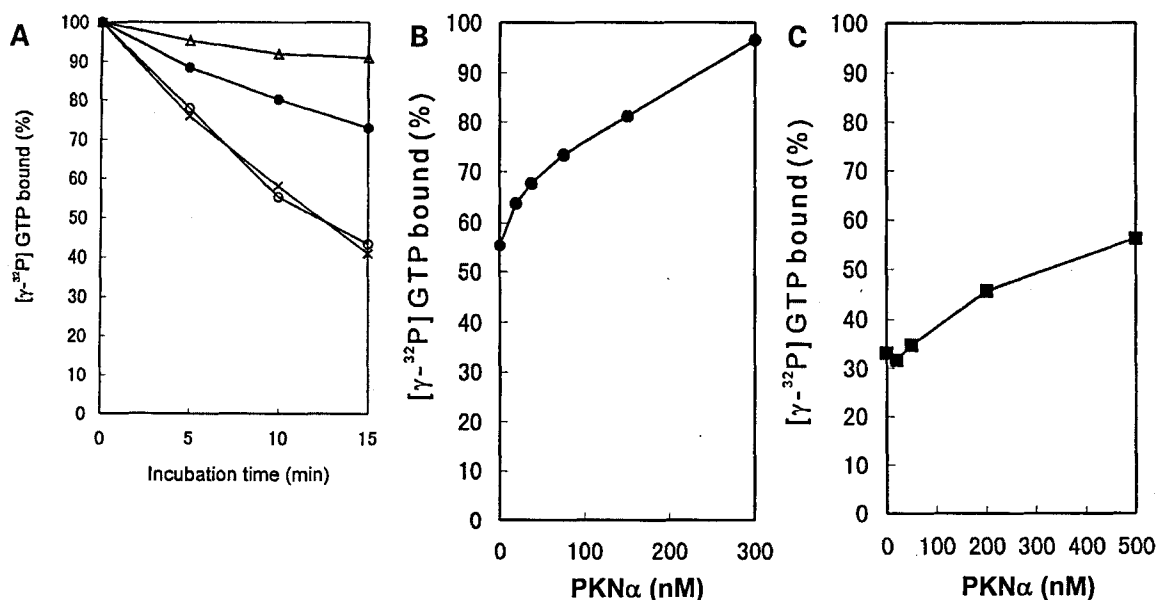


Figure 5 Effects of PKNα on the GTPase activity of RhoA

(A) Effects of added protein on the endogenous GTPase activity of RhoA. Two hundred nM of [γ-³²P] GTP-bound form of RhoA (●, ○, ×) or RhoA^{val14} (△) were incubated with (●) or without (○) 150 nM of the amino-terminal region of PKNα fused to GST or incubated with GST alone (×) for indicated time at 30 °C, and the residual bound radioactivity was determined by a filter binding assay. The remaining [γ-³²P] GTP bound to each protein was expressed as a percent of that measured at 0 min of incubation. (B) Dose dependent effect of PKNα. Two hundred nM of [γ-³²P] GTP-bound form of RhoA fused to GST was incubated for 10 min with the various concentration of the amino-terminal region of PKNα fused to GST. The residual bound radioactivity of RhoA fused to GST preloaded with [γ-³²P]GTP was measured as described in the legend to (A). (C) Effects of PKNα on the GAP-stimulated GTPase activity of RhoA. One hundred nM of [γ-³²P] GTP-bound form of RhoA fused to GST was incubated for 2 min at 25°C in the presence of 50 nM of p122 Rho GAP fused to GST and the various concentration of the amino-terminal region of PKNα fused to MBP. The residual bound radioactivity of RhoA fused to GST preloaded with [γ-³²P]GTP was measured as described in the legend to (A). The results shown are representative of three independent experiments.

Yeast two-hybrid screening using the amino-terminal region of PKN α as bait

To identify potential components of the PKN signaling pathway, the amino-terminus of PKN α (amino acid residues 1-540) was used as bait in a yeast two-hybrid screen of a human brain cDNA library. The 82 plasmids were isolated from approximately one million transformants screened, representing 15 different cDNAs as judged by DNA sequences (Table I). These clones resulted in high β -galactosidase levels upon co-transformation with the PKN α bait construct in the original yeast host strain YGH1. The specificity of this interaction was tested further by measuring the stability of other combinations of two-hybrid construct, LexAbd (instead of Gal4bd)-PKN α and Gal4ad-positive clone to support *lacZ* expression in L40 cells.

Clone#21 encoded the head-rod domain of the neurofilament-L protein, a subunit of neuron-specific intermediate filament. The amino-terminal regulatory region of PKN α was associated with the head-rod domains of other subunits of neurofilament, neurofilament proteins M and H (Mukai et al., 1996b). PKN α phosphorylated each subunit of the neurofilament and the phosphorylation of neurofilament-L protein by PKN α inhibited the polymerization of neurofilament-L protein *in vitro* (Mukai et al., 1996b). Clones#4, #10, and #25 encoded the skeletal muscle type α -actinin, HuActSk1 (Beggs et al., 1992). Clone#10 and #25 were the identical isolates encoding HuActSk1 from amino acid residue 344 to the carboxyl-terminus. Clone#4 encoded HuActSk1 from amino acid residue 333 to the carboxyl-terminus. The amino-terminal region of PKN α outside the RhoA-binding domain was sufficiently shown to associate with α -actinin (Fig. 6, Mukai et al., 1997). *In vitro* binding analyses revealed that full-length α -actinin hardly bound to PKN α , but the addition of phosphatidylinositol 4,5-bisphosphate, which is implicated in actin reorganization, stimulated the binding activity of the full-length α -actinin with PKN α (Mukai et al., 1997). Clone#2 and #11 were identical and contained a 1.3-kilobase pair cDNA insert encoding a novel and partial amino acid sequence. The full-length cDNA of this clone encoded a novel 450-kDa coiled-coil protein, CG-NAP (centrosome and Golgi localized PKN-associated protein) (Takahashi et al., 1999). CG-NAP

interacted with various signaling enzymes including protein kinases (PKN α , PKA, and PKC ϵ) and protein phosphatases (PP1 and PP2A) (Takahashi et al., 1999; Takahashi et al., 2000). Clone#6 and #8 were the identical isolates encoding a neural antigen PCD17, which is recognized by characteristic antibodies of patients with paraneoplastic cerebellar degeneration, from amino acid residue 75 to 443. PCD17, as well as α -actinin and CG-NAP, preferred to interact with the amino-terminal region of PKN α outside the Rho-binding domain in the yeast two-hybrid system (Fig. 6). PCD17 was phosphorylated by PKN α in an arachidonic acid-dependent manner *in vitro* (Takanaga et al., 1998). PCD17 fused to the Gal4 DNA binding domain showed the transcriptional transactivation of the chloramphenicol acetyltransferase reporter gene linked to 5 Gal4 binding sites and minimal promoter in rat C6 glioma cells, indicated that PCD17 have a potential transcriptional activity (Takanaga et al., 1998). It is possible that PKN α serves as a mediator of cellular signals to the nucleus and a regulator of the activity of transcription factors. Indeed, clone#43 contained an open reading frame of amino acid positions from 41 to 381 of human NDRF/NeuroD2, a neuron specific basic Helix-Loop-Helix (bHLH) transcription factor. The detailed analyses of the physiological relevance of the interaction between PKN α and NDRF/NeuroD2 were described below. Other clones are currently being investigated.

Table I Positive clones in the yeast two-hybrid screening using the amino-terminal region of PKN α as bait

A human brain cDNA library was screened in the yeast two-hybrid system using amino acid residues 1-540 of PKN α as bait. Positive clones were sequenced and the obtained sequences were analyzed using the BLAST program on the World Wide Web.

Group	positive clone	coding region
neurofilament-L	#21	a.a. ^a 1-349
α -actinin ^b	#4	a.a. 333-894
(skeletal muscle type)	#10, #25	a.a. 344-894
PCD17	#6, #8	a.a. 75-443
NDRF/NeuroD2	#43	a.a. 43-381
CG-NAP	#2, #11	a.a. 735-1280
Group I	#7, #69	
Group II ^b	#3, #5, #12, #13, #16, #19, #28	
	#14, #17, #22, #23, #26, #29, #31, #33, #37, #45, #50, #52, #61	
	#20, #38	
	#32, #57	
Group III	#24	
Group IV	#27	
Group V	#30	
Group VI	#65	

^a amino acid residues

^b These clones contained the different length inserts derived from the same cDNA.

Interaction between PKN α and the full-length of NDRF/NeuroD2 in the yeast two-hybrid system

To test the interaction of the amino-terminus of PKN α with the full-length of NDRF/NeuroD2, the full-length protein-coding sequence of NDRF/NeuroD2 was constructed from the insert of clone#43 and the 5' portion of the cDNA obtained by PCR. This product was cloned in frame to the DNA binding domain of LexA (LexAbd-NDRF/NeuroD2) and the activation domain of Gal4 (Gal4ad-NDRF/NeuroD2), and the resulting constructs were tested in various combinations for the yeast two-hybrid system. As shown in Fig. 7, co-transformation with both the amino-terminus of PKN α - and NDRF/NeuroD2-fusion constructs resulted in an expression of the β -galactosidase activity. In contrast, neither the combination of LexAbd-p53 and GAL4ad-NDRF/NeuroD2 nor LexAbd-NDRF/NeuroD2 and GAL4ad-largeT could exhibit the β -galactosidase activity. These results confirm the specificity of the association between the amino-terminus of PKN α and the full-length of NDRF/NeuroD2 in the yeast two-hybrid system.

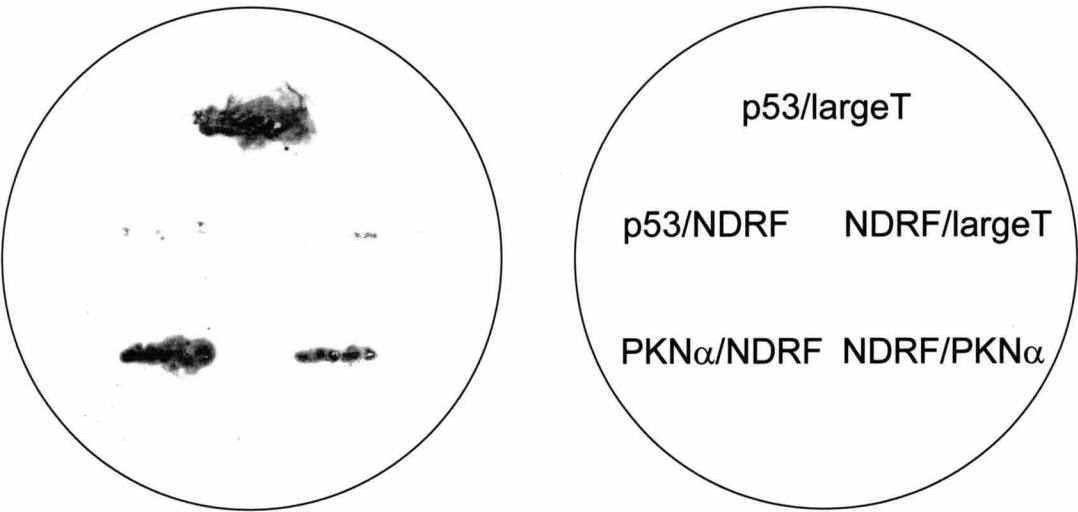


Figure 7 Interaction between PKN α and NDRF/NeuroD2 in the yeast two-hybrid system

L40 cells were co-transfected with expression plasmids encoding various proteins fused to LexAbd (left) and those fused to Gal4ad or VP16ad (right) as indicated in right panel. Left panel shows developments of blue color 2 h after initiating filter lift assays. Murine tumor suppressor p53 and SV40 large T antigen were used as controls.

PKN α binds to NDRF/NeuroD2 in vivo

To investigate whether PKN α interacts with NDRF/NeuroD2 in mammalian cells, COS-7 cells were co-transfected with expression vectors encoding the full-length of PKN α and encoding the full-length of NDRF/NeuroD2 tagged with FLAG epitope. As shown in Fig. 8, in the total cell lysates anti-FLAG antibody reacted with several bands of slightly different mobility, consistent with previous analyses indicating that KW8 (a rat homologue of NDRF/NeuroD2) are variously modified *in vivo* (Kume et al., 1998). Cell lysates were subjected to immunoprecipitation with anti-PKN α antibody, and immunoprecipitates were then probed with anti-FLAG antibody. FLAG-NDRF/NeuroD2 proteins were co-precipitated with PKN α by anti-PKN α antibody (α N2) but not by a control normal rabbit serum (NRS). Reciprocal experiments in which proteins immunoprecipitated with anti-FLAG antibody were blotted with an anti-PKN α antibody confirmed the association of PKN α with NDRF/NeuroD2.

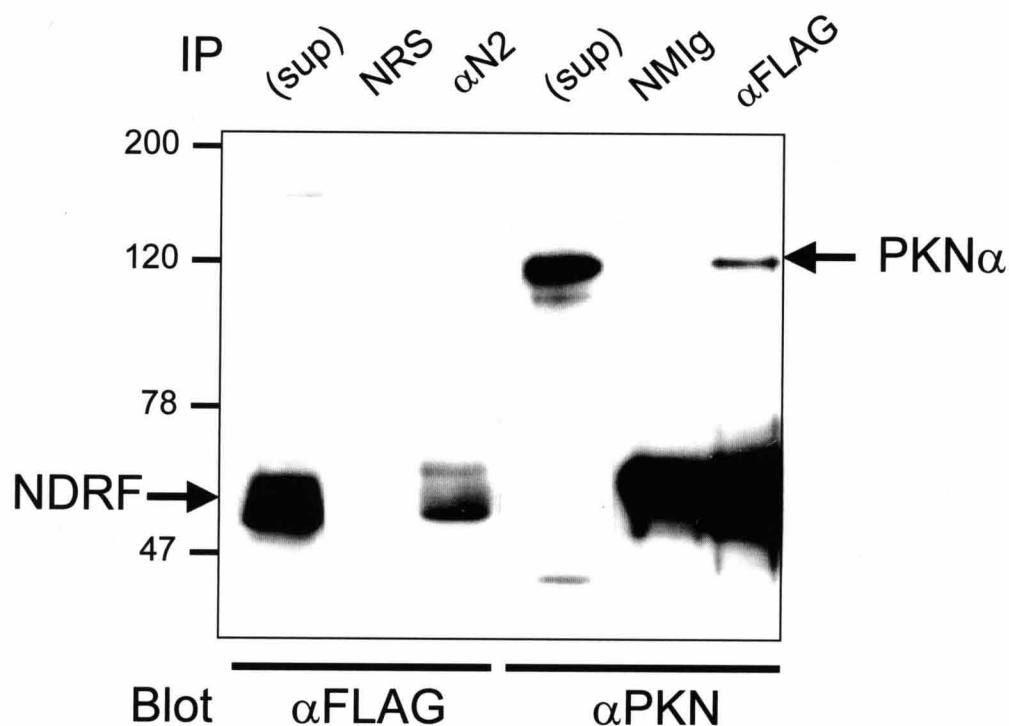


Figure 8 *In vivo* interaction between PKN α and NDRF/NeuroD2

FLAG-tagged full-length of NDRF/NeuroD2 and the full-length of PKN α were co-expressed in COS7 cells and immunoprecipitated (IP) with anti-PKN (α N2), normal rabbit serum (NRS), anti-FLAG M2 (α FLAG), or normal mouse immunoglobulin (NMIg). FLAG-NDRF/NeuroD2 and PKN α in immunoprecipitates and in cell lysates (sup) were visualized by immunoblotting with anti-FLAG M2 (α FLAG) and anti-PKN PRK1/PKN (α PKN), respectively. The results shown are representative of three independent experiments. Molecular weight markers are indicated in kDa on the left.

PKN α binds to NDRF/NeuroD2 in vitro

To delineate the region of NDRF/NeuroD2 recognized by PKN α , GST pull-down studies were performed using GST-PKN α purified from Sf9 cells and a series of *in vitro*-translated [^{35} S] NDRF/NeuroD2 proteins. As shown in Fig. 9A, GST-PKN α pulled down the full-length of NDRF/NeuroD2. This binding was not due to the GST portion of the fusion proteins, because NDRF/NeuroD2 did not bind to the glutathione beads bound with GST only. The carboxyl-terminal truncation mutant of NDRF/NeuroD2 (amino acid residues 1-209) was able to interact with GST-PKN α , but further deletion mutant of NDRF/NeuroD2 (amino acid residues 1-120) resulted in a loss of interaction with GST-PKN α . The internal region of NDRF/NeuroD2 (amino acid residues 120-209) and the amino-terminal truncation mutant of NDRF/NeuroD2 (amino acid residues 120-381) interacted with GST-PKN α . These results suggest that the region between amino acid residues from 120 to 209 of NDRF/NeuroD2 containing the complete bHLH domain is necessary and sufficient for the interaction with PKN α .

To investigate whether PKN α directly binds to NDRF/NeuroD2, another *in vitro* binding system was performed, in which GST-PKN α was mixed with purified carboxyl-terminal fragment of NDRF/NeuroD2 (amino acid residues 120-381) fused to MBP. As shown in Fig. 9B, Western blotting revealed that GST-PKN α was retained on MBP-NDRF/NeuroD2 (amino acid residues 120-381) attached to amylose resin, but not on control MBP only. These results suggest that PKN α could directly interact with NDRF/NeuroD2.

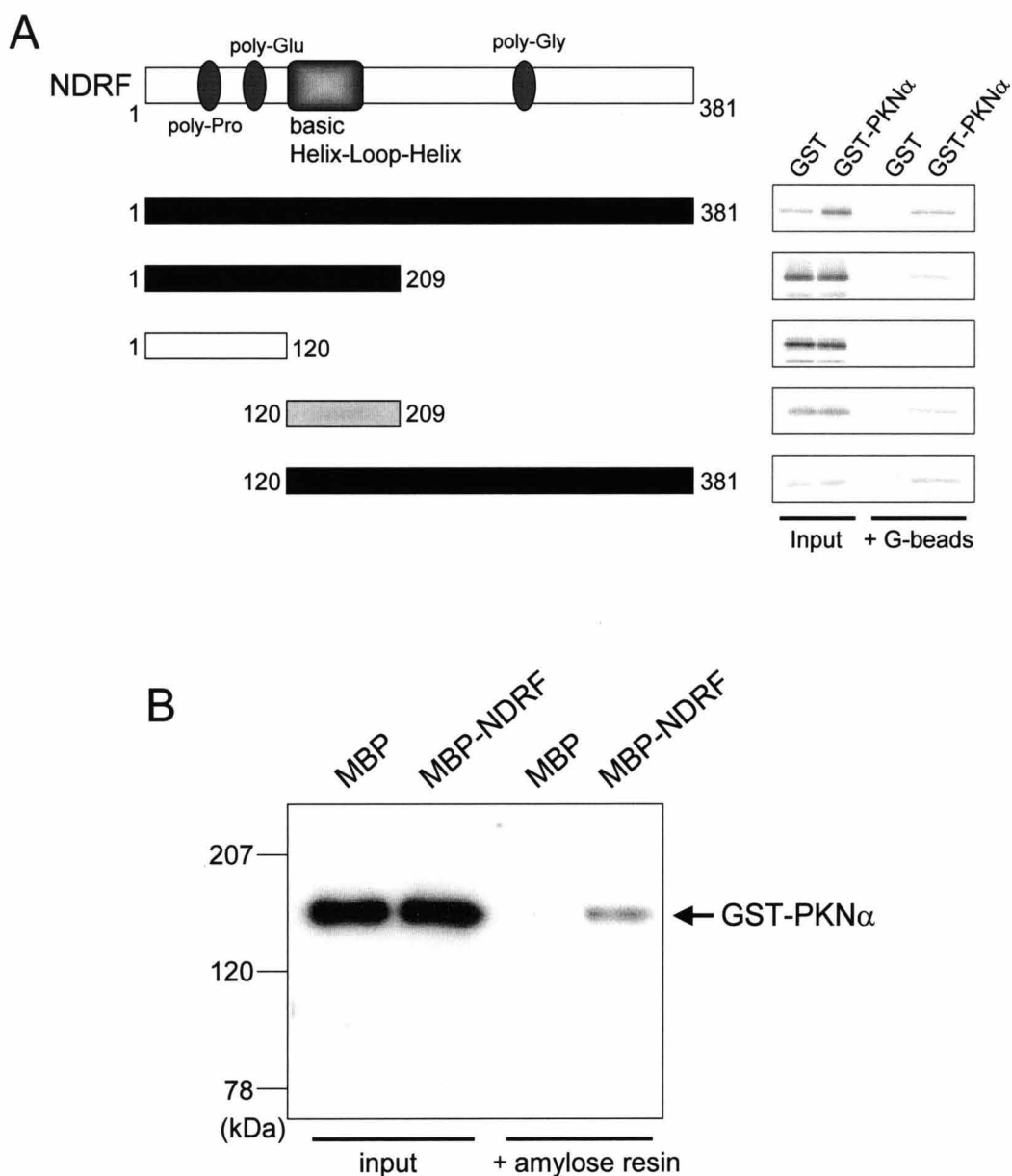


Figure 9 *In vitro* interaction between PKN α and NDRF/NeuroD2

(A) Analysis of PKN α -interacting region in NDRF/NeuroD2. Left panel represents schematics of the NDRF/NeuroD2 deletion mutants. The numbers denote the amino acid positions of each clone. NDRF/NeuroD2 polypeptides described in left panel were translated *in vitro* in the presence of [^{35} S]methionine and incubated with GST or GST-PKN α . After removing aliquots (input), proteins bound to glutathione-Sepharose beads were collected (+G-beads). Proteins in “input” and “+G-beads” preparations were analyzed by autoradiography after SDS-PAGE. **(B)** GST-PKN α was incubated with MBP or MBP-NDRF/NeuroD2 (amino acid residues 120-381). After removing aliquots (input), proteins bound to amylose resin were collected (+amylose resin). Proteins in “input” and “+amylose resin” preparations were analyzed by immunoblotting with anti-PKN α antibody, α C6. The results shown are representative of three independent experiments. Molecular weight markers are indicated in kDa on the left.

Overexpression of Catalytically Active Form of PKN α increases NDRF/NeuroD2-mediated Transcription

To examine whether PKN α could affect the transactivation activity of NDRF/NeuroD2, a useful assay for detection of the transactivation activity of NDRF/NeuroD2 was established. Namely, the luciferase reporter plasmids driven by the chicken ovalbumin minimal promoter nucleotide sequence -50 to +43 (ova) with or without four tandem repeats of the RIPE3 (Hwung et al., 1990) were constructed. Previously Naya et al. revealed using hamster insulinoma cell line HIT that co-expression of BETA2 (a hamster homologue of NeuroD) and widely expressed factor, E47, leads to the activation of the RIPE3 enhancer (Naya et al., 1995). Here, these reporter constructs and various mammalian expression vectors were transiently transfected into murine embryonal carcinoma cell line P19 in undifferentiated condition, in which endogenous NeuroD and NDRF/NeuroD2 are not detected (McCormick et al., 1996). Western blot analyses using anti-T7 tag monoclonal antibody confirmed the expression of NDRF/NeuroD2 tagged with T7 in P19 cells (data not shown). As shown in Fig. 10A, the control plasmid, pGL3-ova, produced a low level of the luciferase activity and showed no response to NDRF/NeuroD2 or NeuroD. Including the four tandem repeats of the RIPE3 (pGL3-4xRIPE3-ova) increased the basal level of the luciferase activity about 1.5-fold. NDRF/NeuroD2, as well as NeuroD, increased the luciferase activity 12-fold in P19 cells transfected with pGL3-4xRIPE3-ova. These results indicate that pGL3-4xRIPE3-ova works as a sensitive tool for detection of the transactivation activity of NDRF/NeuroD2.

Using this assay system, the effect of co-expression of either catalytically active form of PKN α (AF3) or kinase-deficient form of PKN α (AF3 K644E) (Takahashi et al., 1998) on the transactivation activity of NDRF/NeuroD2 was analyzed. Western blot analyses indicated that the expression vectors for AF3 or AF3 K644E produced the predicted protein in P19 cells (data not shown). As shown in Fig. 10B, AF3 slightly increased NDRF/NeuroD2-independent transcription from the minimal promoter with the RIPE3 enhancer. Co-expression of AF3, but

not AF3 K644E consistently enhanced NDRF/NeuroD2-dependent transcription 3-fold. However, overexpression of AF3 had no effect on the expression of reporter gene driven by SV40 promoter, HSV-thymidine kinase promoter or chicken β -actin promoter (data not shown). These results indicate that the protein kinase activities of PKN α specifically contributed to the enhancement of NDRF/NeuroD2-mediated transcriptional activation.

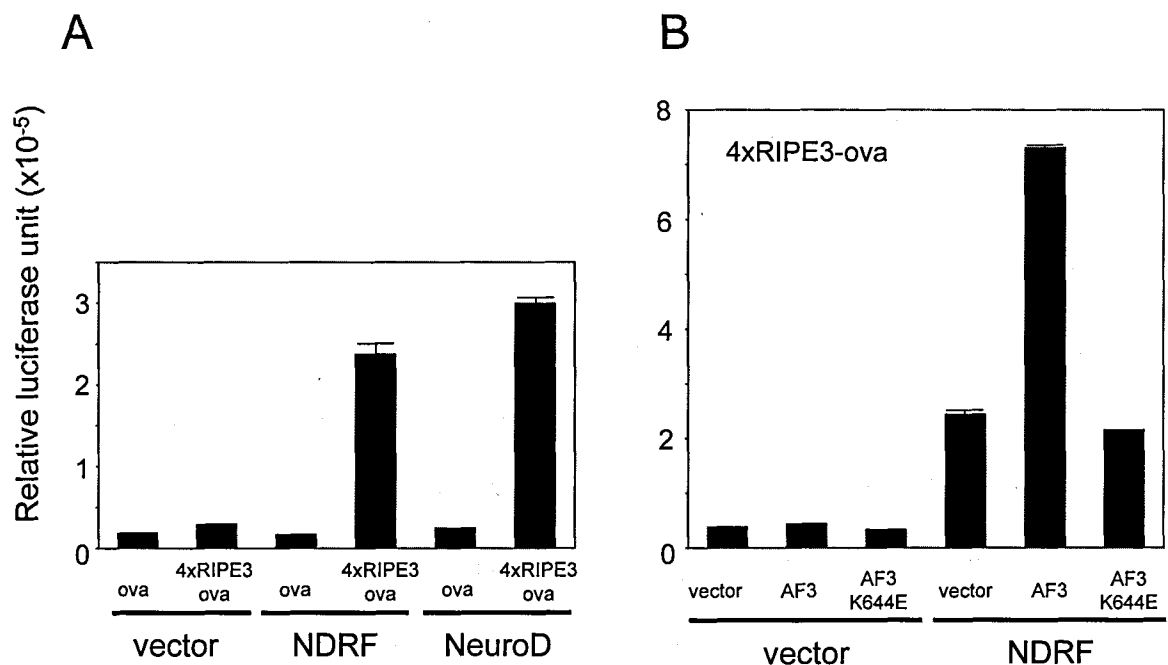


Figure 10 Catalytically active form of PKN enhances NDRF/NeuroD2-dependent transactivation

Representative results are presented as relative luciferase units (mean of triplicate experiments $\pm$ S.D.). Similar results were obtained in at least three independent experiments. (A) Effects of NDRF/NeuroD2 on the RIPE3 enhancer in P19 cells. Cells were transfected with the reporter construct (ova or 4xRIPE3-ova) and the empty control plasmid or the expression plasmid encoding NDRF/NeuroD2 or NeuroD as indicated. (B) Effects of catalytically active form of PKN α on NDRF/NeuroD2-dependent transactivation. P19 cells were transfected with the reporter plasmid (4xRIPE3-ova), the empty control plasmid or the expression plasmid encoding T7-tagged NDRF/NeuroD2 and the expression plasmid encoding PKN α mutants (AF3 or AF3 K644E) as indicated.

Yeast two-hybrid screening for PKN β interacting proteins

PKN β and PKN γ /PRK2, not PKN α , possess proline-rich motifs in their linker regions between the D region and the catalytic domain (Oishi et al., 1999; Palmer et al., 1995b). The proline rich motif in the PKN γ /PRK2 linker region fits consensus for adaptor protein Nck SH3-binding sequences, PxxPxRxxSL, and directly binds to the middle SH3 domain of Nck *in vitro*, suggesting a specific role for PKN γ /PRK2 in Nck-mediated signaling (Quilliam et al., 1996). Thus, the linker regions of the PKN family appear to interact with the specific partners and promote differential regulation and/or subcellular localization of the PKN family.

The PKN β linker region (amino acid residues 465-557, Fig. 2) was used as bait in a yeast two-hybrid screening of a human kidney embryonic 293 cell cDNA library. A total of 1.5×10^6 transformants were plated and 6 clones were strong positive for *His3*, *Ade2*, and *Mel1* reporter constructs. Clones K3 and K4 were the identical isolates encoding the SH3 domain of Graf (GAP for Rho associated with focal adhesion kinase) (Borkhardt et al., 2000). Two of the other clones, K1 and K2, had an insert of 1.3 kbp and 1.7 kbp, respectively. Sequence analysis of these clones revealed that they contained the different length inserts derived from the same cDNA (Fig. 11). Database searching of clone K2 suggested that this cDNA encodes a novel protein with an SH3 domain homologous to Graf (Fig. 12C). As shown in Table II, the yeast cells co-transformed with both the PKN β linker region-fusion construct (Gal4bd-linkPKN β) and clone K2 or K3 were prototrophic for histidine and adenine and exhibited the α -galactosidase activity. In contrast, neither the combination of Gal4bd-linkPKN α and K3 nor Gal4bd-linkPKN α and K2 could grow on SD/-Leu/-Trp/-His/-Ade plate containing 5 mM 3-AT and exhibit the α -galactosidase activity. These results confirm the specificity of the association between the PKN β linker region and the carboxyl-terminal region covering the SH3 domain of Graf and Graf-related protein in the yeast two-hybrid system.

Table II Interaction of the PKN β linker region with Graf and Graf2 within transformed yeast

Interaction of the proteins encoded by the Gal4 DNA binding domain and activation domain constructs (bait and pray) within transfected AH109 yeasts was assessed by growth and development of blue color on SD/-Trp/-Leu/-His/-Ade media containing 5 mM 3-AT and X- α -gal. p53 and largeT served as positive controls. “+” indicates growth and development of blue color within 3 days. “-“ indicates no growth within 7 days.

bait	pray		
	largeT	clone K3 Graf (626-759 ^a)	clone K2 Graf2 (506-786 ^a)
p53	+	-	-
PKN α (511-632 ^a)	-	-	-
PKN β (465-557 ^a)	-	+	+

^a amino acid residues

Characterization of a novel Graf-related protein, Graf2

To obtain the full-length coding sequence of Graf-related protein, 5'-RACE protocols were employed. Fig. 11 shows the nucleotide and amino-acid sequences of the full-length cDNA for Graf-related protein obtained by 5'-RACE. This cDNA clone is predicted to encode a polypeptide of 787 amino acid residues with a calculated molecular mass of 89,327 Da. The deduced amino acid sequence was closely related to but clearly distinct from the sequences of previously characterized Graf, indicating that this cDNA encoded a new member of the Graf family proteins. This newly identified protein was designated as Graf2. Sequence analysis of other cDNA clones by 5'-RACE revealed variations in the region corresponding to the amino-terminus adjacent to the SH3 domain, suggesting the presence of alternative splicing variants (data not shown). As shown in Fig. 12A, the Pfam database analysis (Bateman et al., 2000) revealed that Graf2 contains putative PH domain, a RhoGAP domain and an SH3 domain. The overall architecture and size of Graf2 are very similar to those of human Graf and *C. elegans* T04C9.1 gene product. As shown in Fig. 12B and 12C, the RhoGAP and the SH3 domain of Graf2 demonstrate high degrees of homology to other proteins in database. Chicken Graf (Hildebrand et al., 1996) possesses a RhoGAP domain and an SH3 domain, which are highly homologous to Graf2, but the amino-terminal region of chicken Graf is shorter than that of other Graf family proteins. Human oligophrenin 1 (Billuart et al., 1998) and *Drosophila* CG8948 gene product share high sequence homology to Graf2 from their amino-terminus to the RhoGAP domain, whereas the carboxyl-terminal are divergent from Graf2 and an SH3 domain is absent in these molecules.

ATGGGGCTGCAGCCCTGGAGTTCAGCGACTGCTACCTCGACAGCCGCTGGTTCCGGGAGAGGATCCGCGCTCAGGAAGCGGAACCTCGAGAGGACCAACAAGTTCATCAAGAGCTCATT	120
M G L Q P L E F S D C Y L D S P W F R E R I R A H E A E L E R T N K F I K E L I	40
AAGGACGGGAAGAACCTCATCGTGCAGACGAAAAGTCTGTCACTGGCCAGCGGAAGTTTGCTCATTCACTCAGAGACTTTAAGTTTGAGTTTATCGGTGATGCTGTGACAGATGATGAA	240
K D G K N L I A A T K S L S V A Q R K F A H S L R D F K F E F I G D A V T D D E	80
CGATGCATAGATGCTTCTTACGTGAATTTTCAAATTTTGAAGAACTGGAGGAACAGAGAGAAATTTGGCATTAAAGTGAAGTGAACCTGATTAACCCCTGGAAAAATTCAGA	360
R C I D A S L R E F S N F L K N L E E Q R E I M A L S V T E T L I K P L E K F R	120
AAAGAGCAACTTGGAGCTGTAAAGGAAGAAAAAGAAGTTTGACAAAGAGACAGAAAAGATTATAGTCTAATTGATAAACATTGTAATTTATCAGCAAAAAAGAAAGACTCACATTTA	480
K E Q L G A V K E E K K K F D K E T E K N Y S L I D K H L N L S A K K K D S H L	160
CAAGAGGCAGATATCCAAGTAGAGCAGAACCGGCAACACTTCTATGAAGTGTCTCTCGAGTATGTGTGAAGCTGCAGGAAATCCAAGAAAGAAAGTGTGAGTTTGGAACTATG	600
Q E A D I Q V E Q N R Q H F Y E L S L E Y V C K L Q E I Q E R K K F E F V E P M	200
CTGTCTATTTTTAGGGGATGTTTACCTTCTATCATCAGGGCCATGAAGTGGCAAGACTTCAATCACTACAAAATGGAAGTCAAGATCAACATTGAGAATACACGGAATCGATTGAA	720
L S F F Q G M F T F Y H Q G H E L A K D F N H Y K M E L Q I N I Q N T R N R F E	240
GGAACAAGGTGAGAAGTGAAGAGCTCATGAACAAAATCAGACAGAATCCCAAGGACCACAAAGCAGCAAGTCAAGTTTACAGCCGGAAGGCTACCTGTATGTCAGGAAAAAGGCTGCT	840
G T R S E V E E L M N K I R Q N P K D H K R A S Q F T A E G Y L Y V Q E K R P A	280
CCGTTTGGTTCCAGTTGGGTCAAACACTATTGCATGTATCGAAAAGCAGCAAGAAGTTCAACATGATCCCATTTGAGCACAGATCTGGAGGGAAACTGGGGACGGAGAGGTGTTCTTT	960
P F G S S W V K H Y C M Y R K A A K K F N M I P F E H R S G G K L G D G E V F F	320
TTGAAAGATGTACCAAGAGGCATACTGACTCCATTGACAGAAGGTTTGTGTTTACATAGAAGTGTGATCGGCTGGCGTTTCTTGACCATGCAGGCATTTCGGAAGAGGAAAGG	1080
L K E C T K R H T D S I D R R F C F D I E A A D R P G V S L T M Q A F S E E E R	360
AAGCAGTGGTTGAAGCTCTGGGTGGAAGGAAGCTCTGTCCCATAGTTTAAATACAGCCATCATCCAAGACCAGAAGGAATGCACAGTTGGATAAGATGGGGTTCACAATTATCAGA	1200
K Q W L E A L G G K E A L S H S F N T A I I P R P E G N A Q L D K M G F T I I R	400
AAATGCATCAGTGGCGTTGAACACAGAGGTATAAATGACCAAGGATTGTACAGAGTTGTGGGGGTGAGTTCAAGGTCCAGAGACTTCTGAGTATGTTGATGGATGTAACCAATGCAAT	1320
K C I S A V E T R G I N D Q G L Y R V V G V S S K V Q R L L S M L M D V K T C N	440
GAGGTGGACCTGGAGAATTCTGCAGATTTGGGAAGTGAAGACAATAACAAGTGCCCTGAAACAGTATTGAGGAGTCTTCCAGAGCCTCTCATGACCTATGAGTTACATGGAGATTTTCATT	1440
E V D L E N S A D W E V K T I T S A L K Q Y L R S L P E P L M T Y E L H G D F I	480
GTTCCAGCCAAAAGCGGCAGTCCAGAAATCTCGTGTTAATGCGATCCATTCTTGGTACACAACTGCCAGAGAAGAATAAAGAGATGTTGGATATTTTGGTGAACACTTAACAAATGTT	1560
V P A K S G S P E S R V N A I H F L V H K L P E K N K E M L D I L V K H L T N V	520
TCAATCACTCCAAGCAGAACCTGATGACTGTGGCAAACCTAGGAGTGGTGTGGACCAACTCTGATGAGGCCACAGGAAGAACTGCGCTGCCCTCATGACTTGAAGTTTCAGAAT	1680
S N H S K Q N L M T V A N L G V V F G P T L M R P Q E E T V A A L M D L K F Q N	560
ATTGTTGTGGAATCTTAATTGAAAACCATGAAAAGATTTTTCGAGCGCGCCGATACTACATTCCTGAGCCCACTGCCTGTGACATCACCCCCAAATGCGCCACCAAGGCAGTCG	1800
I V V E I L I E N H E K I F R T P P D T T F P E P T C L S A S P P N A P P R Q S	600
AAGAGACAAGGCCAGAGAACCAAGAGGCCCTGGGcGCTACAATCTTTGTCTGGAGCTGGAAGATGGTGACAATCCTTACCCTTCCAAGGAGGACACCCCTACCAGCAGTCTGGACTCA	1920
K R Q G Q R T K R P V A V Y N L C L E L E D G D N P Y P S K E D T P T S S L D S	640
CTTTCTCCCGTCTCCGTTGACTACAGCTGTCCCTGGGCTCTGGACCAGACAAAACACCTTCTGGCAGATGGAGGGAGCTTTGGAGACTGGGCATCCACTATCCAGGCCAGACC	2040
L S S P S P V T T A V P G P P G P D K N H L L A D G G S F G D W A S T I P G Q T	680
CGATCGTCTGTGGTCCAGTGGCTTAACCCACAGTCTCCAACCACAACAAGCTCCAACCTCAGCTGTGACACCTCTTTCACCCGGTGTGTCCTTTCCCTTTTCTCCTCTGCTACTGTA	2160
R S S V V Q W L N P Q S P T T T S S N S A V T P L S P G S S P F P F S P P A T V	720
GCGGACAAGCCACCTGAAAGCATCCGAGTCGGAAGGCTCGAGCCGTGTATCCGTGTGAAGCAGAACACAGCTCGGAATTATCTTTGATATAGGAGCAATTTTGGAGATGTACAAAC	2280
A D K P P E S I R S R K A R A V Y P C E A E H S S E L S F D I G A I F E D V Q T	760
TCCAGGGAACCTGGCTGGCTAGAAGGGACTCTGAACGGCAAGAGGGGGCTGATTCCACAGAACTACGTCAAGCTGCTGTAG	2361
S R E P G W L E G T L N G K R G L I P Q N Y V K L L *	787

Figure 11 Sequence of the cDNA and deduced amino acid sequences of human Graf2
The amino acid sequence is shown in single-letter code. The stop codon is indicated by an asterisk. The clones isolated in the two-hybrid screening were fused to the Gal4 activation domain at the position indicated by arrows (clone *K1* and *K2*). The putative PH domain is underlined. Gray and shaded boxes indicate the RhoGAP domain and the SH3 domain, respectively.

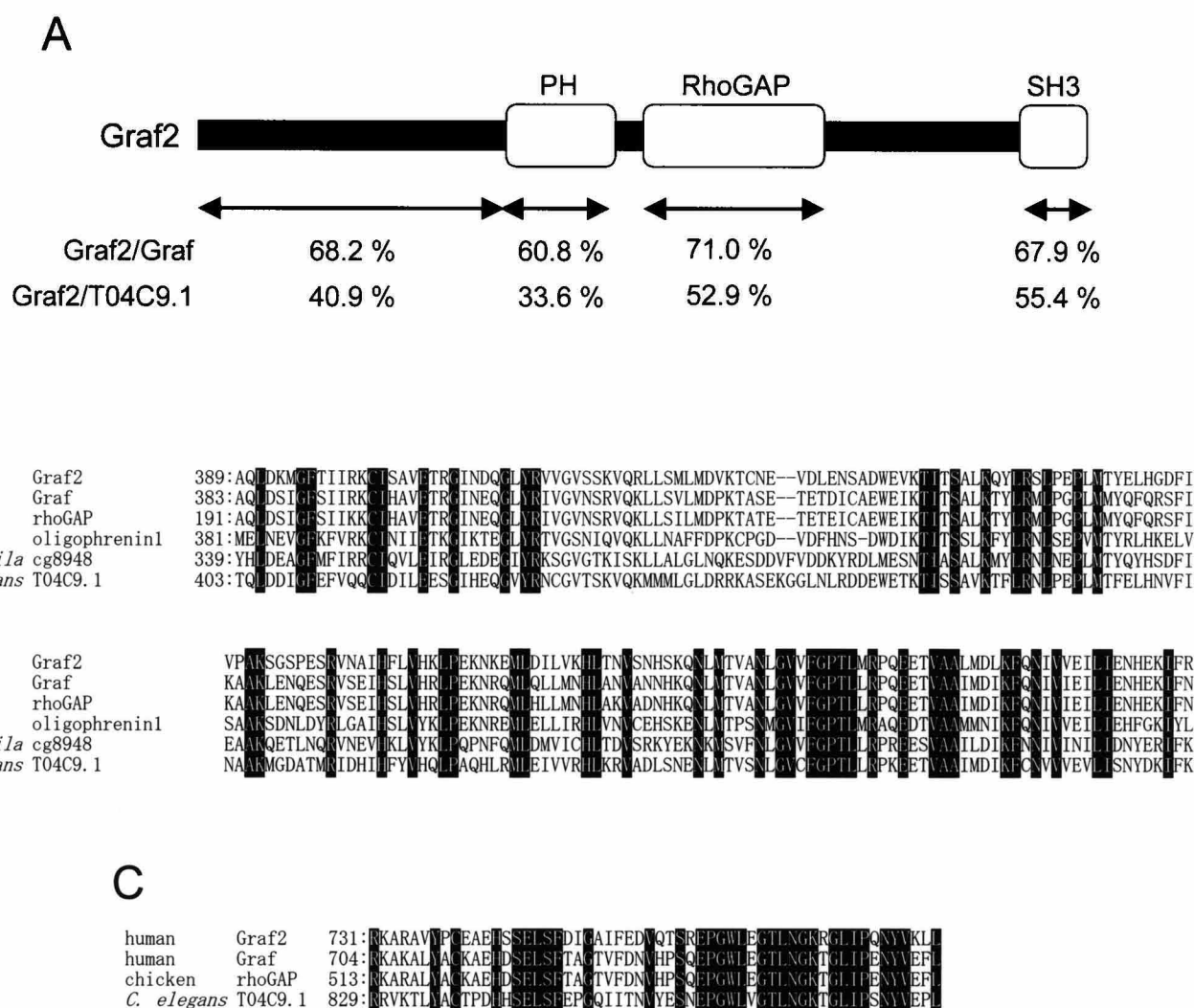


Figure 12 Structure of human Graf2 and sequence comparison

(A) Schematic representation of human Graf2. The percentage values of identity of Graf2 with Graf and with *C. elegans* T04C9.1 gene product are shown underneath. (B) Sequence alignment of the putative RhoGAP domain from Graf2 and related proteins. The position of the first residues of the protein sequence is indicated at the beginning of each sequence. Introduced gaps are shown as hyphens, and aligned identical residues are black boxed. The DDBJ/GenBankTM/EMBL Data Bank accession number: of the sequences are as follows: human Graf, Y10388; chicken Graf, U36309; human oligophrenin 1 AJ001189; *Drosophila* CG8948, AE003500; *C. elegans* T04C9.1, U80955. (C) Sequence alignment of the SH3 domain from Graf2 and homologous proteins. Aligned identical residues are black boxed.

Northern blot analysis of human RNAs revealed a major Graf2 transcript of approximately 3.8 kb in length that was present at different levels depending on the tissue (Fig. 13A, middle panel). High level expression was detected in heart and skeletal muscle. Graf2 mRNA level was low but still detectable in placenta, lung, kidney, and pancreas. In liver and skeletal muscle, a second transcript of approximately 1.8 kb in length was also detected that might represent either alternatively spliced mRNA or a closely related but different gene product. The distribution of the transcript of Graf2 contrasted with that of Graf, which is predominantly expressed in heart, brain, and placenta (Fig. 13A, top panel).

To characterize the Graf2 proteins, a polyclonal antibody against the SH3 domain of Graf2 was produced. First, we tested the specificity of this antibody for Graf proteins by immunoprecipitation of HA-tagged Graf (HA-Graf) or Graf2 (HA-Graf2) expressed in COS-7 cells. As shown in Fig. 13B, HA-Graf was expressed as a doublet band, consistent with previous analyses indicating that Graf proteins are phosphorylated in cells (Taylor et al., 1998). In immunoprecipitates from this lysate by the antibody α G2SH3, anti-HA immunoreactivity was not detected. While anti-HA antibody detected HA-Graf2 in immunoprecipitates by α G2SH3 from COS-7 cells expressing HA-Graf2. Neither protein was precipitated when normal rabbit serum was used. These results indicate that the α G2SH3 antibody selectively recognizes HA-Graf2 but not HA-Graf. In order to identify endogenous Graf2 protein, the immunoprecipitation was performed using several rat tissue extracts. As shown in Fig. 13C, a protein migrating with a molecular mass of ~100 kDa was detected by α G2SH3 antibody in immunoprecipitates from brain, skeletal muscle, and testis. Low level of immunoreactivity was seen in heart, lung, and thymus. In skeletal muscle, a doublet band for Graf2 was observed. The upper band may represent a post-translationally modified form of Graf2, possibly a phosphorylated form akin to Graf. The faster migrating bands in liver and testis presumably correspond to the alternatively spliced forms or degradation products of Graf2.

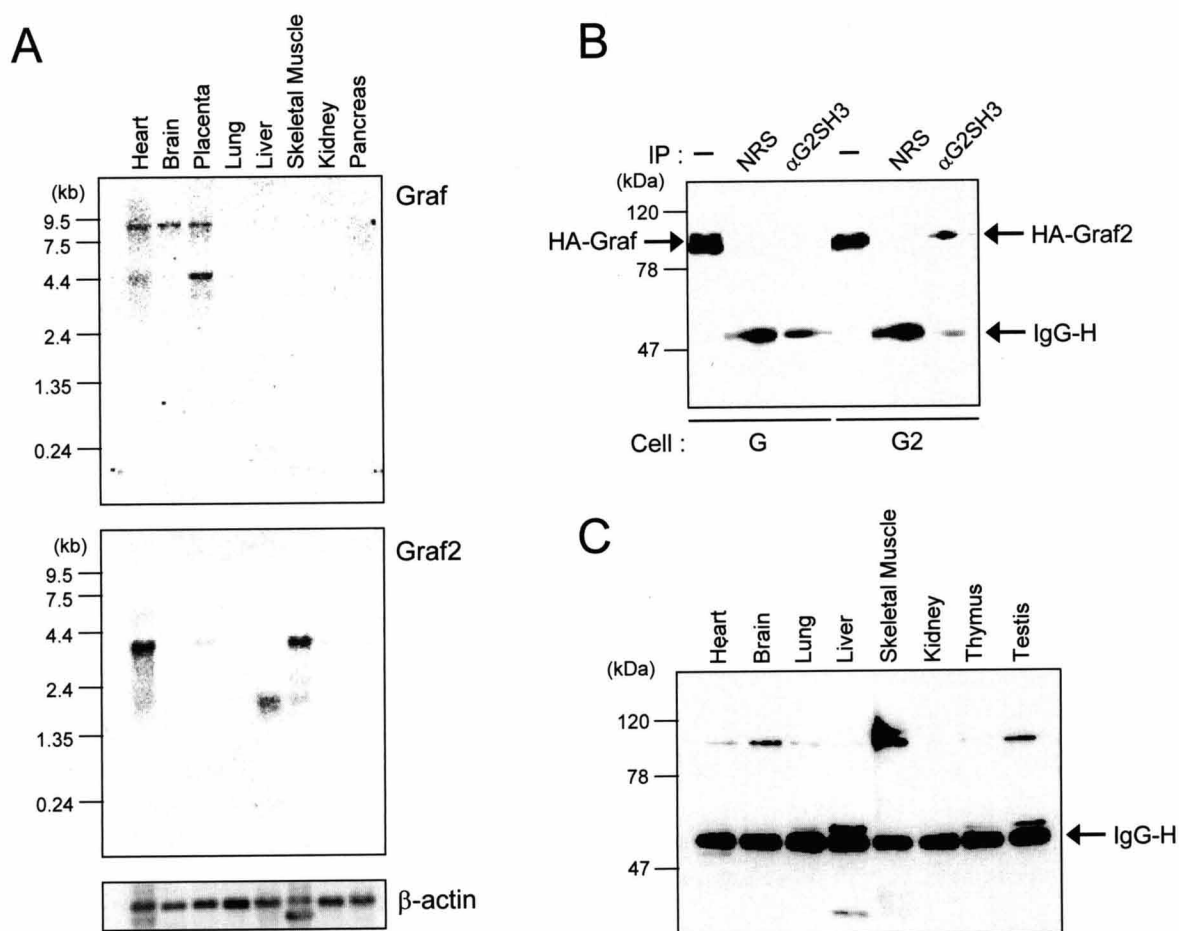


Figure 13 Expression pattern of Graf2 mRNAs and proteins

(A) mRNA distributions of human Graf and Graf2. Human Multiple Tissue Northern blot was hybridized with Graf (top), Graf2 (middle), and β -actin (bottom)-specific cDNA probes. Positions of the RNA size markers are shown at the left. (B) Specificity of α G2SH3 antibody. Lysates prepared from COS-7 cells transiently expressing HA-Graf (G) or HA-Graf2 (G2) were immunoprecipitated with normal rabbit serum (NRS) or rabbit antibody against Graf2 (α G2SH3). After washing the immunoprecipitate, proteins were resolved by SDS-PAGE, transferred to membrane, and probed with anti-HA antibody. Positions of the protein size markers are shown at the left. Black arrows indicate the position of each protein. (C) Protein distributions of Graf2. Lysates prepared from rat tissues were immunoprecipitated with antibody α G2SH3. After washing the immunoprecipitate, proteins were resolved by SDS-PAGE, transferred to membrane, and probed with antibody α G2SH3. Positions of the protein size markers are shown at the left.

To examine that the protein encoded by Graf2 cDNA actually displays a GAP activity, the RhoGAP domain (amino acid residues 381-587) of Graf2 fused to GST was expressed in *E. coli* and examined its ability to stimulate the GTPase activities of Rho family proteins. As shown in Fig. 14, the RhoGAP domain of Graf2 (GST-Graf2GAP), as well as that of Graf (GST-GrafGAP), exhibited the GAP activities toward RhoA and Cdc42Hs but appeared much less active on Rac1.

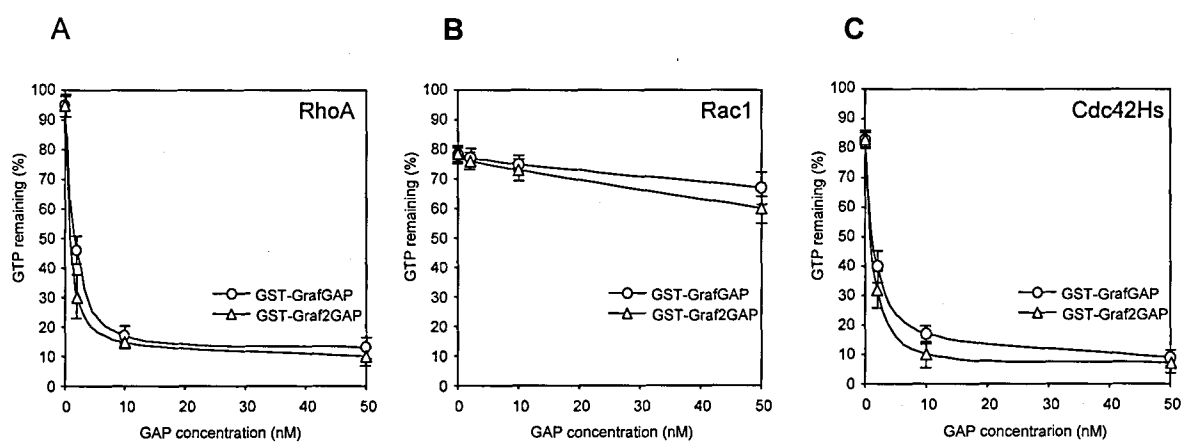


Figure 14 The GAP activity of Graf and Graf2

GTPase activity was analyzed by a filter binding assay. GST-RhoA (A), GST-Rac1 (B), and GST-Cdc42Hs (C) were loaded with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ for 10 min at 30 °C in GTP binding buffer. MgCl_2 and unlabelled GTP were added. The indicated concentrations of GAP domain of Graf (GST-GrafGAP) or Graf2 (GST-Graf2GAP) expressed in *E. coli* were added to each GTPases (100 nM) and incubated for 2 min at 25 °C. Shown is the remaining GTP at 2 min as percent of bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ before incubation at 25 °C. Each bar represents the mean (+SD) of three independent experiments.

Association of PKN β with Graf and Graf2

To investigate whether PKN β interacts directly with Graf and Graf2, an *in vitro* binding analysis using the recombinant proteins purified from *E. coli* was carried out. As shown in Fig. 15A, the carboxyl-terminal region (amino acid residues 596-759) covering the SH3 domain of Graf fused to GST (GST-GrafCterm) and the SH3 domain (amino acid residues 734-786) of Graf2 fused to GST (GST-Graf2SH3) pulled down PKN β (amino acid residues 301-610) fused to MBP (MBP-PKN β). Reciprocal experiments confirmed the association of PKN β with the SH3 domain of Graf or that of Graf2 (Fig. 15B). The association between the purified proteins from *E. coli* suggests that these interactions do not require any intermediate proteins and eukaryote-specific post-translational modifications.

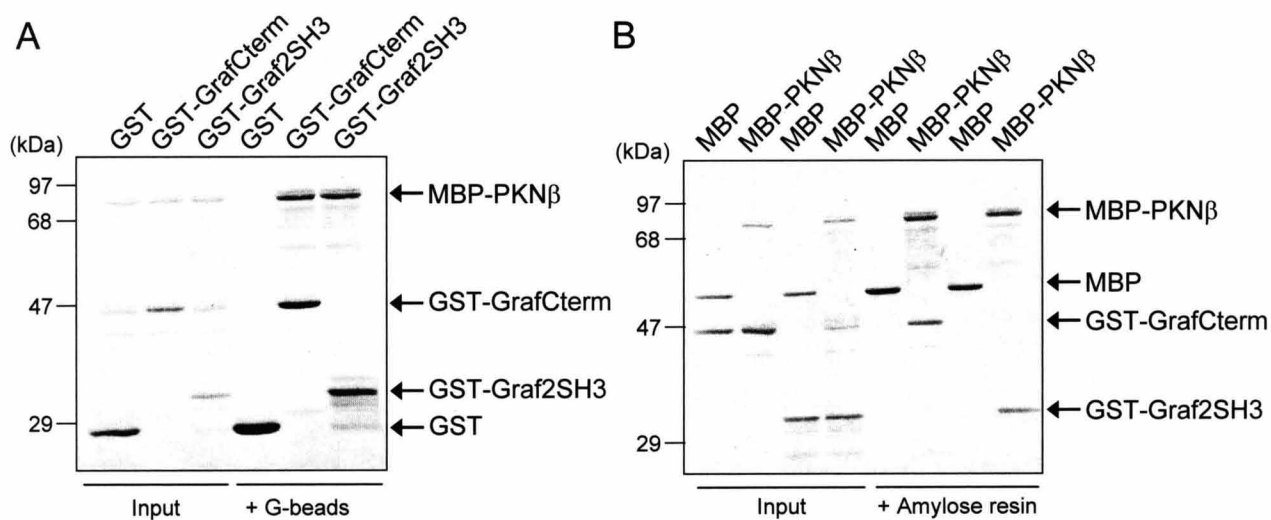


Figure 15 The interaction of PKN β with Graf and Graf2 *in vitro*

Proteins in each preparation were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The results shown are representative three independent experiments. Positions of the protein size markers are shown at the left. Black arrows indicated the position of each protein. (A) GST-pull down assay. MBP-PKN β (amino acid residues 301-610) was incubated with GST, GST-GrafCterm (amino acid residues 596-759), or GST-Graf2SH3 (amino acid residues 734-786). After removing aliquots (Input), proteins bound to glutathione-Sepharose were collected (+G-beads). (B) MBP-pull down assay. GST-GrafCterm or GST-Graf2SH3 was incubated with MBP or MBP-PKN β . After removing aliquots (Input), proteins bound to amylose resin were collected (+Amylose resin).

To investigate whether PKN β interacts with Graf and Graf2 in mammalian cells, COS-7 cells were co-transfected with the expression plasmids encoding the full-length of PKN β tagged with FLAG epitope (FLAG-PKN β) and encoding HA-Graf or HA-Graf2. Cell lysate was subjected to immunoprecipitation with anti-FLAG antibody, and immunoprecipitates were then probed with anti-HA antibody. As shown in Fig. 16, HA-Graf2 was co-precipitated with FLAG-PKN β by anti-FLAG antibody but not by normal mouse immunoglobulin. HA-Graf was also co-precipitated with FLAG-PKN β . However, the co-precipitated Graf contained only fast migrated species, whereas Graf in cell extracts appear as a doublet band. It is reported that phosphorylation of Graf leads the mobility shift (Taylor et al., 1998). Taken together, association between PKN β and Graf may be regulated by phosphorylation.

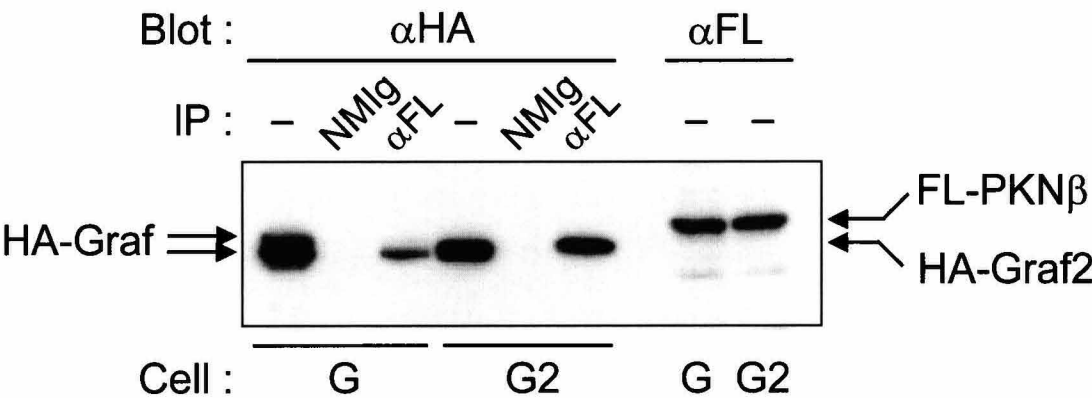


Figure 16 The interaction of PKN β with Graf and Graf2 in COS-7 cells
 FLAG-PKN β and HA-Graf (G) or HA-Graf2 (G2) were co-expressed in COS-7 cells (Cell) and immunoprecipitated (IP) with anti-FLAG (α FL) or normal mouse immunoglobulin (NM Ig). Immunoprecipitates and extracts (-) were analyzed by immunoblotting with anti-HA (α HA) for Graf and Graf2, or with anti-FLAG (α FL) for PKN β . Black arrows indicate the position of each protein.

Phosphorylation of Graf and Graf2 by PKN β

To test the ability of PKN β to phosphorylate Graf or Graf2, the full-length GST fusion proteins purified from Sf9 cells were subjected to *in vitro* phosphorylation by PKN β . As shown in Fig. 17, the constitutively active form of PKN β (GST-PKN β cat) phosphorylated both GST-Graf and GST-Graf2 *in vitro*.

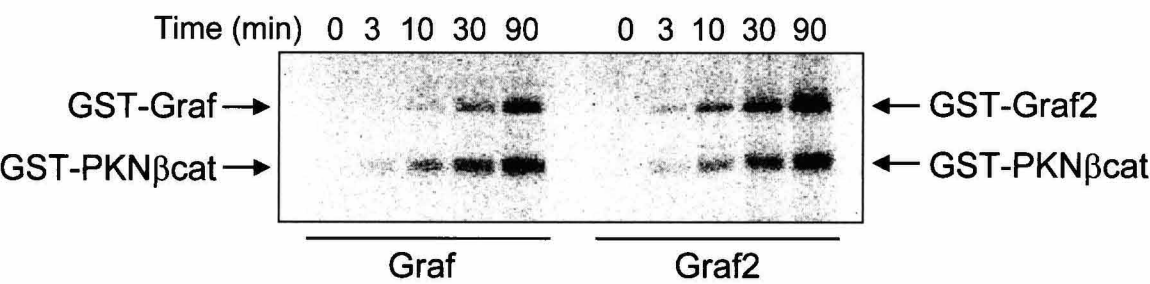


Figure 17 Phosphorylation of Graf and Graf2 by PKN β

Shown is the autoradiograph of SDS-PAGE of GST-Graf and GST-Graf2 incubated with GST-PKN β cat, the constitutively active form of PKN β purified from Sf-9 cells, for the indicated time at 30 °C. Black arrows indicate the position of each protein.

Discussions

Multiple regions of the PKN family contribute to the distinct interaction surfaces for various interacting proteins (Fig. 18). The amino-terminal regions of the PKN family from the CZ1 region to the D region are highly conserved (Fig. 2) and most interacting proteins that recognize this region of PKN α , such as α -actin, CG-NAP, and PCD17, also interacted with amino-terminal regions of PKN β in the yeast two-hybrid system (data not shown). The ability of each amino-terminal region to interact with common binding partners suggests the conformational similarity of the amino-terminal region of the PKN family. On the other hand, the linker regions of the PKN family are structurally divergent. PKN β specifically binds to the SH3 domains of Graf and Graf2 (Table II, Fig. 15). PKN γ /PRK2 also binds specifically to the middle SH3 domain of Nck (Quilliam et al., 1996). Since PKN α could not interact with the SH3 domains of Graf, Graf2 and NCK in yeast two-hybrid system (data not shown), these kinases may be differentially regulated in protein kinase activity and subcellular localization. It is possible that after the each member of the PKN family is localized at the specific subcellular sites by the interaction with specific targeting proteins or by the unknown mechanism, they are retained by the interaction with common partners for the PKN family, such as actin-cross linking protein α -actin and Golgi and centrosome associated protein CG-NAP.

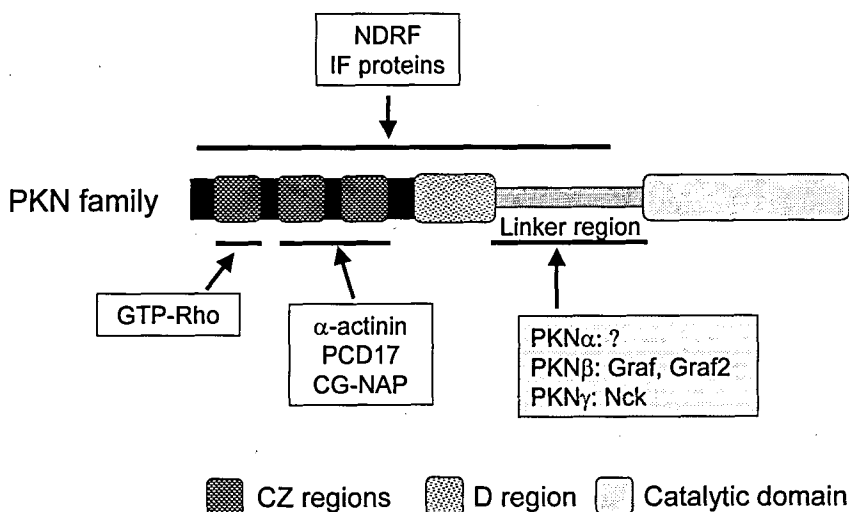


Figure 18 Interacting proteins for the PKN family and their binding surface

Interaction of PKN α with cytoskeletal proteins

Dynamic rearrangements of the cytoskeleton are required for various cellular processes, such as shape changes, motility, chemotaxis, and cell division (Stossel, 1993; Fishkind and Wang, 1995; Zigmond, 1996; Mitchison and Cramer, 1996). The cytoskeleton consists mainly of three components: actin filaments, microtubules, and intermediate filaments (IFs) (Stossel, 1993; Zigmond, 1996; Mitchison and Cramer, 1996). The actin dynamics are governed by Rho family GTPases, including Rho, Rac, and Cdc42 (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Rho promotes the formation of actin stress fibers and focal adhesions (Ridley and Hall, 1992). In this study, Rho is shown to interact with PKN α in a GTP-dependent manner, and PKN α could associate with actin cross-linking protein α -actinin. The Rho-binding site maps on the amino acid residues 33-111 of PKN α that corresponds to the CZ1 region, whereas α -actinin very weakly interacts with this region of PKN α in the yeast two-hybrid system. By contrast, α -actinin strongly interacts with amino acid residues 136-306 of PKN α , whereas no interaction was detected between the dominant-active form of Rho and PKN α . Thus PKN α provides the distinct region for the interaction with RhoA and with α -actinin. These results raise the possibility that PKN α binds simultaneously to RhoA and α -actinin. The interaction between PKN α and the full-length of α -actinin is dependent on phosphatidylinositol 4,5-bisphosphate (PIP₄,5P₂) (Mukai et al., 1997). Recently it has shown that Rho interacts physically and regulates the activity of PI(4)P5-kinase and thereby regulates the cellular levels of PI₄,5P₂ (Chong et al., 1994; Ren et al., 1996). It is possible that the interaction between PKN α and α -actinin is stimulated by Rho-signaling in PI₄,5P₂-dependent fashion and α -actinin serve as a scaffolding protein that targets PKN α to specific cytoskeletal substrates. PKN α actually phosphorylates actin cytoskeletal protein caldesmon and G-actin *in vitro* (Mukai et al., 1997). In addition, phosphoinositide including PI₄,5P₂ is one of the activator for the protein kinase activity of PKN α (Palmer et al., 1995a). PKN α could be activated by Rho via direct and/or indirect mechanism and be implicated in mediating some effects of Rho on the actin cytoskeleton.

Intermediate filaments (IFs) proteins are grouped into the five major types (Steinert and Roop, 1988; Fuchs and Weber, 1994). In this screening, only neurofilament-L, one of the type IV IF proteins that express specifically in neuronal cells, were identified as the interacting IF protein for PKN α . PKN α interacts with not only the head rod domains of other type IV IF proteins, neurofilament M and neurofilament H (Mukai et al., 1996b), but also vimentin (Matsuzawa et al., 1997), one of the type III IF filament protein that is the most common intermediate protein, and cytokeratin 18, one of the type I IF protein (data not shown). Since the expression of PKN α is ubiquitous, the interaction between PKN α and various IF proteins suggest the participation of PKN α in the rearrangement of IF proteins in various cell types. Rho kinase, one of the target protein kinase for Rho, accumulates with Rho at the cleavage furrow when cells undergo cytokinesis (Takaishi et al., 1995; Kosako et al., 1999). Rho-kinase can phosphorylate IF proteins, such as vimentin and GFAP (Goto et al., 1998; Kosako et al., 1997), and the expression of GFAP mutated at its Rho-kinase phosphorylation sites results in impaired glial filament segregation into postmitotic daughter cells (Yasui et al., 1998). These results suggest that Rho-kinase is essential not only for cytokinesis but also for segregation of GFAP filaments into daughter cells. PKN α phosphorylates these proteins and neurofilament-L and vimentin phosphorylated by PKN α lost the polymerization activity *in vitro* (Mukai et al., 1996b; Matsuzawa et al., 1997). Like Rho-kinase, it is possible that PKN α regulates the IF proteins in Rho-mediated pathway. Alternatively, because the phosphorylation site of GFAP by PKN α is distinct of that by Rho-kinase, the regulatory mechanism of PKN α may be different from that of Rho-kinase, and PKN α and Rho-kinase could function as the regulator of IF proteins at the distinct subcellular sites.

Interaction of PKN α with a transcription factor, NDRF/NeuroD2

Transcription factors characterized by the bHLH domain play essential roles in the cell specific expression of many different genes and in some cases, the tissue-specific determination

and differentiation. The basic region is required for specific DNA binding, and the bHLH region is involved in protein-protein interaction (Davis et al., 1990). In the well-studied case of mammalian myogenesis, a critical part is played by MyoD family, including MyoD, MRF4, Myf5 and myogenin (Olson, 1990). These tissue-specific factors function *in vivo* as heterodimers with widely expressed factors such as E12, E47 and HEB and bind to a conserved DNA sequence known as an E-box. Recently, an abundance of experimental evidence has accumulated to indicate that serine/threonine protein kinases are implicated in the regulation of the transcriptional activity of these myogenic transcription factors. Phosphorylation of the DNA binding domain of myogenin by purified PKC *in vitro* inhibits DNA binding, and overexpression of constitutively active form of PKC α in the C2 muscle cell line blocks the transactivation by myogenin (Li et al., 1992b). Overexpression of catalytic subunit of PKA represses the transactivation activities of the all member of MyoD family through an indirect mechanism (Hardy et al., 1993; Li et al., 1992a; Winter et al., 1993). In contrast, overexpression of catalytic α -subunit of casein kinase II increases the transcriptional activity of MyoD and MRF4 in C3H10T1/2 fibroblast (Johnson et al., 1996). The MAPK pathway is activated in differentiating muscle cells and expression of constitutively active form of MEK1 or Raf1 enhances the transcriptional activity of MyoD (Gredinger et al., 1998). Overexpression of Mos kinase, the product of the *mos* proto-oncogene, induces increasing expression of muscle-specific genes expression whereas inhibition of Mos expression by antisense RNA results in blockage of myogenesis (Leibovitch et al., 1995). Mos protein physically interacts with MyoD homodimer, but not with the MyoD-E12 heterodimer, and phosphorylation of MyoD by activated Mos kinase inhibits the DNA-binding activity of MyoD homodimers and promotes functional MyoD-E12 heterodimer formation (Lenormand et al., 1997). Thus, the activity of bHLH transcription factors is likely to be extensively regulated by a number of different positive and negative signals through protein kinases.

The NDRF/NeuroD2-dependent transcriptional activation could be enhanced by catalytically active form of PKN α but not by kinase-deficient mutant. These results extend the

possible regulatory pathways that depend on PKN α -mediated phosphorylation of transcriptional machinery. NDRF/NeuroD2 possesses consensus phosphorylation sites for PKA, PKC and casein kinase II and previous studies have demonstrated that KW8 (a rat homologue of NDRF/NeuroD2) is phosphorylated *in vivo* (Kume et al., 1998). Recombinant PKN α purified from Sf9 cells could phosphorylate NDRF/NeuroD2 *in vitro* (data not shown). By analogy with the enhancement of the transactivation activity of MyoD by Mos kinase, PKN α could enhance the formation and/or stabilization of functional heterodimer composed of NDRF/NeuroD2 and ubiquitous partner *in vivo* by interacting with NDRF/NeuroD2 and by phosphorylating transcriptional factor. Alternatively, it is conceivable that NDRF/NeuroD2 may facilitate PKN α to target substrates of the transcriptional machinery, where PKN α can phosphorylate and activate other transcription components, such as coactivators.

Our *in situ* hybridization studies using adult brain sections reveals that NDRF/NeuroD2 mRNA is abundantly expressed in neurons of the hippocampal pyramidal cell layer, granule cell layer of the dentate gyrus, cerebral cortex and cerebellar Purkinje cells (Yasunami et al., 1996). Recent studies from our laboratory have shown that the distribution of PKN α mRNA is also observed in these regions (Hashimoto et al., 1998). The similar distribution of these transcripts suggests that interaction between PKN α and NDRF/NeuroD2 could be physiologically relevant. Further studies on the characterization of the signaling system via PKN α may provide new insights in the control of NDRF/NeuroD2-mediated transcription, which will lead to the elucidation of the molecular mechanisms involved in neuronal terminal differentiation and in the neuronal plasticity in adulthood.

Interaction of PKN β with the GAP proteins for Rho family

PKN β , as well as PKN α and PRK2, contains the Rho effector motif class I in its CZ1 region (Oishi et al., 1999) and interacts with RhoA in GTP dependent manner (data not shown). In the present study, it was demonstrated that PKN β associates with the GTPase regulators, Graf and

Graf2. These interactions may provide new insight into the role of PKN β in Rho signaling.

A large number of studies by expressing constitutively active (GTP hydrolysis-defective) mutants and dominant-negative mutant (mutant that sequesters GEFs) of Rho family GTPases suggest that they behave as simple on-off switches. However, some instances have been reported that overexpression of either constitutively active or dominant-negative mutants of Rho inhibits the same Rho-mediated biological functions, such as PDGF-stimulated Rat1 cell invasion into collagen matrices (Banyard et al., 2000) and gate and fence functions of the tight junctions in MDCK cells (Jou et al., 1998). One interpretation of these findings is that the cycle between GTP- and GDP-bound states are necessary steps to exert these biological effects. Alternatively, the modest accumulation of GTP-Rho may be required to the optimal level of activation of downstream pathways to regulate complex cellular behaviors, such as cell migration. It is possible that the association of PKN β with the GAP proteins, Graf and Graf2, is important for the feedback mechanism that regulates the balance between GDP- and GTP-Rho to promote biological responses in Rho-mediated signaling.

Activation of Rho in neuronal cells induces growth cone collapse and neurite retraction (Kozma et al., 1997; Postma et al., 1996; Tigyi et al., 1996; Katoh et al., 1996). In PC12 cells, overexpression of Graf enhances the apparent rate of Rho-mediated neurite retraction and the GAP activity of Graf is required for this effect (Taylor et al., 1999). Since Graf2 was expressed in brain (Fig. 13C) and possessed the GAP activity that was comparable to that of Graf (Fig. 14), Graf2 also may promote the Rho-mediated neurite retraction. Furthermore, the direct interaction of PKN β with Grafs opens the possibility that PKN β participates in the regulation of the retraction rate. The complex between PKN β and Graf or Graf2 may play a modulatory role for Rho signaling in neurite.

Graf and Graf2 enhanced the GTPase activities of RhoA and Cdc42Hs *in vitro* (Fig. 14). It is reported that overexpression of Graf in Swiss 3T3 cells blocks Rho-mediated stress fibers, but does not inhibit Cdc42-mediated filopodial extensions (Taylor et al., 1999), suggesting that Graf

does not behave as a GAP for Cdc42 in certain cell type. This difference in the selectivity for Rho family of the GAP activity between *in vitro* and *in vivo* is assumed to reflect the critical regulation of Graf *in vivo*, such as post-translational modification and subcellular localization. PKN β may alter the specificities and/or the targeting sites of Graf and Graf2 via direct interaction and phosphorylation in response to the upstream signaling including Rho.

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