Binding and Phosphorylation of a Novel Male Germ Cell-specific cGMP-dependent Protein Kinase-anchoring Protein by cGMP-dependent Protein Kinase Iα*

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cGMP-dependent protein kinase (cGK) is a major cellular receptor of cGMP and plays important roles in cGMP-dependent signal transduction pathways. To isolate the components of the cGMP/cGK signaling pathway such as substrates and regulatory proteins of cGK, we employed the yeast two-hybrid system using cGK-Iα as a bait and isolated a novel male germ cell-specific 42-kDa protein, GKAP42 (42-kDa cGMP-dependent protein kinase anchoring protein). Although the N-terminal region (amino acids 1–66) of cGK-Iα is sufficient for the association with GKAP42, GKAP42 could not interact with cGK-Iβ, cGK-II, or cAMP-dependent protein kinase. GKAP42 mRNA is specifically expressed in testis, where it is restricted to the spermatocytes and early round spermatids. Endogenous cGK-I is co-immunoprecipitated with anti-GKAP42 antibody from mouse testis tissue, suggesting that cGK-I physiologically interacts with GKAP42. Immunocytochemical observations revealed that GKAP42 is localized to the Golgi complex and that cGK-Iα is co-localized to the Golgi complex when coexpressed with GKAP42. Although both cGK-Iα and -Iβ, but not cAMP-dependent protein kinase, phosphorylated GKAP42 in vitro, GKAP42 was a good substrate only for cGK-Iα in intact cells, suggesting that the association with kinase protein is required for the phosphorylation in vitro. Finally, we demonstrated that the kinase-deficient mutant of cGK-Iα stably associates with GKAP42 and that binding of cGMP to cGK-Iα facilitates their release from GKAP42. These findings suggest that GKAP42 functions as an anchoring protein for cGK-Iα and that cGK-Iα may participate in germ cell development through phosphorylation of Golgi-associated proteins such as GKAP42.

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such as substrates and regulatory proteins of cGK. Because AKAPs have been associated with the regulatory units of cAK in the inactive state, we used the regulatory region located in the N terminus of cGK-I ς as a bait. Here we report evidence that cGK-I ς directly interacts with and phosphorylates the novel male germ cell-specific protein GKAP42 in vitro and in vivo. The interaction of cGK-I ς with GKAP42 facilitated the translocation of cGK-I ς to the Golgi complex, and cGK-I ς was released in response to intracellular cGMP accumulation. In the male germ cells, the Golgi complex is considered to be important for the formation of the acrosomic system and chromatoid body. These findings suggest that GKAP42 functions as an anchoring protein for cGK-I ς and that cGK-I ς might have functions through the interaction with Golgi-associated proteins during spermatogenesis.

EXPERIMENTAL PROCEDURES

Materials—The MATCHMAKER II two-hybrid system and the mouse 17-day embryo MATCHMAKER cDNA library were obtained from CLONTECH. Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). COS-7 cells were from Dainippon Pharmaceutical Co. (Osaka, Japan). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Life Technologies (Grand Island, NY). [32P]ATP and Hybond-N+ were products of Amersham Pharmacia Biotech.

Plasmid Construction—The full-length bovine cGK-I ς cDNA was a gift from Dr. Thomas M. Lincoln (University of Alabama at Birmingham). A cDNA encoding full-length cGK-I ς or its N-terminal region (amino acids 1–418) was subcloned into the BamHI (made blunt with T4 DNA polymerase) site of the yeast expression vector pAS2-1 (CLONTECH) in frame with the DNA-binding domain of the yeast transcriptional activator GAL4, generating pAS2-1-cGK-I ς or pAS2-1-cGK-Iα, respectively. Deletion mutants of cGK-I ς were constructed by digestion of pAS2-1-cGK-I ς with appropriate restriction enzymes. Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratageneco) according to the protocol of the manufacturer as we described previously (14). To introduce the desired mutations, the following primers were used: 5′-ATTCCCCAGAATTCCGCCCATTCCATTTGCAATGAGGCT-3′ and 5′-ATTCCCCAGAAATCGAGAAACAACTGTCGCGGTGTCACGTCCTTTGAGTGACACCGACAGGTTTGTC-3′. The resulting plasmids were transformed into Escherichia coli DH5α and Mut2a, and their sequences were confirmed by DNA sequencing.

The full-length bovine cGK-I ς cDNA was a gift from Dr. Michihiko Fujii (Kihara Institute for Biological Research, Japan). In addition to the cDNA encoding full-length cGK-I ς, a reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using human lung poly(A)-induced cDNA as a template. To clone the human cGK-I ς reverse transcriptase-PCR using a mouse testis poly(A)-induced cDNA as a template was performed as described previously (20). Digoxigenin-labeled cDNA probes (antisense and sense) were made by in vitro transcription using cDNAs subcloned into the pGEM-T vector as templates in the presence of digoxigenin-labeled dUTP (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. When the probe DNA was used for hybridization to cDNAs subcloned into the pGEM-T vector, a 5′-galactosyranoside was added to the culture to a final concentration of 0.2 mm, the culture was incubated for an additional 2 h. The cells were washed once with ice-cold soluble buffer (50 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and resuspended in 5 ml of ice-cold soluble buffer containing 10 mg/ml aprotinin, 10 mM leupeptin, and 1 mM dithiothreitol. After freezing and thawing, suspended cells were sonicated on ice in short bursts. The resulting pellet was collected by centrifugation at 15,000 × g for 15 min at 4 °C. The supernatant was then incubated with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 2 h at 4 °C. The beads were settled by centrifugation at 700 × g, washed five times with ice-cold soluble buffer, and incubated with 10 mM reduced glutathione for 10 min at 4 °C to elute GST-GKAP42 fusion protein from the beads. After centrifugation, the supernatant was dialyzed against phosphate-buffered saline (PBS). Polyclonal antibody raised against GKAP42 was obtained by injecting rabbits with GST-GKAP42 fusion protein in Freund's complete adjuvant. In Situ Hybridization—Freshly dissected 9-week ICR mouse testes were fixed for 6 h in Bouin's solution and embedded in paraffin. Four-μm sections were cut and mounted on Superfrost Plus slides (Fisher). In situ hybridization using digoxigenin-labeled probes was performed as described previously (20). Digoxigenin-labeled cDNA probes (antisense and sense) were made by in vitro transcription using cDNAs subcloned into the pGEM-T vector as templates in the presence of digoxigenin-labeled dUTP (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. After hybridization with a solution of 0.1 M sodium citrate, pH 7.0, 0.5% SDS, 5× Denhardt's solution (1 × Denhardt's solution: 0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll 400), and 100 μg/ml salmon sperm DNA at 65 °C for 16 h. The filters were washed with 2× SSC and 0.5% SDS at room temperature for 10 min, followed by two 30-min washes with 0.1× SSC and 0.5% SDS at 55 °C. The filters were exposed to x-ray film at −70 °C for 1 day.

The interactions of cGK-I ς with its regulatory subunits and regulatory proteins during spermatogenesis.
m NaCl, 0.2% sarcosyl, 0.02% heat-denatured salmon sperm DNA, 1× Denhardt’s solution, and 50% formamide) and then hybridized overnight at 50 °C in hybridization buffer with 100 ng/ml cRNA probe. After being rinsed in 5× SSC at 60 °C for 20 min, the sections were washed with 50% formamide and 2× SSC at 60 °C for 30 min. Next, they were sub- jected to RNase digestion for 20 min at 37 °C in hybridization buffer containing 10× Tris-HCl, 1 mM EDTA, and 0.5 mM NaCl, pH 7.5) and then washed with 50% formamide and 2× SSC at 60 °C for 30 min. For detection of hybridized cRNA probes, anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals) was reacted at 1:500, and color was developed by incubation with 4-nitro blue tetrazolium chloride and X-phosphate solution.

**Immunoprecipitation of Endogenous GKAP42 and cGK-I from Mouse Testis**—ICR mouse testis was homogenized in TNE buffer (10 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 0.15% NaCl, 10 mg/ml aprotinin, 10 mM leupeptin, and 1 mM dithiothreitol). The homogenates were centrifuged at 50,000 × g for 30 min at 4 °C. The resulting supernatants were filtrated with a 0.45-μm filter (Millipore) and immunoprecipitated with 10 μg of either anti-GKAP42 polyclonal antibod- y or normal rabbit IgG with protein G-Sepharose overnight at 4 °C by rotation. The beads were washed five times with TNE buffer, and immune complexes were eluted by heating at 95 °C in 2× SDS sample buffer, subjected to SDS-PAGE, and analyzed by immuno blotting with anti-FLAG antibody M5 (Kodak).

**Results**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. The full-length cGK-Iα cDNA and GKAP42 cDNA in the expression vector pFLAG-CMV-2 (Eastman Kodak Co.) were transiently expressed in COS-7 cells using LipofectAMINE Plus reagent (Life Technologies, Inc.) following the manufacturer’s instructions. Twenty-four h after transfection, cells were washed twice with ice-cold PBS and scraped into ice-cold TNE buffer. Cell extracts were centrifuged at 16,000 × g for 15 min at 4 °C to remove cellular debris. The supernatants were immunoprecipitated with anti-FLAG antibody M5 (Kodak) with protein G-Sepharose overnight at 4 °C by rotation. The beads were washed five times with TNE buffer, and the immunoprecipitated samples were used for the in vitro kinase assay.

Protein kinase assays were performed in kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 0.2 mM γ-[32P]ATP, 2 μM protein kinase A inhibitor peptide (5–24), 5 mM glycerophosphoric acid, and 1 mM sodium orthovanadate) in the presence of 100 μM BPDEide (Calbiochem), a synthetic substrate selective for cGK. Reactions were performed in the presence or absence of cGMP (5 mM final concentration). Assays were conducted at 30 °C for 30 min and termi- nated by centrifuging and aliquoting the peptide onto phosphocellulose P-81 paper (Whatman). The phosphocellulose pads were washed five times with 0.5% phosphoric acid and counted using a Fuji BAS2000 imaging analyzer. The values represent the -fold activation over the basal activity of the lysates from mock-transfected cells taken as 1 and are expressed as the mean of the three independent experiments. Phospho- rylated proteins of cGK in vitro was performed in kinase reaction buffer with or without 5 μM cGMP. Phosphorylation by cAK was performed in the same buffer, but in the presence or absence of 2 μM protein kinase A inhibitor peptide (5–24). The samples were incubated at 30 °C for 30 min and centrifuged at 16,000 × g for 4 °C. The beads were mixed with an equal volume of 2× SDS sample buffer and heated at 95 °C for 5 min, and the denatured proteins were loaded onto SDS-polyacrylamide gels. The gels were dried and subjected to autoradiog- raphy at ~80 °C.

**Immunofluorescence Microscopy**—COS-7 cells grown on cover glasses were cotransfected with the full-length GKAP42 cDNA in the expression vector pCMV-EGFPpC1 (CLONTECH) and/or pHAG-cGK-Iα. After 24 h, the cells were directly fixed with 4% paraformaldehyde and 4% sucrose in 0.2× NaPO4, pH 7.2, for 30 min and blocked with 1.5% normal goat serum in PBS for 1 h at room temperature. Cells were treated with anti-FLAG antibody M5 (diluted 1:500 in PBS with 0.1% Tween 20) for 1 h at room temperature and washed five times with PBS. The primary antibody was visualized with FluoroLinkTM CyTM3-labeled goat anti-mouse IgG (Amersham Pharmacia Biotech). Two monoclonal antisera raised against the Golgi zone were purchased from Biogenes (Bourgogne, United Kingdom), Chemicon International, Inc. (Temecula, CA) and used for immuno fluorescence microscopy.

**Phosphorylation of GKAP42 in Vivo**—COS-7 cells were cotransfected with pHAG-cGK-Iα and pFLAG-GKAP42 as described above. After treatment in the presence or absence of 8-CPT-cGMP (Sigma) for 30 min, cells were scraped into ice-cold radioimmuno precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.15× NaCl, 0.1% SDS, 1% Nonidet P-40, 2 mM EDTA, 10 μg/ml aprotinin, 10 μM leupeptin, 25 mM glycophosphoric acid, and 1 mM sodium orthovanadate). The cell extracts were centrifuged at 16,000 × g for 15 min at 4 °C to remove cellular debris, and the supernatants were subjected to SDS-PAGE and analyzed by immunoblotting using anti-FLAG antibody M5. The full-length cGK-Iα cDNA in pHAG-cGK-Iα and the full-length GKAP42 cDNA in pCDNA-1His (In vitro) were transiently expressed in COS-7 cells as described above. After treatment in the presence or absence of 8-CPT-cGMP for 30 min, cells were scraped into ice-cold TNE buffer. Cell extracts were centrifuged at 16,000 × g for 15 min at 4 °C to remove cellular debris. The supernatants were immunoprecipitated with anti-Xpoxyl antibody (Santa Cruz Biotechnology) with protein G-Sepharose in the presence or absence of 8-CPT-cGMP for 4 h at 4 °C by rotation. The beads were washed five times with TNE buffer, and immune complexes were eluted by heating at 95 °C in 2× SDS sample buffer, subjected to SDS-PAGE, and analyzed by immunoblotting with anti-FLAG antibody M5.

The recent reports (9, 10) that the type II regulatory subunits of cAK (cAK-RII) are able to associate with AKAPs raise the possibility that the regulatory domain located in the N terminus of cGK interacts with cGK- specific anchoring proteins. To identify potential components of cGK signaling, we performed yeast two-hybrid screening. An expression vector that encodes the N-terminal region of bovine cGK-Iα (amino acids 1–416) fused to the GAL4 DNA-binding domain (GAL4-BD) was used as a bait in the yeast two-hybrid screening of a mouse 17-day embryo cDNA library. From the 1 × 107 transformants screened, three colonies were positive as determined by activation of the His3 reporter gene and the β-galactosidase assay. As expected, two of the isolated cDNAs encoded mouse slow skeletal troponin T (14). Furthermore, we obtained one cDNA encoding a novel protein (GKAP42) as a true positive clone. To examine the specificity of the interaction between cGK-Iα and GKAP42 in the yeast two-hybrid system, full-length cGK-Iα, cGK-II, cAK-RIIa, and cAK-Cα were fused to GAL4-BD and cotransformed with GKAP42. The yeast two- hybrid analysis demonstrated that only cGK-Iα can associate with GKAP42 (Fig. 1A). Expression of each GAL4-BD-fused protein was confirmed by immunoblot analysis using anti- GAL4-BD antibody (data not shown).

To identify the region of cGK-Iα responsible for the interaction with GKAP42, we constructed a series of cGK-Iα deletion mutants and tested them for their ability to associate with GKAP42 in the yeast two-hybrid system. As shown in Fig. 1B, we found that the cGK-Iα domain required for interaction with GKAP42 is restricted to the N-terminal region between amino acids 1 and 66, containing the leucine zipper motif, a sequence of heptad repeats of leucines and isoleucines forming an α-helical structure.

The full-length cDNA of GKAP42 was isolated from a λ-phage mouse lung cDNA library by conventional hybridization screening and contained an open reading frame of 1098 nucleotides that encodes a polypeptide of 366 amino acids with a calculated molecular mass of 41.8 kDa (Fig. 2A). The first methionine is sur- rounded by a Kozak consensus sequence. In the 3′-untranslated region of GKAP42, a typical polyadenylation signal, AATAAA (31), is located 3′ upstream of the poly(A) tail. A search of the GenBankTM Data Bank using the nucleotide and amino acid sequences revealed no significant similarity to other reported sequences. In addition, the predicted protein sequence does not contain a characteristic signal sequence at the N terminus or a transmembrane sequence as determined by hydropathy analysis. Secondary structure analysis for coiled-coils (19) predicted four stretches of coiled-coil structure (Fig. 2B). These structural fea- tures are thought to be involved in association with other proteins and/or homodimerization/homo-oligomerization.
Stage-specific Expression of GKAP42 in Testis—Northern blot analysis was performed using poly(A)\(^{+}\) RNA derived from various mouse tissues and the GKAP42 cDNA fragment as a probe. A strong hybridization signal of 1.5 kilobase pairs was predominantly or solely observed in testis (Fig. 3A). To identify the cell types in which GKAP42 mRNA is expressed, sections of adult mouse testis were hybridized in situ with the antisense RNA probe complementary to GKAP42 mRNA. Intense hybridization signals were detected in testicular germ cells at the stages of spermatocytes and round spermatids (Fig. 3B). No or little signal was detected in spermatogonia, late spermatids, and somatic Sertoli and Leydig cells. Furthermore, when the sense RNA was used as a control probe, no positive staining was observed (Fig. 3C).

Next, we prepared a polyclonal antibody against GKAP42 using a bacterially synthesized GST-fused fragment corresponding to amino acids 1–337 of GKAP42. The specificity of the anti-GKAP42 antibody was tested by immunoblot analysis of total proteins from COS-7 cells transfected with an expression vector encoding FLAG epitope-tagged GKAP42 (FLAG-GKAP42). Although no signal was observed in the mock-transfected cells, a specific band of 42 kDa was detected in the cells transfected with a plasmid encoding FLAG-GKAP42 using both anti-GKAP42 and anti-FLAG antibodies (Fig. 4A). Furthermore, to determine whether the GKAP42 mRNA detected in germ cell is translated into a protein, immunoblot analysis was performed with total protein from mouse adult testis. Although the anti-GKAP42 antibody recognized an immunoreactive polypeptide of 42 kDa in the soluble fraction of the testis homogenate, GKAP42 protein was absent in the mature sperm prepared from mouse epididymis (Fig. 4B). Furthermore, attempts were made to determine whether endogenous cGK-I associates with GKAP42 under the physiological condition. Soluble fractions from adult mouse testis were immunoprecipitated with anti-GKAP42 antibody, and coprecipitating cGK-I was detected by immunoblot analysis using a polyclonal antibody against cGK-I. In this assay, GKAP42 was able to coprecipitate cGK-I (Fig. 4C), confirming the in vivo interaction detected by the yeast two-hybrid analysis.
that GKAP42 was phosphorylated by both cGK-I kinase assay using the immunoprecipitation complex showed in vitro whether cGK-I would phosphorylate GKAP42.

Does not stimulate or inhibit cGK activity.

Northern blot of poly(A) was hybridized using a 32P-labeled fragment of GKAP42 cDNA as described under “Experimental Procedures.”

The sizes (in kilobase pairs (kb)) and positions of mRNA size markers are shown. B and C, in situ localization of GKAP42 mRNA in mouse testis. Paraffin sections of adult mouse testis were hybridized with digoxigenin-labeled antisense (B) or sense (C) RNA probe complementary to GKAP42 mRNA as described “Experimental Procedures.” For detection of hybridized cRNA probes, anti-digoxigenin antibody conjugated to alkaline phosphatase was reacted, and color was developed by incubation with 4-nitro blue tetrazolium chloride and X-phosphate solution.

Co-localization of cGK-I with GKAP42 in Mammalian Cells—To identify whether cGK-Iα can form complexes with GKAP42 in mammalian cells and to determine the intracellular localization of cGK-Iα with or without GKAP42, the plasmid DNA encoding green fluorescence protein fused to full-length GKAP42 was constructed and cotransfected with FLAG epitope-tagged cGK-Iα into COS-7 cells. Immunofluorescence microscopy showed that in cells transfected with green fluorescence protein-GKAP42, GKAP42 was concentrated at juxtanuclear regions corresponding to the Golgi complex (Fig. 5, A and C) because of co-staining with anti-Golgi zone antibody (Fig. 5D). Although cGK-Iα was localized to the cytoplasm in cells transfected with FLAG-cGK-Iα alone (Fig. 5B), cGK-Iα was co-localized to the Golgi complex when coexpressed with GKAP42 (Fig. 5, E and F). These observations were consistent with the ability of cGK-Iα to interact with GKAP42 in vitro.

GKAP42 Is Specifically Phosphorylated by cGK-I—Next, we examined the ability of GKAP42 protein to modulate cGK activity in vitro. cGK activity was measured by immune complex kinase assay using a synthetic substrate selective for cGK, BPDEide (22). However, overexpression of GKAP42 did not affect cGK activity independent of the presence or absence of cGMP (Fig. 6A), suggesting that the association with GKAP42 does not stimulate or inhibit cGK activity.

Because many AKAPs are phosphorylated by cAK, we examined whether cGK-I would phosphorylate GKAP42. In vitro kinase assay using the immunoprecipitation complex showed that GKAP42 was phosphorylated by both cGK-Iα and cGK-Iβ in a cGMP-dependent manner (Fig. 6B). Although cAK activity was confirmed by phosphorylation of cardiac troponin I, one of the substrates for both cGK and cAK, GKAP42 was not phosphorylated by cAK. A kinase-defective mutant of cGK-Iα, cGK-Iα D502A, in which aspartic acid 502 is changed to alanine (23), could not phosphorylate GKAP42, indicating that GKAP42 is a good substrate for only cGK-I isozymes in vivo (Fig. 6C). Additionally, we found that phosphorylation by cGK-Iα caused a slight shift in the mobility of GKAP42 protein during SDS-PAGE by in vitro kinase assay (data not shown). Because some substrates have been shown to be good substrates in vitro but not in vivo (24), we investigated the phosphorylation of GKAP42 by an in vivo kinase assay. For this purpose, we used FLAG-cGK-I and FLAG-GKAP42 and treated the cotransfected COS-7 cells with 8-CPT-cGMP, a cell-permeable analogue of cGMP. The cell extracts were subjected to SDS-PAGE and analyzed by immunoblotting using anti-FLAG antibody. The shift in the mobility of GKAP42 protein was cGMP-dependent only when cotransfected with wild-type cGK-Iα (Fig. 7A). Interestingly, cGK-Iβ failed to phosphorylate GKAP42 in vivo. Taken together with the observation that cGK-Iβ could not interact with GKAP42 in the yeast two-hybrid system, the direct interaction with the kinase protein is required for the phosphorylation in intact cells.

Previous studies with peptide libraries defined optimal amino acid sequences (RXXST), X is any amino acid) for phosphorylation by cGK (25). Although GKAP42 is preferentially phosphorylated by cGK-I in vitro and in vivo, a typical cGK phosphorylation site was not identified in GKAP42. To identify the cGK-I phosphorylation site of GKAP42, we constructed a series of GKAP42 deletion mutants and performed an in vitro kinase assay. cGK-Iα was shown to phosphorylate
amino acids 71–199 of GKAP42 (data not shown). cGK-Iα was previously demonstrated to autophosphorylate primarily at threonine 58, which is an atypical cGK substrate sequence (IGPRTT58RAQGI). Glass and Smith (26) reported that the recognition site specificity of cGK requires an arginine located on the C-terminal side (underlined) of the phosphorylated residue, X(S/T)RX. Because GKAP42 also has this motif (PAQKES106REEN) in the region of amino acids 71–199, we prepared a mutant of GKAP42 (GKAP42 S106A) in which serine 106 is changed to alanine and tested it in both in vitro and in vivo kinase assays. The mutation S106A was shown to completely abolish the band shift in vitro (data not shown) and in vivo (Fig. 7B). In addition, mutation of a nearby residue, namely S86A or T178A, could not disrupt the potential phosphorylation by cGK-Iα.

In Vivo Interaction between cGK-I and GKAP42—To determine whether cGK-Iα can interact with GKAP42 in mammalian cells, we carried out co-immunoprecipitation assays. An expression vector encoding Xpress epitope-tagged GKAP42 (Xpress-GKAP42) was transfected alone or with an expression vector encoding FLAG-cGK-Iα into COS-7 cells. The cell lysates were immunoprecipitated using anti-Xpress antibody, and co-precipitating cGK-Iα was detected by immunoblot analysis using anti-FLAG antibody. The ability of the anti-Xpress antibody to precipitate a complex of cGK-Iα and GKAP42 suggests that these two proteins strongly interact in vivo (Fig. 8). Next, to test whether kinase activity affects the interaction with GKAP42, we compared the interactions between GKAP42 and
cell extracts were subjected to SDS-PAGE and analyzed by immuno- 
 blotting. Taken together with the observation that GKAP42 is 
detected in vivo, the phosphorylation might result in the reduction of the stability of the interaction between the two proteins.

Recent reports have demonstrated that cGK-I undergoes a conformational change when cGMP binds to the cGMP-binding domains of cGK-I (27, 28). Finally, we examined the effect of the conformational change produced by cGMP on the interaction between cGK-I and GKAP42. When COS-7 cells were cotransfected with cGK-I and GKAP42 and treated with 8-CPT-cGMP, the stability of the interaction between cGK-I and GKAP42 was reduced compared with no treatment. These results indicate that the binding of cGMP to cGK-I enables them to release from GKAP42.

DISCUSSION

In this study, a cDNA encoding a novel germ cell-specific protein was identified as a cGK-I-interacting protein by yeast two-hybrid screening using the N-terminal regulatory region of cGK-I as a bait, and we demonstrated the physiological interaction of these two proteins in testis tissue. We also showed that GKAP42 selectively associates with cGK-I and that the phosphorylation of GKAP42 seems to be dependent on direct interaction with the kinase protein. Very recently, we showed that troponin T functions as an anchoring protein for cGK-I and that cGK-I may participate in the regulation of muscle contraction through phosphorylation of troponin I (14). Similarly, troponin T protein does not interact with cGK-II or cAMP-depend-
defined optimal amino acid sequences for phosphorylation by cGK (RKX(S/T)) (25). Additionally, Glass and Smith (26) reported that the recognition site specific to cGK requires an arginine on the C-terminal side (underlined) of the phosphorylated residue, X(S/T)RX. In fact, an autophosphorylation site (IGPRPT58RAQGI) in cGK-Iα fits this motif. Although GAKP42 does not contain a typical cGK phosphorylation site (RKX(S/T)), an arginine located on the C-terminal side of serine 106 (PAQKES106REEN) might be involved in the potential phosphorylation at serine 106 in vivo. Furthermore, we demonstrated that the association with GAKP42 is selective for cGK-Iα in a family of cyclic nucleotide-dependent protein kinases. More important, two isoforms of cGK-I (Iα and Iβ), produced by alternative splicing, differ in their N-terminal domains (amino acids 1–89 and 1–104, respectively), resulting in the specificity of interaction with GAKP42. Previous reports on AKAPs described differential subcellular localization of the isoforms likely due to specificity of the anchoring proteins. cAK-RIIα has a 6-fold increased affinity for microtubule-associated protein 2, and cAK-RIIβ has a 2-fold affinity for AKAP75 (33). Furthermore, a few AKAPs, dual-specificity AKAPs, interact with both type I and II regulatory subunits (34, 35), whereas most AKAPs interact specifically with type II regulatory subunits. An added level of specificity in cAK signaling may be achieved through the differential localization of regulatory subunit isoforms by association with isoform-selective AKAPs. Likewise, the N-terminal domain of cGK-I isoforms, through the interaction with their anchoring proteins, may play an important role in the determination of subcellular localization, leading to the mediation of isoyme-specific functions.

One of the intriguing observations of this study is that GAKP42 is located in the Golgi complex. Recently, a number of Golgi-associated proteins have been isolated by antibodies from patients with autoimmune diseases and by antibodies prepared in animals. These include giantin or GCP372 (36), golgin-245 or p230 (37), GCP230 (38), GM130 or golgin-95 (39), and GCP170 (golgin-160) or male-enhanced antigen-2 (40). Although giantin/GCP372 is an integral membrane protein anchored to the membrane by the C-terminal hydrophobic domain, all the other proteins have no hydrophobic domain that could function as a signal sequence or participate in membrane localization. The common characteristic of all these proteins is a very large domain, enabling the formation of coiled-coil structures analogous to the myosin family. One of the predicted structural features of GAKP42 is a long helical domain that is very likely to form a coiled-coil structure. To date, the Golgi complex is considered to play a crucial role in the formation of acrosomes and the chromatoid body in male germ cells. The acrosome is a specialized membrane-bound organelle located in the head of sperm cells that contains a rich store of hydrolytic enzymes and that is crucial for fertilization (41). Very recently, male mice bearing a null mutation of golgin-160 (male-enhanced antigen-2) were reported to be sterile due to a block of spermatogenesis, showing apparent scarcity of spermatids and spermatozoa (42). Although spermatogenesis in the homozygotes proceeded at least to meiotic metaphase I, deformation of condensed nuclei was evident in elongated spermatids, suggesting the important roles of Golgi-associated proteins during spermatogenesis. On the other hand, although a large number of studies have reported that NO or natriuretic peptides/cGMP pathways play an important role in the nervous, cardiovascular, and immune systems of various species, information concerning the cGMP-dependent signaling pathway in germ cells is still limited. Recently, a cyclic nucleotide-gated channel was identified in mammalian sperm, and cyclic nucleotide-gated channels have been demonstrated to be directly opened by either cGMP or cAMP and are permeable to Ca2+ ions (43). Although a rise in the cellular Ca2+ concentration has been proposed to alter sperm motility and to trigger the acrosomal reaction, the acrosomal exocytosis of spermatozoa induced by natriuretic peptides is dependent on Ca2+ influx and the protein kinase C signaling pathway, but not on the cGK signaling pathway (44). Recently, several works have focused on the cAMP signaling pathway during sperm differentiation. Although studies on mice lacking the transcription factor CREM, a nuclear target of cAK, have indicated an important role for the cAMP-dependent pathway in spermatid differentiation (45, 46), the adenylyl cyclase (47) and type 4 cAMP-specific phosphodiesterase (48) are co-localized in the acrosomal membrane, suggesting one functional region for the cAMP signaling pathway during spermatogenesis. Furthermore, a variety of AKAPs, including AKAP82 (49), AKAP84 (50), AKAP110 (51), and AKAP121 (52), have been identified in testis. The introduction of anchoring inhibitor peptides designed to disrupt the interaction of cAK with AKAPs inhibited sperm motility (53). AKAP82 and AKAP84 are localized in the flagellum, suggesting that they are involved in the assembly of the fibrous sheath surrounding the axoneme and in sperm motility. Taken together with the previous immunohistochemical showing the presence of cGK-I protein in rat spermatocytes (54), the current study suggests that cGK-Iα might participate in germ cell development through phosphorylation of Golgi-associated proteins such as GAKP42.

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