Characterization of a Novel Giant Scaffolding Protein, CG-NAP, That Anchors Multiple Signaling Enzymes to Centrosome and the Golgi Apparatus

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A novel 450-kDa coiled-coil protein, CG-NAP (centrosome and Golgi localized PKN-associated protein), was identified as a protein that interacted with the regulatory region of the protein kinase PKN, having a catalytic domain homologous to that of protein kinase C. CG-NAP contains two sets of putative RII (regulatory subunit of protein kinase A)-binding motif. Indeed, CG-NAP tightly bound to RIIα in HeLa cells. Furthermore, CG-NAP was coimmunoprecipitated with the catalytic subunit of protein phosphatase 2A (PP2A), when one of the B subunit of PP2A (PR130) was exogenously expressed in COS7 cells. CG-NAP also interacted with the catalytic subunit of protein phosphatase 1 in HeLa cells. Immunofluorescence analysis of HeLa cells revealed that CG-NAP was localized to centrosome throughout the cell cycle, the midbody at telophase, and the Golgi apparatus at interphase, where a certain population of PKN and RIIα were found to be accumulated. These data indicate that CG-NAP serves as a novel scaffolding protein that assembles several protein kinases and phosphatases on centrosome and the Golgi apparatus, where physiological events, such as cell cycle progression and intracellular membrane traffic, may be regulated by phosphorylation state of specific protein substrates.

Stimulation of various signaling cascades results in activation of protein kinases and phosphatases, which alter phosphorylation states of their respective substrates, leading to diverse physiological responses. Many serine/threonine protein kinases and phosphatases have relatively broad and overlapping substrate specificity. One of the mechanisms to organize such enzymes into individual signaling pathways is targeting them to discrete subcellular locations by anchoring proteins. For instance, type II cyclic AMP (cAMP)-dependent protein kinase (PKA) is targeted to intracellular compartments through association of its regulatory subunit RII with protein kinase A anchoring proteins (AKAPs) (1, 2). Three types of targeting proteins for protein kinase C (PKC) have been described (3–5). Three classes of phosphatase-targeting subunits have been identified that are specific for protein phosphatase 1 (PP1), PP2A, and PP2B (6).

Recently, a new class of multivalent adapter proteins that coordinate the location of multienzyme signaling complexes has been identified. For example, the pheromone mating response in yeast proceeds efficiently by clustering the successive members in the mitogen-activated protein kinase cascade on the scaffold protein STE5 (7). AKAP79 anchors not only PKA but also PKC and PP2B at the postsynaptic densities of mammalian synapses (8). AKAP250 (gravin) targets both PKA and PKC to the membrane cytoskeleton and filopodia of cells (9).

PKN is a serine/threonine protein kinase, having a catalytic domain homologous to the PKC family in the C-terminal region and a unique regulatory region in the N-terminal region (10). PKN is activated by a small GTPase Rho (11–13), unsaturated fatty acids such as arachidonic acid (14, 15), and by truncation of the N-terminal regulatory region (14, 16). Since PKN represents broad substrate specificity in vitro (10), PKN function may be regulated by intracellular targeting as well as by specific interaction with its substrates. We previously demonstrated that PKN associates with and phosphorylates intermediate filament proteins in vitro (17, 18), which may be physiological substrates for PKN. PKN interacts with the actin cross-linking protein α-actinin, but does not efficiently phosphorylate it in vitro (19), suggesting that α-actinin serves as a scaffolding protein that targets PKN to specific cytoskeletal substrates.

In the present study, a cDNA encoding a novel coiled-coil protein with predicted molecular mass of 450 kDa was identified as a PKN-interacting protein by a yeast two-hybrid screen using the N-terminal regulatory region of PKN as bait. This protein was localized to centrosome throughout the cell cycle and the Golgi apparatus at interphase. Therefore, it was designated CG-NAP (centrosome and Golgi localized PKN-associated protein). CG-NAP interacted with various signaling enzymes including protein kinases (PKN and PKA) and phosphatases (PP1 and PP2A), and thus, may function as a novel multivalent adapter protein at these organelles.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EMBL Data Bank with accession number AB019691.

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§ The abbreviations used are: PKA, protein kinase A; AKAP, protein kinase A anchoring protein; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; CG-NAP, centrosome- and Golgi-localized PKN-associated protein; GST, glutathione S-transferase; aa, amino acid(s); BFA, brefeldin A; HA, hemagglutinin; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); Pipes, 1,4-piperazinediethanesulfonic acid; ad, activation domain; bd, binding domain; DTAF, dichlorotriazinyl amino fluorescein.
addition of glutathione-Sepharose 4B, the reaction was continued for an additional 30 min. The resin was extensively washed with the same buffer, then bound proteins were eluted, resolved by SDS-PAGE, and the radioactive bands were visualized using a Fuji BAS1000 imaging analyzer.

For in vitro homodimer formation, the purified deletion mutants (His)_6-tagged HH and GST-fused HH were incubated in a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% Triton X-100, 3 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol at 4°C for 1 h. Then proteins bound to glutathione-Sepharose 4B were analyzed by immunoblotting with anti-His antibody.

Cell Culture, Transfection, and Drug Treatments— COS7 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Mammalian expression plasmids for HA- and FLAG-tagged proteins were constructed by inserting the corresponding cDNA fragments into pTBT701-HA and pTBT701-FLAG (20), respectively. Plasmids pMbPKN3 (10) and pRC/CMV/PKN-FL (16) were used to express PKN and FLAG-tagged PKN, respectively. For transient expression studies, COS7 cells were transfected with expression plasmids by electroporation using GenePulser II (Bio-Rad). To disrupt the Golgi structure, HeLa cells were treated with 20 μg/ml nocodazole (Sigma) for 90 min or 10 μg/ml brefeldin A (BFA) (Wako, Japan) for 15 min.

Antibodies— Polyclonal antisera against CG-NAP designated as αEE and αN2 were prepared by immunizing rabbits with bacterially synthesized GST-fused fragments of 423-542 and 2875-2979, respectively (see Fig. 3). For immunofluorescence analysis, αEE was affinity-purified using antigen-coupled Sepharose beads according to the manufacturer’s instruction (Amersham Pharmacia Biotech). Polyclonal antisera against PKN, αC6 and αN2, were previously described (21). The following antibodies were purchased: anti-α-tubulin GTU88, anti-Golgi 58K protein, and anti-α-tubulin DM1A (SIGMA); anti-PKA-RIIα, anti-P2A-C, and anti-PP1 (Transduction Laboratories); mouse anti-α-HA 12CA5, and rat anti-HA 3F10 (Roche Molecular Biochemicals); and anti-PKA-C (Eastman Kodak); anti-His(6)-tagged His antibody (Qiagen); rhodamine-conjugated anti-rabbit IgG, and DTAF-conjugated anti-mouse IgG (Chemicon International); peroxidase-conjugated secondary antibodies (Santa Cruz).

Immunoprecipitation and Immunoblotting— Cells were lysed with a buffer containing 20 mM Tris-HCl at pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin. Cleared lysates were incubated with the appropriate antibody at 4°C for 2 h, then Protein G-Sepharose (Amersham Pharmacia Biotech) was added and the reaction was continued for another 1 h. After extensively washing the resin with the same buffer, the bound proteins were resolved by SDS-PAGE and then subjected to immunoblotting as described (10). Blots were visualized by enhanced chemiluminescence method.

Northern Blotting— Polyadenylated RNA was prepared from HeLa cells using QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). The blot of HeLa mRNA (3 μg) was incubated with the [32P]-labeled probe prepared from the cDNA insert of clone 2-43, followed by extensive washing. The radioactive band was then visualized using a Fuji BAS1000.

Immunofluorescence Microscopy— Cells grown on cover glasses were fixed with 4% paraformaldehyde and washed with PBS, then permeabilized with 0.1% Triton X-100, then incubated with the relevant antibody for 1 h at room temperature. Cells were then stained with secondary antibodies and visualized using a confocal laser scanning fluorescent microscope (Zeiss), the same microscope equipped with a 40× water immersion objective. The images were analyzed with the MetaMorph software (Universal Imaging Corporation).

For in vivo homodimer formation, the purified deletion mutants (His)₆-tagged HH and GST-fused HH were incubated in a buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 150 mM NaCl, 0.05% Triton X-100, 1 mM EDTA, and 1 μM leupeptin at 4°C for 1 h. After

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**— The N-terminal region (amino acids 1–540) of PKN fused to the Gal4 DNA binding domain (Gal4bd) was used as bait to screen a million clones of a human brain cDNA library fused to the Gal4 transcription activation domain (Gal4ad) as described (17). Screening to isolate PR130 was performed using a human fetal kidney cDNA library fused to the Gal4ad (CLONTECH) with a fragment of PKN (aa 136–306) fused to the LexA DNA binding domain (LexAbd) as bait. The yeast expression plasmids for proteins fused to the LexAbd and those to the VP16 transcription activation domain (VP16ad) were constructed by subcloning the corresponding cDNA fragments into pBTM116 and pVP16, respectively.

**Isolation of cDNA Clones**— The cDNA clones encoding CG-NAP were isolated by screening human neuroblastoma and HeLa cDNA libraries with a [32P]-labeled probe prepared from the insert of clone 2-43. To obtain the entire cDNA sequence, 5’-RACE and 3’-RACE methods were employed using a Marathon-Ready cDNA library (human hippocampus) according to the manufacturer’s instruction (CLONTECH). Mammalian expression plasmid for full-length CG-NAP was constructed by assembling these clones into pTBT701-HA (20).

Full-length cDNAs of human RIAs and human PR130 were obtained by PCR cloning using cDNA libraries of a human lung cancer cell line and human fetal kidney, respectively.

**Preparation of Recombinant Proteins in Escherichia coli**— Expression plasmids for proteins fused to glutathione S-transferase (GST) were constructed by subcloning the corresponding fragments into pGEX4T-2 (Amersham Pharmacia Biotech). An expression plasmid for the deletion mutant HH tagged with (His)₆-epitope was constructed by subcloning the corresponding fragment into pRSET A (Invitrogen). GST-fused and (His)₆-tagged recombinant proteins were expressed in E. coli and purified by using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and nickel-NTA-agarose (Qiagen), respectively, according to the manufacturer’s instruction.

**In Vitro Binding Assay**— [35S]Methionine-labeled PKN2 (aa 1–474 of PKN/17) was incubated with GST-P#2-43 in a buffer containing 20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol, 150 mM NaCl, 0.05% Triton X-100, 1 mM EDTA, and 1 μM leupeptin at 4°C for 1 h. After

**FIG.1. Interaction between PKN and P#2-43.** A, analysis by yeast two-hybrid system. L40 cells were cotransfected with expression plasmids encoding various proteins fused to LexAbd (left) and those fused to VP16ad (right) as indicated in left panel. Right panel shows development of blue color 1 h after initiating filter assays. Murine tumor suppressor p53 and SV40 large T antigen were used as controls. B, analysis by in vitro binding assay. 2S-Labeled in vitro translated PKN2 (aa 1–474 of PKN) was incubated with bacterially synthesized GST or GST-fused P#2-43. After removing aliquots (Input), proteins bound to glutathione-Sepharose 4B were collected (Output). Proteins in “Input” and “Output” preparations were separated on SDS-PAGE followed by visualization of radioactive bands using a Fuji BAS1000. C, analysis by immunoprecipitation. HA-tagged P#2-43 and full-length PKN were coexpressed in COS7 cells and immunoprecipitated (IP) with anti-PKN (αN2), normal rabbit serum (NRS), anti-HA 12CA5 (αHA), or normal mouse immunoglobulin (NMIg). P#2-43 and PKN in immunoprecipitates and in extracts (−) were visualized by immunoblotting with anti-HA 3F10 (αHA) and αN2, respectively.
RESULTS

Yeast Two-hybrid Screen for PKN Interacting Proteins—The N-terminal region (aa 1–540) of PKN was used as bait to screen a human brain cDNA library by yeast two-hybrid system as described (17). Clone 2-43 contained a 1.3-kilobase pair cDNA insert encoding a novel and partial amino acid sequence, which was named P#2-43. Other combinations of two-hybrid constructs further confirmed the specific interaction between the N-terminal region of PKN and P#2-43 (Fig. 1). Binding of PKN to P#2-43—To investigate whether PKN directly interacts with P#2-43 in vitro, GST pull-down assay was performed. In vitro translated PKNN2 (aa 1–474 of PKN) specifically bound to GST-fused P#2-43 (Fig. 1B, lane 4). To confirm the interaction between PKN and P#2-43 within the cellular context, immunoprecipitation was performed using COS7 cells expressing HA-tagged P#2-43 and full-length PKN (Fig. 1C). Anti-PKN antibody (αN2) coimmunoprecipitated P#2-43 (lane 3) with PKN, and conversely, anti-HA 12CA5 coimmunoprecipitated PKN (lane 5) with P#2-43. These results indicate that PKN directly interacts with P#2-43 through the N-terminal region.

Primary Structure of CG-NAP—We obtained the presumptive full-length coding sequence from human cDNA libraries of neuroblastoma, hippocampus, and HeLa cells by conventional hybridization screening in combination with 5’- and 3’-RACE methods. The combined cDNA sequence contained an open reading frame of 11,700 bp encoding a polypeptide of 3,899 amino acids with a predicted molecular mass of 451,803 daltons (Fig. 2A). We designated this giant protein as CG-NAP (centrosome- and Golgi-localized PKN-associated protein). CG-NAP was a novel protein; however, BLAST search yielded two proteins that are highly homologous to partial regions of CG-NAP: human yotiao (22) and rabbit AKAP120 (23), corresponding to aa 1–1626 and 2049–3060, respectively (Fig. 3). CG-NAP also represents limited and relatively weak homology with pericentrin (Fig. 3), a centrosomal protein (24). CG-NAP contains four leucine zipper-like motifs (Fig. 2A) and many stretches of coiled-coil structure (Fig. 2B). These structural features are thought to be involved in association with other proteins and/or homodimerization/homo-oligomerization (25).

Homodimerization of P#2-43 was suggested by the yeast two-hybrid assay using a combination of P#2-43-LexAbd and P#2-43-VP16ad (data not shown). Therefore, homodimerization of the N-terminal region of CG-NAP was examined in vitro using a deletion mutant HH (aa 17–859). (His) 6-tagged HH was copurified with GST-fused HH by glutathione-Sepharose (Fig. 4A).

FIG. 2. Primary structure and expression of CG-NAP. A, primary structure of CG-NAP. Predicted amino acid sequence of full-length CG-NAP is shown with dark boxes indicating leucine residues in leucine zipper-like motifs. The coding region of the original clone 2-43 is shaded. B, coiled-coil analysis of CG-NAP by COILS program (43). C, homodimerization of the N-terminal region of CG-NAP. GST or GST-fused HH (aa 17–859 of CG-NAP) was incubated with (His) 6-tagged HH. After removing aliquots (Input), proteins bound to glutathione-Sepharose 4B were collected (Output). Proteins in “Input” and “Output” preparations were analyzed by immunoblotting with anti-His antibody. D, Northern blots of CG-NAP. Polyadenylated RNA from HeLa cells was probed with cDNA insert from clone 2-43. Position of mRNA is indicated by arrowhead. E, immunoblots of recombinant and endogenous CG-NAP. HA-tagged CG-NAP expressed in COS7 cells was immunoprecipitated with 12CA5 (αHA) or with normal mouse immunoglobulin (NMIg). Immunoprecipitates and extracts of HeLa cells (H) and U937 cells (U) were separated on 4.5% SDS-PAGE, followed by immunoblotting with αEE, αBH, or control rabbit serum (NRS) as indicated.
Two-hybrid clone
P#2-43

Antigen

Deletion Mutants
HH
ES
MB
BB
BS
BLAST
yotiao
AKAP120
Pericentrin

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Fig. 3. Schematic representation of various constructs of CG-NAP and the corresponding positions of cDNA sequences yielded by BLAST search. Schematic representation of the structure of CG-NAP is shown on the top. LZ, leucine zipper-like motif; PP1, putative PP1 binding motif; RII, putative RII binding motif. Aligned below are locations of polypeptide P#2-43 encoded by the original clone 2-43, the EE and BH fragments bacterially expressed as GST-fused proteins to generate rabbit polyclonal antisera aEE and aBH, respectively, and various deletion constructs of CG-NAP. cDNAs representing high sequence homology obtained by BLAST search, yotiao (22), AKAP120 (23), and pericentrin (24) are also shown at corresponding regions of CG-NAP with percentage of amino acid sequence homology in parentheses. Start and end positions of each fragment are indicated.

2C), suggesting the homodimerization (or homo-oligomerization) of this protein. Furthermore, we independently confirmed the homodimerization by coimmunoprecipitation experiment using different epitope-tagged constructs of CG-NAP deletion mutant (aa 1–1280) coexpressed in COS7 cells (data not shown).

Expression of CG-NAP mRNA and Protein—Northern blots using polyadenylated RNA of HeLa cells revealed a single band longer than 12 kilobase pairs (Fig. 2D). CG-NAP mRNA of the identical size was ubiquitously expressed at low abundance in human tissues (data not shown).

To examine whether the cDNA sequence obtained here encoded the complete CG-NAP, we performed immunoblotting using antisera aEE raised against a CG-NAP fragment EE (Fig. 3). Recombinant CG-NAP expressed in COS7 cells (Fig. 2E, lane 2) co-migrated with the endogenous CG-NAP in HeLa (lane 3) and U937 (lane 4) cells. The size of the band appeared to agree with the calculated molecular mass of 450 kDa. Another antisera, aBH, raised against a different part of CG-NAP (Fig. 3) also detected a band of the same size (Fig. 2E, lane 6). These results indicate that the cDNA sequence obtained here encodes full-length CG-NAP.

Localization of CG-NAP to Centrosome, the Midbody, and the Golgi Apparatus—Subcellular localization of CG-NAP was examined by immunofluorescence analysis using aEE in HeLa cells at various phases of the cell cycle (Fig. 4A). Cells were extracted with nonionic detergent before fixation to visualize proteins of low abundance associated with intracellular structures. In interphase cells, CG-NAP was localized to one spot at the perinuclear region and to a dispersed network near the spot (Fig. 4A, a), presumably corresponding to centrosome and the Golgi apparatus, respectively. In mitotic cells, CG-NAP was localized to the spindle poles (Fig. 4A, d, g, and j), and to extremities of the midbody in the cells at telophase/cytokinesis (Fig. 4A, j). Antiserum aBH also gave the identical staining (Fig. 4B, c), whereas normal rabbit serum did not (data not shown). Subcellular distribution of CG-NAP was similar in other cell lines, such as SaOS2, TIG1, HEK293, and NIH3T3 cells (data not shown). Localization of CG-NAP to centrosome and the midbody was confirmed by double-staining, using aEE or aBH with an antibody against a centrosomal protein γ-tubulin (26) (Fig. 4B). CG-NAP was also co-stained with Golgi 58K protein at perinuclear area (Fig. 4C, a and b). We further examined the relationship between CG-NAP and the Golgi apparatus using the microtubule-destabilizing agent nocodazole (27) and the fungal metabolite BFA (28), which are known to disrupt the Golgi apparatus by distinct mechanisms. Nocodazole treatment of HeLa cells dispersed the perinuclear CG-NAP staining into scattered pattern throughout the cytoplasm (Fig. 4C, d), which is characteristic for the Golgi staining of nocodazole-treated cells. BFA treatment disrupted the perinuclear CG-NAP staining (Fig. 4C, e). BFA-induced Golgi
disruption is reversible (28), and the CG-NAP staining was also recovered by incubation in the absence of the drug (Fig. 4C, f). Either treatment did not change the centrosomal staining of CG-NAP. These results indicate that CG-NAP is localized to centrosome throughout the cell cycle, the midbody at telophase, and the Golgi apparatus at interphase. Immunostaining of recombinant CG-NAP expressed in COS7 cells detected an intense spot at the perinuclear area (Fig. 4D, a), which was obvious when cells were fixed after detergent extraction (Fig. 4D, b). This spot was colocalized with γ-tubulin (data not shown), suggesting that recombinant CG-NAP is predominantly localized to centrosome.

Interaction of PKN with Full-length CG-NAP—We next examined the interaction between PKN and full-length CG-NAP. PKN was coimmunoprecipitated with full-length CG-NAP when both proteins were exogenously expressed in COS7 cells (Fig. 5A). Immunofluorescence analysis revealed that a certain population of PKN was localized to centrosome in the cells fixed after detergent extraction (Fig. 5B), although this protein is predominantly located in the soluble fraction of the cells (21). These results suggest that PKN is associated with CG-NAP under physiological condition.

CG-NAP as AKAP—AKAP120 (23) shows high sequence homology with a part of CG-NAP (Fig. 6A), and a putative RII-binding motif forming an amphipathic helix (29) was conserved in CG-NAP at aa 2540–2558 (Motif 2 in Fig. 6A). Another RII-binding motif was also found at aa 1438–1455 (Motif 1 in Fig. 6A). We therefore examined whether these regions could bind to RIIα. Deletion mutants ES (aa 1229–1917, Fig. 3) and MB (aa 2380–2876, Fig. 3) were coimmunoprecipitated with RIIα (Fig. 6B). Furthermore, interaction between RIIα and full-length CG-NAP was observed using exogenously expressed proteins in COS7 cells (Fig. 6C, lane 3), and using endogenous proteins in HeLa cells (Fig. 6C, lane 5). These data indicate that CG-NAP binds to RIIα at high affinity and constitutively, and thus, is a novel AKAP. Immunofluorescence analysis de-
detected RIIα in cytosol and in other organelles, when cells were fixed without detergent extraction (Fig. 6D, b). On the other hand, RIIα was found to be colocalized with CG-NAP in the cells fixed after detergent extraction (Fig. 6D, c and d).

**Association of CG-NAP with PP2A**—Several AKAPs associate not only with PKA but also with other signaling enzymes including PP2B (8). We have isolated another cDNA clone encoding aa 203–1150 of PR130 as PKN-interacting protein by yeast two-hybrid screen (Fig. 7A). PR130 is one of the B subunits of PP2A, and its mRNA is expressed ubiquitously at low levels (30); however, a heterotrimeric PP2A holoenzyme containing PR130 has not been described. We found that a complex consisting of PR130, the A subunit (PP2A-A), and the catalytic subunit (PP2A-C) was formed in insect cells in an equal molar ratio and exhibited phosphatase activity.2 These data indicate that PR130 is a functional B subunit of the PP2A holoenzyme.

Next, we examined the interaction between PKN and PR130 by coimmunoprecipitation using full-length proteins. PR130 and PKN associated weakly but significantly (Fig. 7B). Since the efficiency of coimmunoprecipitation was relatively low and

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The present study discovered a novel 450-kDa protein CG-NAP ubiquitously expressed in human tissues. CG-NAP is localized to centrosome throughout the cell cycle, the midbody at telophase, and the Golgi apparatus at interphase in cultured cell lines. We have demonstrated that CG-NAP interacts with PKN, RIIα subunit of PKA, PP2A through its regulatory B subunit PR130, and the catalytic subunit of PP1. Therefore, CG-NAP may function as a scaffolding protein for the subcellular targeting of these enzymes, and thus may be a novel multivalent adapter protein for signaling enzymes as well as a new AKAP.

AKAPs localized to centrosome (AKAP350) (32) and the Golgi apparatus (AKAP85) (33) have been identified by RI overlay of human lymphoblast lysates. The relationship be-

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**FIG. 7.** Interaction of PP2A or PP1 with CG-NAP. A, interaction of PKN and PR130, a regulatory subunit of PP2A, in yeast two-hybrid assay. Yeast L40 cells were cotransfected with combinations of expression vectors as indicated: PKN (aa 136–296 of PKN) or p53 fused to LexAbd (left) and PR130 (aa 203–1150 of PR130) or SV40 large T antigen fused to VP16ad (right). Blue color developed 1 h after initiation of the filter assay on YPA complementation strips (left) and blue color developed 1 h after initiation of the filter assay on YPA complementation strips (right). B, coimmunoprecipitation of PR130 with PKN. HA-tagged full-length PKN (HA-PK\#2-43) and FLAG-tagged full-length PKN (FL-PKN) were coexpressed in COS7 cells and immunoprecipitated with M2 (αFL), 12CA5 (αHA), or normal mouse immunoglobulin (NMIg). Extracts (−) and immunoprecipitates were analyzed by immunoblotting with M2 (αFL) or 3F10 (αHA). C, coimmunoprecipitation of PR130 with CG-NAP. FLAG-tagged PR130 (FL-PK\#2-43) was expressed in COS7 cells. Endogenous CG-NAP in cell extracts was immunoprecipitated with αBH or normal rabbit serum (NRS) as a control. FL-PR130 (FL-PK\#2-43) was coexpressed in COS7 cells (Fig. 7F, lane 1), and furthermore, with endogenous CG-NAP in HeLa cells (Fig. 7F, lane 2). These results indicated that PP1-C associates with CG-NAP under physiological condition.

**DISCUSSION**

The endogenous catalytic subunit of PP1 (PP1-C) interacted with PK\#2-43 (containing the putative PP1 binding motif) expressed in COS7 cells (Fig. 7F, lane 3), and furthermore, with endogenous CG-NAP in HeLa cells (Fig. 7F, lane 2). These results indicated that PP1-C associates with CG-NAP under physiological condition.

**DISCUSSION**

The present study discovered a novel 450-kDa protein CG-NAP ubiquitously expressed in human tissues. CG-NAP is localized to centrosome throughout the cell cycle, the midbody at telophase, and the Golgi apparatus at interphase in cultured cell lines. We have demonstrated that CG-NAP interacts with PKN, RIIα subunit of PKA, PP2A through its regulatory B subunit PR130, and the catalytic subunit of PP1. Therefore, CG-NAP may function as a scaffolding protein for the subcellular targeting of these enzymes, and thus may be a novel multivalent adapter protein for signaling enzymes as well as a new AKAP.

AKAPs localized to centrosome (AKAP350) (32) and the Golgi apparatus (AKAP85) (33) have been identified by RI overlay of human lymphoblast lysates. The relationship be-

direct interaction between PKN and PR130 in vitro was hardly detected by GST-pull down assay (data not shown), we speculated that this interaction is indirect and mediated by some adapter protein such as CG-NAP. Thus, we examined whether PR130 interacted with CG-NAP using COS7 cells expressing full-length PR130, 12CA5 (αHA), or normal mouse immunoglobulin (NMIg) as a control. Extracts (−) and immunoprecipitates were analyzed by immunoblotting with 3F10 (αHA). E, coimmunoprecipitation of PP2A-C with CG-NAP through binding with PR130. FL-PR130 and HA-tagged BS were coexpressed in COS7 cells and immunoprecipitated with M2 (αFL), 12CA5 (αHA), or normal mouse immunoglobulin (NMIg). The extracts (−) and the immunoprecipitates were examined for the presence of endogenous PP2A-C by immunoblotting. PP2A-C in lane 6 implies that PP2A-C interacted with HA-BS through binding with PR130. F, interaction of the catalytic subunit of PP1 with CG-NAP. FLAG-tagged PK\#2-43 expressed in COS7 cells was immunoprecipitated with M2 (lane 3). HeLa cell extracts were immunoprecipitated with αBH (lane 2) or normal rabbit immunoglobulin (NRS, lane 1) as a control. Immunoprecipitates and extracts (−) of the COS7 cells (C, lanes 5 and 6) and HeLa cells (H, lane 4) were analyzed by immunoblotting with anti-PP1-C (αPP1) for endogenous PP1-C, or with M2 (αFL) for PK\#2-43.
tween these AKAPs and CG-NAP remains unclear, since amino acid sequences of these AKAPs are currently unknown. In the course of this study, two novel proteins, rabbit AKAP120 (23) and human yotiao (22), were discovered, both of which represent high sequence homology with partial regions of CG-NAP (Fig. 3). In addition, contiguous BAC clones of human genome, RG293F11 (from chromosome 7q21–22) and GS541B18 (from chromosome 7q21), were found to cover aa 20–1639 and 1793–3768, respectively, of CG-NAP, suggesting that CG-NAP is encoded by a single gene located at chromosome 7q21–22. AKAP120 may be coded by a rabbit homolog of CG-NAP. Yotiao might be a partial clone or the product of the alternative splicing or post-translational proteolytic processing of CG-NAP.

Phosphorylation of centrosomal proteins is suggested to be involved in the regulation of centrosomal function (34). Various protein kinases and phosphatases are localized to centrosome (6), and some of the kinases are implicated in the regulation of centrosome separation (35, 36) and microtubule nucleation (37). Protein phosphorylation is also implicated in mitotic Golgi fragmentation (38) and separation (35, 36) and microtubule nucleation (37). Protein phosphorylation is also implicated in mitotic Golgi fragmentation (38) and separation (35, 36) and microtubule nucleation (37). Protein phosphorylation is also implicated in mitotic Golgi fragmentation (38) and separation (35, 36) and microtubule nucleation (37). Protein phosphorylation is also implicated in mitotic Golgi fragmentation (38) and separation (35, 36) and microtubule nucleation (37).

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REFERENCES

1. Hirsch, A. H., Glantz, S. B., Li, Y., You, Y. & Rubin, C. S. (1992) J. Biol. Chem. 267, 2131–2134
2. Dell’Acqua, M. L. & Scott, J. D. (1997) J. Biol. Chem. 272, 12881–12884
3. McMillan-Rosen, D., Khaner, H. & Lopez, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3997–4000
4. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsu, T., Hamajima, K., Okawa, K., Iwamatsu, A. & Kaibuchi, K. (1996) Science 271, 645–650
5. Chou, K. Y., Satterberg, B., Lyons, D. M. & Elion, E. A. (1994) Cell 78, 499–512
6. Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S. & Scott, J. D. (1996) Science 271, 1589–1592
7. Tsukada, J., Kuroda, S., Tokunaga, C., Kiyohara, Y., Higuchi, O., Konishi, H., Mizuno, K., Mukai, H., Ono, Y., Kakizuka, A. & Narumiya, S. (1998) Science 271, 645–648
8. 13. Shibata, H., Mukai, H., Inagaki, Y., Homma, Y., Kinura, K., Kaibuchi, K., Narumiya, S. & Ono, Y. (1996) FEBS Lett. 385, 221–224
14. Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., Miyahara, M., Hori, K. & Ono, Y. (1994) Biochem. Biophys. Res. Commun. 204, 348–356
15. Kitagawa, M., Mukai, H., Shibata, H. & Ono, Y. (1995) Biochem. J. 310, 657–664
16. Takahashi, M., Mukai, H., Toshimori, M., Miyamoto, M. & Ono, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11566–11571
17. Mukai, H., Toshimori, M., Shibata, H., Kitagawa, M., Shimakawa, M., Miyahara, M., Sunakawa, H. & Ono, Y. (1996) J. Biol. Chem. 271, 9816–9822
18. Matsuzawa, K., Kosako, H., Inagaki, N., Shibata, H., Mukai, H., Ono, Y., Amano, M., Kaibuchi, K., Matsuura, Y., Aruma, I. & Inagaki, M. (1997) Biochem. Biophys. Res. Commun. 234, 621–625
19. Mukai, H., Toshimori, M., Shibata, H., Takanaga, H., Kitagawa, M., Miyahara, M., Shimakawa, M. & Ono, Y. (1997) J. Biol. Chem. 272, 4740–4746
20. Kuroda, S., Tokunaga, C., Kiyochara, Y., Higuchi, O., Konishi, H., Mizuno, K., Gill, G. N. & Kikkawa, U. (1996) J. Biol. Chem. 271, 31029–31032
21. Mukai, H., Miyahara, M., Sunakawa, H., Shibata, H., Toshimori, M., Kitagawa, M., Shimakawa, M., Takanaga, H. & Ono, Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10385–10389
22. Lin, J. W., Wyszynski, M., Madhavan, R., Sealock, R., Kim, J. U. & Sheng, M. (1997) J. Neurosci. 17, 1001–1109
23. Rogalski, A. A., Bergman, J. E. & Singer, S. J. (1984) J. Cell Biol. 99, 1101–1109
24. Lippeincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. & Klausner, R. D. (1989) J. Cell Biol. 106, 801–813
25. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, R. G. & Scott, J. D. (1991) J. Biol. Chem. 266, 14188–14192
26. Hendrix, P., Mayer-Jaekel, R. E., Cron, P., Goris, J., Holstein, J., Mettnever, W. & Hemmings, B. A. (1993) J. Biol. Chem. 268, 15267–15276
27. Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T. & Barford, D. (1997) EMBO J. 16, 1876–1887
28. Kerner, G., Rios, R. H., Landmark, B. F., Skaffell, B., Lohmann, S. M. & Bornens, M. (1993) Exp. Cell Res. 204, 230–240
29. Vandre, D. D., Davis, F. M., Rao, P. N. & Borisy, G. G. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4439–4443
30. Glover, D. M., Leibowitz, M. H., McLean, D. A. & Parry, H. (1995) Cell 81, 95–105
31. Fry, A. M., Meraldi, P. & Nigg, E. A. (1998) EMBO J. 17, 470–481
32. Verde, F., Dogterom, M., Stelzer, E., Karsenti, F. & Leibler, S. (1992) J. Cell Biol. 116, 1097–1108
33. Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jansen, E., Rahman, D., Rappin, D. J. C. & Warren, G. (1998) Cell 94, 783–793
34. Davidson, H. W., McGowan, C. H. & Balch, W. E. (1992) J. Cell Biol. 116, 1343–1355
35. Muniz, M., Alonso, M., Hidalgo, J. & Velasco, A. (1996) J. Biol. Chem. 271, 30935–30941
36. Kikkawa, T., Taniguchi, T., Mukai, H., Kitagawa, M., Hashimoto, T., Maeda, K., Ono, Y. & Tanaka, C. (1998) J. Neurosci. 18, 7402–7410
37. Kashishian, A., Howard, M., Loh, C., Gallatin, W. M., Hoekstra, M. F. & Lai, Y. (1998) J. Biol. Chem. 273, 27412–27419
38. Lupas, A. (1996) Methods Enzymol. 266, 513–525