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PKNβ interacts with the SH3 Domains of Graf and a Novel Graf Related Protein, Graf2, Which Are GTPase Activating Proteins for Rho Family[1]

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PKNβ is a novel isoform of PKNα, which is one of the target protein kinases for the small GTPase Rho. By yeast two-hybrid screening of a human embryonic kidney 293 cell cDNA library with the PKNβ linker region containing proline-rich motifs as a bait, clones encoding Graf (GAP for Rho associated with focal adhesion kinase) and a novel Graf-related protein, termed Graf2, were isolated. The full length of Graf2 contains a putative PH domain, a RhoGAP domain, and an 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 *Escherichia coli* bound directly to PKNβ. Graf or Graf2 was co-immunoprecipitated with PKNβ in COS-7 cells transiently transfected with Graf or Graf2 and PKNβ expression constructs. The catalytically active form of PKNβ phosphorylated Graf and Graf2 *in vitro*. The interplay of PKNβ and the GTPase-activating proteins, Graf and Graf2, may offer a novel mechanism regulating the Rho-mediated signaling.

Key words: GTPase activating protein, PKN, protein kinase, protein–protein interaction, Rho.

Protein kinases are responsible for the regulation of a variety of physiological processes via phosphorylation of structural and/or functional proteins. In some protein kinases, the catalytic activities and subcellular localization are regulated by direct interaction with different types of interacting proteins. The PKN family of protein kinases, comprising PKNα/PRK1/PAK1, PKNβ, and PKNγ/PRK2/PAK2, constitutes a subclass of lipid- and proteolysis-activated serine/threonine protein kinases with a catalytic domain highly homologous to those of PKC family members in the carboxyl-terminal region (*1–8*). The amino-terminal region of the PKN family contains three repeats of the CZ region (CZ1–CZ3, which correspond closely to HR1a–HR1c) and the D region (HR2), and is assumed to restrict the protein kinase activity of the catalytic domain in the absence of activators (*7–12*). The amino-terminal region of PKNα also functions as a binding interface to various proteins. Small GTPase Rho interacts with the CZ1 region of PKNα in a GTP-dependent fashion and activates the catalytic activity of PKNα (*13–15*). Cytoskeletal proteins such as intermediate filament proteins (*16, 17*) and actin cross-linking protein α-actinin (*18*) associate with PKNα, suggesting the participation of PKNα in cytoskeletal reorganization. The amino-terminal region of PKNα also interacts with the potential transcription factor PCD-17 (*19*) and the basic Helix-Loop-Helix transcription factor NDRF/NeuroD2 (*20*), raising the possibility that PKNα controls the gene expression. In addition, PKNα binds to centrosome and Golgi-localized giant anchoring protein CG-NAP, which assembles such protein kinases and phosphatases as PKA, PKCε, PP1, and PP2A (*21, 22*).

PKNβ and PRK2, but not PKNα, possess proline-rich motifs in their linker regions between the D region and the catalytic domain (*3, 6*). The proline-rich motif in the PRK2 linker region possesses the consensus sequence for adaptor protein Nck SH3-binding sequences, PxxPxRxxSL, and

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Abbreviations: PKC, protein kinase C; CZ, charged amino acids with a leucine zipper-like sequence; PKA, protein kinase A; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; SH3, src homology 3; X-α-gal, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside; GAP, GTPase-activating protein; GST, glutathione S-transferase; MBP, maltose-binding protein; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor.

directly binds to the middle SH3 domain of Nck *in vitro*, suggesting a specific role for PRK2 in Nck-mediated signaling (*23*). 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.

In the present study, we identified Graf (*24, 25*) and a novel Graf-related protein Graf2 as the binding partners of PKNβ. The PKNβ linker region directly bound to the SH3 domains of Graf and Graf2, and PKNβ phosphorylated Graf and Graf2 *in vitro*. Graf2, as well as Graf, activated the GTPase activities of RhoA and Cdc42Hs, but not of Rac1, *in vitro*.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screening of cDNA Library and Interaction Analysis—The PKNα linker region (amino acid residues 511–632) and the PKNβ linker region (amino acid residues 465–557) were fused in-frame to the carboxyl-terminus of the GAL4 DNA-binding domain in vector pGBKT7 (Clontech). The resulting plasmids were termed pGBKT7-linkPKNα and pGBKT7-linkPKNβ, respectively. pGBKT7-linkPKNβ was used to transform the yeast reporter strain AH109 together with a human kidney embryonic 293 cell library cDNA cloned carboxyl-terminal to the activation domain of GAL4 in vector pACT2 (Clontech). A total of 1.5×10^6 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, six growing clones were picked and streaked on SD/–Trp/–Leu/–His/–Ade plates containing 5 mM 3-AT and X-α-gal. The pACT2 library plasmids were isolated from *His*$^+$, *Ade*$^+$, *Mel1*$^+$ clones and retransformed into the original yeast host strain in combination with the pGBKT7-linkPKNβ, pGBKT7-linkPKNα, or pGBKT7-p53 tumor suppresser (pGBKT7-53, Clontech). At least five independent yeast colonies on SD/–Trp/–Leu plates were streaked onto SD/–Trp/–Leu/–His/–Ade plates 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 (*Mel1* gene activation). Library plasmids that activated marker expression only in the presence of PKNβ were sequenced, and the sequences obtained were analyzed using the BLAST program on the World Wide Web.

Isolation of cDNA Clones—To clone the full-length cDNA of human Graf2, 5′-RACE (5′ rapid amplification of cDNA ends) was employed using Marathon-Ready HeLa cell cDNA according to the manufacturer's instructions (Clontech). The full-length cDNA of human Graf was isolated from a HeLa cell cDNA library by PCR.

Analysis of Graf2 mRNA Expression—Multiple tissue Northern blot (Human MTN blot, Clontech) was hybridized with the ^{32}P-labeled *Eco*RV/*Eco*RI fragment of human Graf cDNA (1318–2388) or the *Eco*RI/*Bgl*II fragment of human Graf2 cDNA (1336–2361), followed by extensive washing. The blot was exposed on an imaging plate, which was then examined using a BAS1000 imaging analyzer (Fuji Film).

Preparation of Recombinant Proteins in E. coli—The cDNA fragment encoding the SH3 domain of human Graf2 (amino acid residues 734–786) was cloned into pRSET (Invitrogen). The cDNA fragment encoding human PKNβ (amino acid residues 301–610) was cloned into pMAL-c2 (New England Biolabs). The cDNA fragments encoding the carboxyl-terminal region containing the SH3 domain of human Graf (amino acid residues 596–759) and human Graf2 (amino acid residues 734–786), and those encoding the RhoGAP domain of human Graf (amino acid residues 375–583) and human Graf2 (amino acid residues 381–587) were each inserted into pGEX4T (Amersham Pharmacia Biotech). The plasmids containing the GST-RhoA, GST-Rac1, and GST-Cdc42Hs constructs were kindly provided by Dr. Kozo Kaibuchi (Nagoya University). 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.

Antibodies—The polyclonal antibody, αG2SH3, 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) was used to affinity-purify the antibody from the whole serum.

Mouse anti-HA 12CA5 (Roche Molecular Biochemicals), anti-FLAG M2 (Eastman Kodak), and peroxidase-conjugated secondary antibodies (Santa Cruz) were purchased.

Analysis of Graf2 Protein Expression—Rat tissues were removed quickly after decapitation and homogenized in 10 volumes of RIPA buffer (50 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 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 ×*g* for 1 h at 4°C, the supernatant was precleared on protein A-Sepharose 4B (Amersham Pharmacia Biotech). Cleared lysate was incubated for 2 h at 4°C in the presence of αG2SH3 antibody. Protein A-Sepharose 4B was then added, and incubation was continued for 1 h 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 h at room temperature. Immunoreactive bands were visualized with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech).

Preparation of Recombinant Proteins in Sf9 Cells—The cDNA fragment encoding the full-length 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 human Graf2 was cloned into pBlueBacHis/GST (*12*). The cDNA fragment encoding the catalytic domain of human PKNβ (amino acid residues 520–889) was cloned into pAcGHLT-C. 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 (*12*). The purified protein was dialyzed against dialysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$).

In Vitro Binding Assays—GST fusion protein and MBP fusion protein were mixed in binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5% Triton X-100, 1 μg/ml leupeptin) and incubated for 2 h at 4°C. Glutathione-Sepharose 4B or amylose resin was then added, and incubation was continued for 1 h at 4°C. After extensive washing of the resin with 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—For analysis of the interaction of PKNβ with Graf or Graf2 in culture cells, the cDNA fragment encoding the full-length human Graf or human Graf2 was cloned into pTB701-HA (*26*). pRc/CMV/hPKNβ/FLAG, for FLAG-tagged full-length PKNβ, was constructed as described (*3*). COS-7 cells were transfected with the expression plasmids by electroporation using a GenePulser II (Bio-Rad). After 2 days, cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Nonidet P-40, 1 μg/ml leupeptin). Cleared lysate was incubated with appropriate antibody at 4°C for 2 h, then protein A–Sepharose (Amersham Pharmacia Biotech) was added and the reaction was continued for a further 1 h. After extensive washing of the resin with the same buffer, the bound proteins were resolved by SDS-PAGE and then subjected to immunoblotting as described (*1*).

GTPase Assays—The GTPase activity of RhoA, Rac1, or Cdc42Hs was assayed by measuring the decreased radioactivity of [γ-^{32}P]GTP-bound form of GTPases. GST-RhoA, GST-Rac1, and GST-Cdc42Hs (400 nM) were incubated at 30°C with [γ-^{32}P]GTP (1.11 TBq/mmol) in 20 μl of GTP binding buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.3% CHAPS, 1 μM GTP), respectively, and the nucleotide exchange was stopped after 10 min by the addition of $MgCl_2$ (final concentration, 10 mM). Preloaded GTPases (final concentration, 100 nM) were diluted with 60 μl of GAP buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 1 mM GTP) and each GAP protein was added to the reaction mixture. After incubation at 25°C, the reaction was terminated by the addition of 2 ml of ice-cold stop buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$) 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.

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, pH 7.5, 8 mM $MgCl_2$, 100 μM ATP, 185 kBq of [γ-^{32}P]ATP, 5 μg/ml of phosphoacceptors, and 0.5 μg/ml of the constitutive active form of PKNβ fused to GST (GST-PKNβcat). 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 use of a BAS1000 imaging analyzer (Fuji Film).

RESULTS

Yeast Two-Hybrid Screening for PKNβ Interacting Proteins—The PKNβ linker region (amino acid residues 465–557, Fig. 1) 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 six clones were strong positive for *His3, Ade2,* and *Mel1* reporter constructs. Two of the clones, K3 and K4, were identical isolates encoding the SH3 domain of Graf (GAP for Rho associated with focal adhesion kinase) (*25*). Two other clones, K1 and K2, had an insert of 1.3 and 1.7 kbp, respectively. Sequence analysis of these clones revealed that they contained inserts of different length derived from the same cDNA (Fig. 2A). A database search for clone K2 suggested that this cDNA encodes a novel protein with a SH3 domain homologous to Graf (Fig. 2D). As shown in Table I, the yeast cells co-transformed with both the PKNβ linker region-fusion construct (pGBKT7-linkPKNβ) and clone K2 or K3 were prototrophic for histidine and adenine and exhibited the α-galactosidase activity. In contrast, the combination of pGBKT7-linkPKNα with K2 or K3 could not 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 PKNβ linker region and the carboxyl-terminal region covering the SH3 domain of Graf and Graf-related protein in the yeast two-hybrid system.

Characterization of a Novel Graf-Related Protein, Graf2—To obtain the full-length coding sequence of Graf-related protein, we performed 5′-RACE protocols. Figure 2A 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. We des-

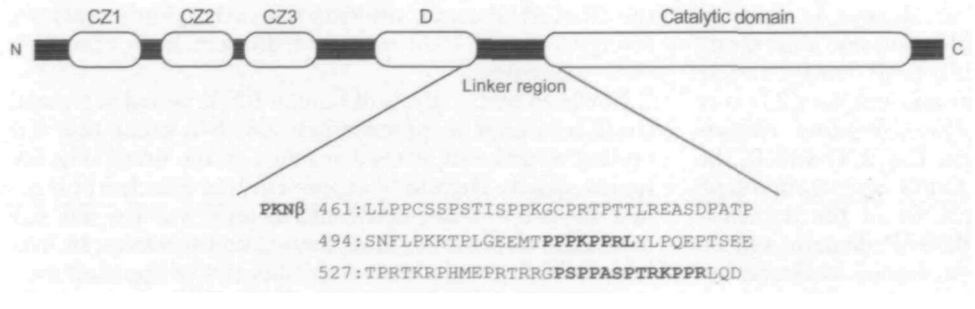

Fig. 1. **Proline-rich motifs in the PKNβ linker region.** A schematic representation of the PKN family is shown at the top. *CZ* (*CZ1–CZ3*) regions, *D* region, and *catalytic domain,* which are conserved among various vertebrates and isoforms of the PKN family, are shown as boxes. Sequence of the PKNβ linker region is shown underneath. Amino acid residues in two proline-rich motifs of PKNβ are in bold.

A

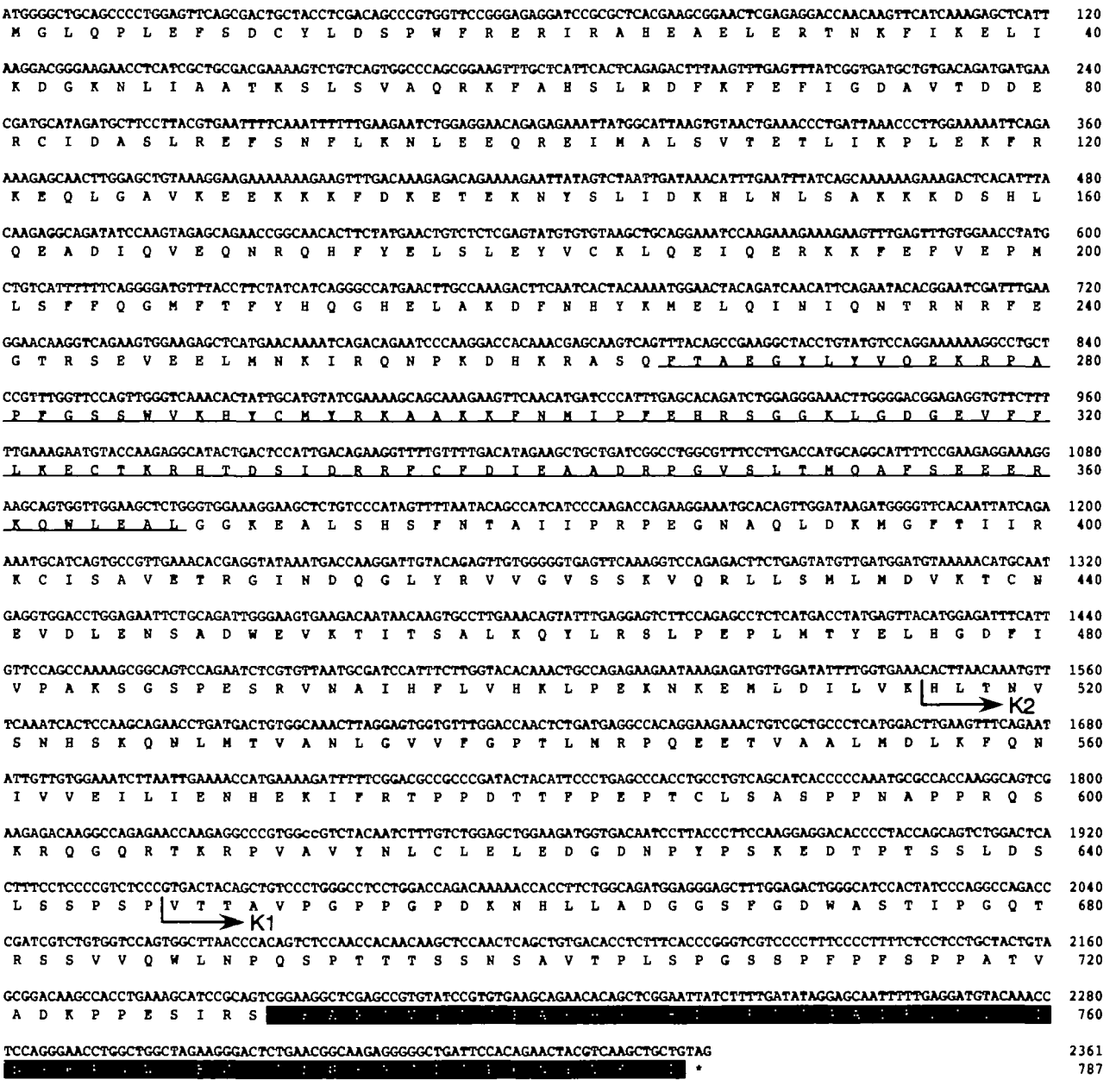

Fig. 2. A

ignated this newly identified protein 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. 2B, Pfam database analysis (*27*) revealed that Graf2 contains a putative PH domain, a RhoGAP domain and an SH3 domain. In overall architecture and size, Graf2 is very similar to human Graf and *Caenorhabditis elegans* T04C9.1 gene product. As shown in Fig. 2, C and D, the RhoGAP and the SH3 domain of Graf2 demonstrate high degrees of homology to other proteins in the database. Chicken Graf (*24*) 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 (*28*) and *Drosophila* CG8948 gene product share high sequence homology to Graf2 from their amino-terminus to the RhoGAP domain, whereas the carboxyl-terminals are divergent from Graf2 and an SH3 domain is absent in these molecules.

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. 3A, 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

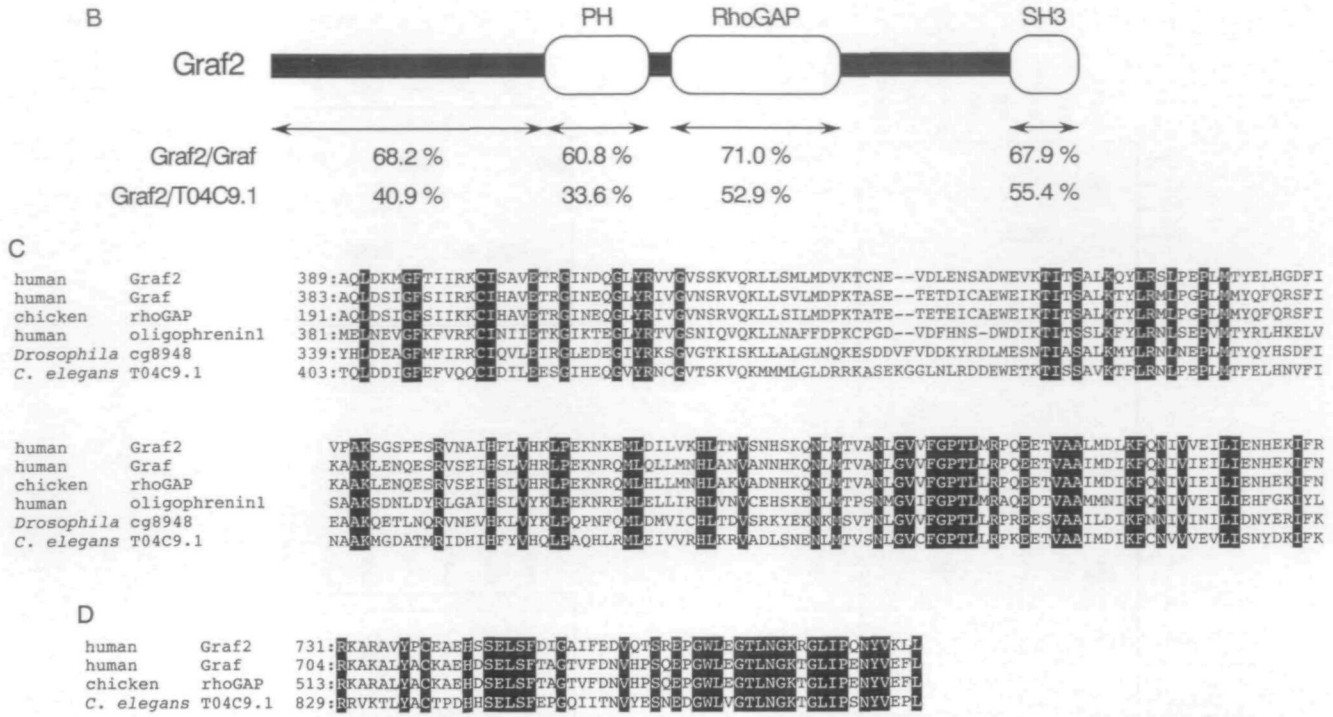

Fig. 2. **Structure of human Graf2 and sequence comparison.** (A) Sequence of the cDNA and deduced amino acid sequences of human Graf2. The amino acid sequence is shown in single-letter code. The stop codon is indicated by an asterisk. The clones isolated in the two-hybrid screening were fused to the Gal4 activation domain at the positions 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. (B) 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. (C) 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/GenBank™/EMBL Data Bank accession numbers of the sequences are as follows: human Graf, Y10388; chicken Graf, U36309: human oligophrenin 1, AJ001189: *Drosophila* CG8948, AE003500; *C. elegans* T04C9.1, U80955. (D) Sequence alignment of the SH3 domain from Graf2 and homologous proteins. Aligned identical residues are black boxed.

TABLE I. **Interaction of the PKNβ 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 prey) 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	Prey		
	LargeT	Clone K3 Graf (626–759)	Clone K2 Graf2 (506–786)
p53	+	–	–
PKNα (511–632)	–	–	–
PKNβ (465–557)	–	+	+

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. 3A, top panel).

To characterize the Graf2 proteins, we produced a polyclonal antibody against the SH3 domain of Graf2. 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. 3B, HA-Graf was expressed as a doublet band, consistent with previous analyses indicating that Graf proteins are phosphorylated in cells (*29*). In immunoprecipitates from this lysate by the antibody αG2SH3, anti-HA immunoreactivity was not detected, but in immunoprecipitates by αG2SH3 from COS-7 cells expressing HA-Graf2, the anti-HA antibody detected HA-Graf2. Neither protein was precipitated when normal rabbit serum was used. These results indicate that the αG2SH3 antibody selectively recognizes HA-Graf2 but not HA-Graf. To identify the endogenous Graf2 protein, the immunoprecipitation was performed using several rat tissue extracts. As shown in Fig. 3C, a protein migrating with a molecular mass of ~100 kDa was detected by αG2SH3 antibody in immunoprecipitates from brain, skeletal muscle, and testis. A 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 alternatively spliced forms or degradation products of Graf2.

To confirm that the protein encoded by Graf2 cDNA actually displays a GAP activity, we expressed the RhoGAP domain (amino acid residues 381–587) of Graf2 fused to GST in *E. coli* and examined its ability to stimulate the GTPase

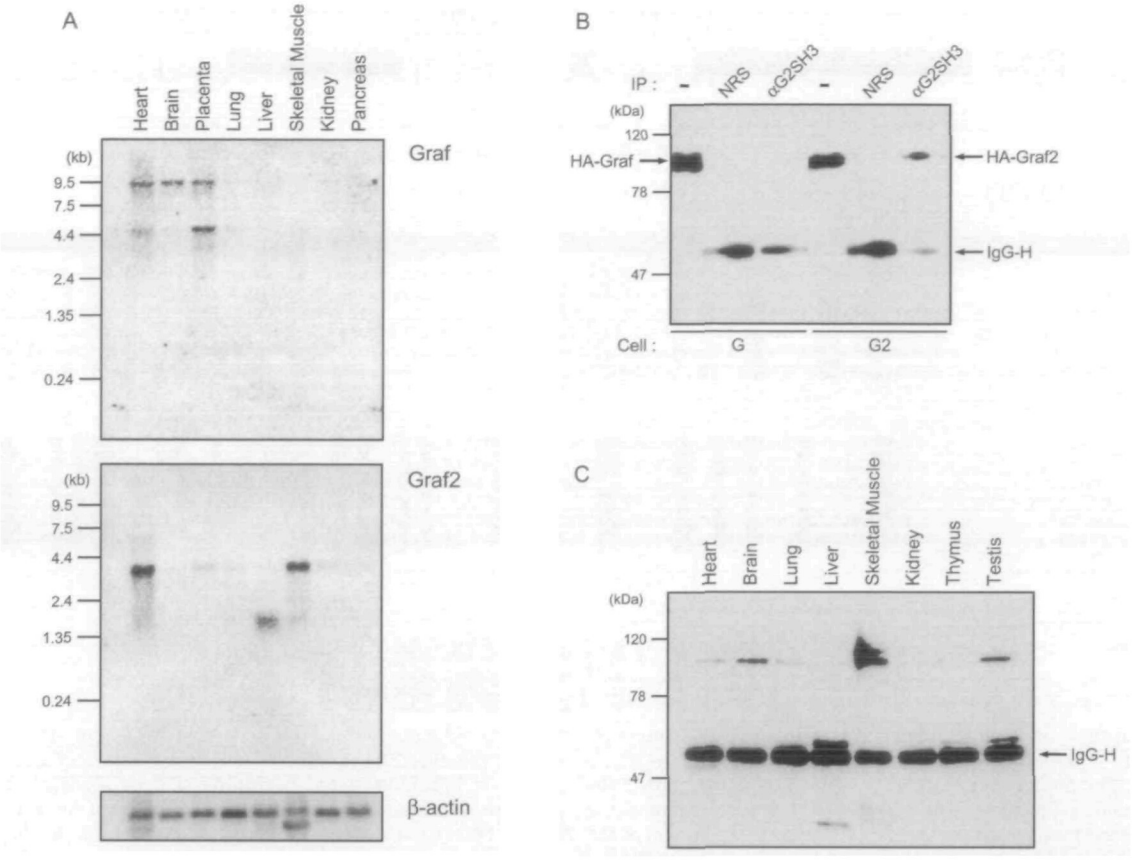

Fig. 3. **Expression pattern of Graf2 mRNAs and proteins.** (A) mRNA distributions of human Graf and Graf2. Human Multiple Tissue Northern blot was hybridized with Graf (top), Graf2 (middle), and β-actin (bottom)–specific cDNA probes. Positions of the RNA size markers are shown at the left. (B) Specificity of αG2SH3 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 (αG2SH3). 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 αG2SH3. After washing the immunoprecipitate, proteins were resolved by SDS-PAGE, transferred to membrane, and probed with antibody αG2SH3. Positions of the protein size markers are shown at the left.

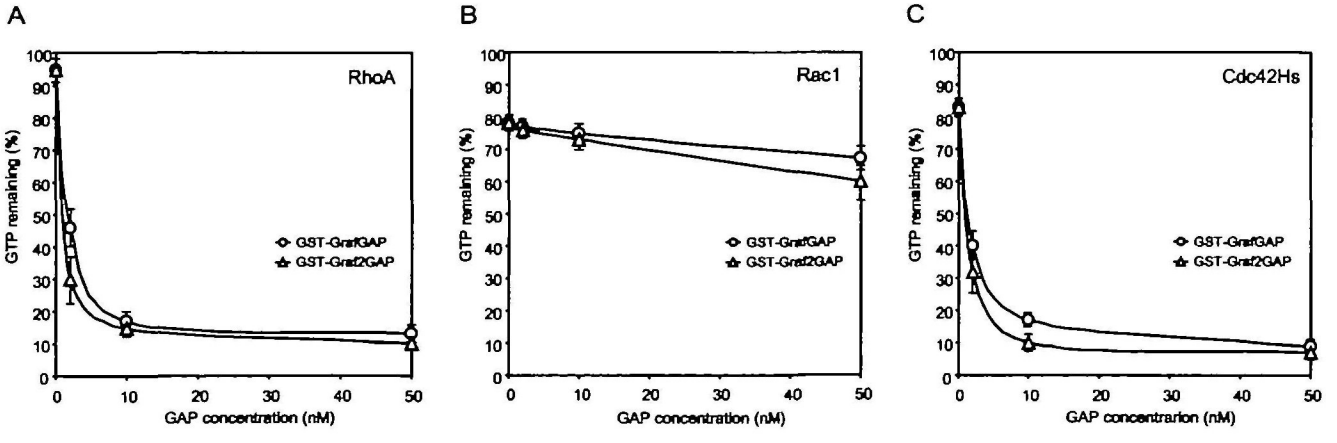

Fig. 4. **The GAP activity of Graf and Graf2.** GTPase activity was analyzed by a filter binding assay. GST-RhoA (A), GST-Rac1 (B), and GST-Cdc42Hs (C) were loaded with [γ-^{32}P]GTP for 10 min at 30°C in GTP binding buffer. $MgCl_2$ and unlabelled GTP were added. The indicated concentrations of GAP domain of Graf (*GST-GrafGAP*) or Graf2 (*GST-Graf2GAP*) expressed in *E. coli* were added to each GTPase (100 nM) and incubated for 2 min at 25°C. Shown is the remaining GTP at 2 min as percent of bound [γ-^{32}P]GTP before incubation at 25°C. Each bar represents the mean (+SD) of three independent experiments.

activities of Rho family proteins. As shown in Fig. 4, 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 toward Rac1.

Association of PKNβ with Graf and Graf2—To investigate whether PKNβ interacts directly with Graf and Graf2, we performed an *in vitro* binding analysis using the recombinant proteins purified from *E. coli*. As shown in Fig. 5A, 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 PKNβ (amino acid residues 301–610) fused to MBP (MBP-PKNβ). Reciprocal experiments confirmed the association of PKNβ with the SH3 domain of Graf or that of Graf2 (Fig. 5B). 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.

To investigate whether PKNβ interacts with Graf and Graf2 in mammalian cells, COS-7 cells were co-transfected with the expression plasmids encoding the full length of PKNβ tagged with the FLAG epitope (FLAG-PKNβ) and encoding HA-Graf or HA-Graf2. The cell lysate was subjected to immunoprecipitation with anti-FLAG antibody, and immunoprecipitates were probed with the anti-HA antibody. As shown in Fig. 6, HA-Graf2 was co-precipitated with FLAG-PKNβ by anti-FLAG antibody but not by normal mouse immunoglobulin. HA-Graf was also co-precipitated with FLAG-PKNβ. However, the co-precipitated Graf contained only the fast-migrating species, whereas Graf in cell extracts appeared as a doublet band. It is reported that phosphorylation of Graf leads to mobility shift (*29*). Thus, the association between PKNβ and Graf may be regulated by phosphorylation.

Phosphorylation of Graf and Graf2 by PKNβ—To test the ability of PKNβ to phosphorylate Graf or Graf2, the full-length GST fusion proteins purified from Sf9 cells were subjected to *in vitro* phosphorylation by PKNβ. As shown

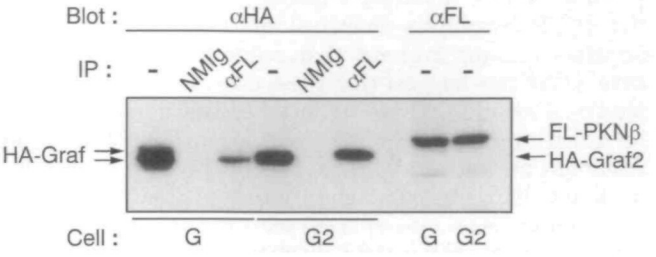

Fig. 6. **The interaction of PKNβ with Graf and Graf2 in COS-7 cells.** FLAG-PKNβ and HA-Graf (G) or HA-Graf2 (G2) were co-expressed in COS-7 cells (Cell) and immunoprecipitated (IP) with anti-FLAG (αFL) or normal mouse immunoglobulin (NMIg). Immunoprecipitates and extracts (–) were analyzed by immunoblotting with anti-HA (αHA) for Graf and Graf2, or with anti-FLAG (αFL) for PKNβ. Black arrows indicate the position of each protein.

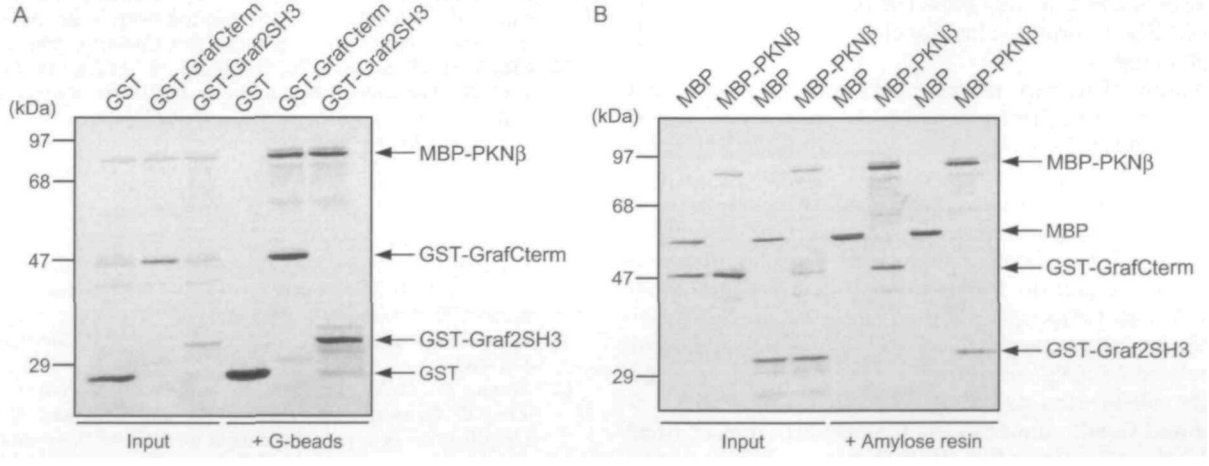

Fig. 5. **The interaction of PKNβ with Graf and Graf2 *in vitro*.** Proteins in each preparation were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The results shown are representative of three independent experiments. Positions of the protein size markers are shown at the left. Black arrows indicate the position of each protein. (A) GST-pull down assay. MBP-PKNβ (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-PKNβ. After removing aliquots (Input), proteins bound to amylose resin were collected (+Amylose resin).

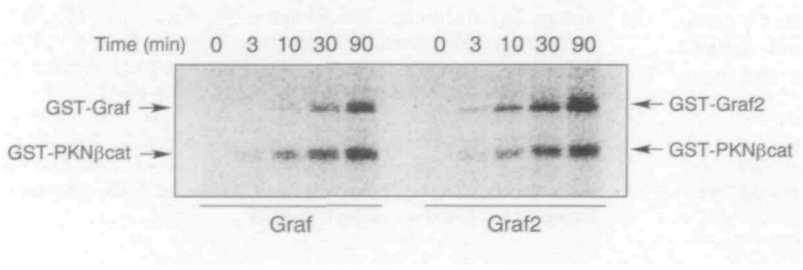

Fig. 7. **Phosphorylation of Graf and Graf2 by PKNβ.** Shown is the autoradiograph of SDS-PAGE of GST-Graf and GST-Graf2 incubated with GST-PKNβcat, the constitutively active form of PKNβ purified from Sf-9 cells, for the indicated time at 30°C. Black arrows indicate the position of each protein.

in Fig. 7, the constitutively active form of PKNβ (GST-PKNβcat) phosphorylated both GST-Graf and GST-Graf2 *in vitro*.

DISCUSSION

PKNβ, as well as PKNα and PRK2, contains the Rho effector motif class I in its CZ1 region (*3*) and interacts with RhoA in GTP-dependent manner (data not shown). In the present study, we demonstrated that PKNβ associates with the GTPase regulators Graf and Graf2. These interactions may provide new insight into the role of PKNβ in Rho signaling.

Many studies involving expression of constitutively active (GTP hydrolysis–defective) mutants and dominant-negative mutant (mutant that sequesters GEFs) of Rho family GTPases suggest that these enzymes behave as simple on-off switches. However, instances have been reported in which 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 (*30*) and gate and fence functions of the tight junctions in MDCK cells (*31*). One interpretation of these findings is that the cycle between GTP- and GDP-bound states is a necessary step to exert these biological effects. Alternatively, the modest accumulation of GTP-Rho may be required to produce the optimal level of activation of downstream pathways in order to regulate complex cellular behavior such as cell migration. It is possible that the association of PKNβ 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 (*32–35*). 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 (*36*). Since Graf2 was expressed in brain (Fig. 3C) and possessed comparable GAP activity to that of Graf (Fig. 4), Graf2 also may promote the Rho-mediated neurite retraction. Furthermore, the direct interaction of PKNβ with Grafs opens the possibility that PKNβ participates in the regulation of the retraction rate. The complex between PKNβ and Graf or Graf2 may play a modulatory role for Rho signaling in neurites.

Graf and Graf2 enhanced the GTPase activities of RhoA and Cdc42Hs *in vitro* (Fig. 4). It is reported that overexpression of Graf in Swiss 3T3 cells blocks Rho-mediated stress fibers, but does not inhibit Cdc42-mediated filopodial extensions (*36*), suggesting that Graf does not behave as a GAP for Cdc42 in certain cell types. 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. PKNβ 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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