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# Molecular Characterization of a Third Member of the Guanylyl Cyclase-activating Protein Subfamily*

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**The mammalian retina contains at least two guanylyl cyclases (GC1 and GC2) and two guanylyl cyclase-activating proteins (GCAP1 and GCAP2). Here we present evidence of the presence of a new photoreceptor-specific GCAP, termed GCAP3, which is closely related to GCAP1. The sequence similarity of GCAP3 with GCAP1 and GCAP2 is 57 and 49%, respectively. Recombinant GCAP3 and GCAP2 stimulate GC1 and GC2 in low $[Ca^{2+}]_{free}$ and inhibit GCs when $[Ca^{2+}]_{free}$ is elevated, unlike GCAP1, which only stimulates GC1. GCAP3 is encoded by a distinct gene present in other mammalian species but could not be detected by genomic Southern blotting in rodents, amphibians, and lower vertebrates. The intron/exon arrangement of the GCAP3 gene is identical to that of the other GCAP genes. While the GCAP1 and GCAP2 genes are arranged in a tail-to-tail array on chromosome 6p in human, the GCAP3 gene is located on 3q13.1, suggesting an ancestral gene duplication/translocation event. The identification of multiple $Ca^{2+}$-binding proteins that interact with GC is suggestive of complex regulatory mechanisms for photoreceptor GC.**

Absorption of light by rhodopsin results in a decrease of [cGMP] within photoreceptor outer segments (1). This leads to the closure of cGMP-gated cation channels and reduction in $Ca^{2+}$ influx (reviewed in Refs. 2 and 3). In addition, $Ca^{2+}$ is continuously extruded from the photoreceptor cells by a light-independent $Na^+/Ca^{2+}$-$K^+$ exchanger (4). The net result of photoactivation is a decrease in intracellular [cGMP] and $[Ca^{2+}]$. Guanylyl cyclase-activating proteins (GCAPs)[1] are activated by this decrease in $[Ca^{2+}]$ and accelerate cGMP production by stimulating GCs. Increase in [cGMP] leads to the opening of the cation channels and restores the dark conditions of photoreceptors (5). Two GCAPs have been identified in photoreceptors, GCAP1 (6) and GCAP2 (7, 8), as well as two guanylyl cyclases, GC1 (9, 10) and GC2 (11). GCAP1 exclusively stimulates GC1, while GCAP2 stimulates both GC1 and GC2 (12). Recently, a third GCAP-like photoreceptor protein, guanylyl cyclase-inhibitory protein, termed GC1P, was identified in frog cone photoreceptors and also was shown to interact with GC (13).

GCAPs are $Ca^{2+}$-binding proteins belonging to the calmodulin superfamily but they only have three functional EF-hand motifs. GCAP1 and GCAP2 genes are organized in a tail-to-tail array in vertebrates (14–16). In humans, the array is located on the short arm of chromosome 6 (p21.1). A defect in the GCAP1 gene (Y99C) has recently been linked to autosomal dominant cone dystrophy (17–19), which affects cones but not rods. The Y99C mutation has been shown to alter $Ca^{2+}$ sensitivity of GCAP1, leading to the constitutive stimulating activity of GC1 at high $[Ca^{2+}]$, where normal GCAP1 inhibits it. GCAP1 has been localized to rod and cone photoreceptor outer segments in the retina of various species (8, 15, 20, 21), while GCAP2 has been detected in cone inner segments (22), rods (7), and the inner retina (15, 21). GCAP1 has been proposed to be a major regulator of GC in the preparations of bovine rod outer segments (ROS) (6, 8). In humans, the most intense labeling with anti-GCAP1 antibodies has been observed in cone outer segments, and weaker labeling has been observed in rod outer segments (8, 21). Since human rods are unaffected in autosomal dominant cone dystrophy, GCAP2 or a third GCAP-like $Ca^{2+}$-binding protein may substitute in part for the mutant GCAP1, or rods are less sensitive to elevated cGMP levels (18). In this paper, we describe molecular cloning and characterization of a third form of GCAP, GCAP3, which by sequence similarity appears to be more closely related to GCAP1 than to GCAP2. This novel isoform provides additional evidence of complex regulations of cGMP production/degradation in photoreceptor cells from different species and of the existence of an evolving family of GCAP genes.

## MATERIALS AND METHODS

*cDNA Cloning of GCAP3*—GCAP3 was amplified from a λgt10 human retina cDNA library in two overlapping fragments using primers designed based on the expressed sequence tag clone (a364442 deposited A. R. Kerlavage, The Institute for Genomic Research, Rockville, MD).

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF109998–AF110001 (exons 1–4 of human genomic sequence, respectively) and AF110002 and AF110003 (for the cDNA sequence of GCAP3 and its splice variant, respectively).*

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[1] The abbreviations used are: GCAP, guanylyl cyclase-activating protein; GC, guanylyl cyclase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BAC, bacterial artificial chromosome; kb, kilobase(s); FISH, fluorescence *in situ* hybridization; ROS, rod outer segment(s).

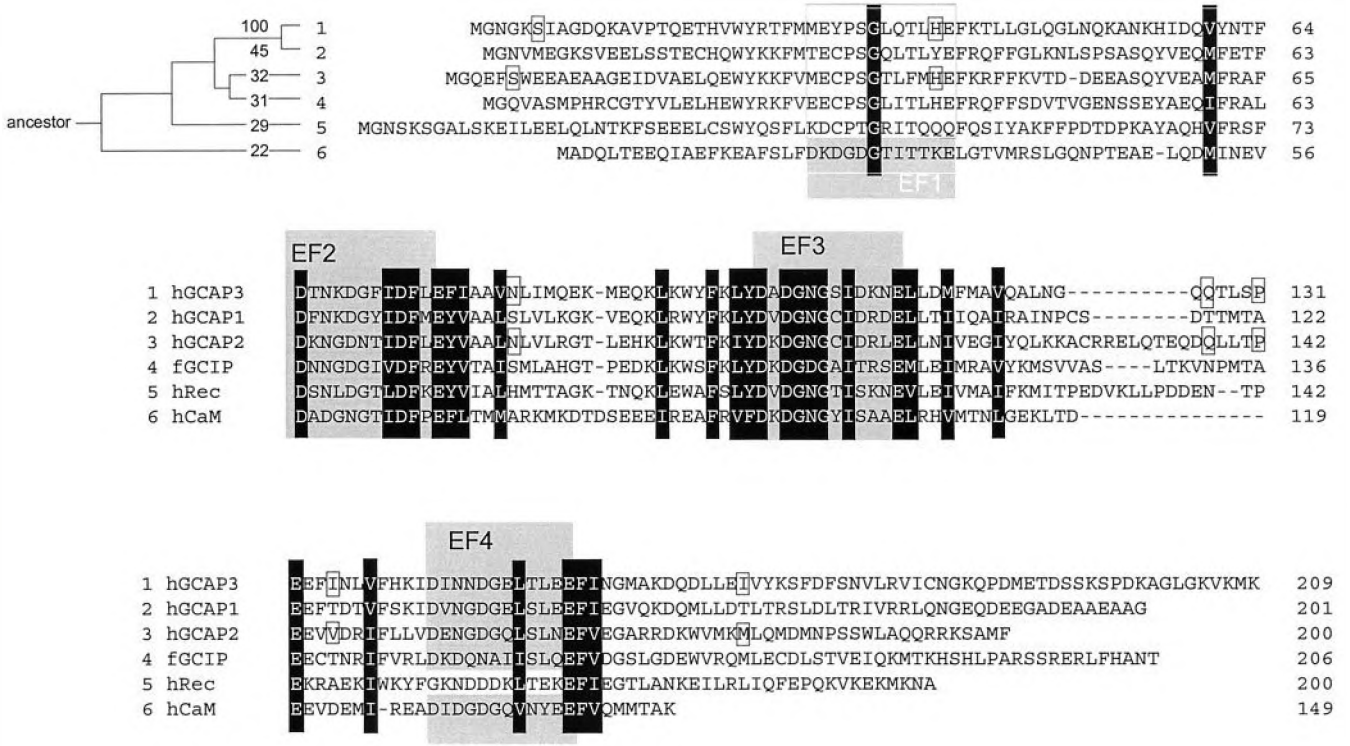


FIG. 1. **Alignment of GCAPs with recoverin and calmodulin.** *1*, human GCAP3; *2*, human GCAP1 (34); *3*, human GCAP2 (14); *4*, frog guanylyl cyclase-inhibitory protein (13); *5*, human recoverin (35); *6*, human calmodulin (36) amino acid sequences. *Top left*, dendrogram generated by PC/Gene (Intelligenetics, Inc.) based on amino acid sequence similarity. The *numbers* inside the dendrogram reflect percentage identities with GCAP3 as 100%. *Shaded areas* denote EF-hand motifs. Amino acid residues conserved in all six sequences are printed *white* on *black*. Amino acids common to GCAP2 and GCAP3, but distinct from GCAP1, are *boxed*.

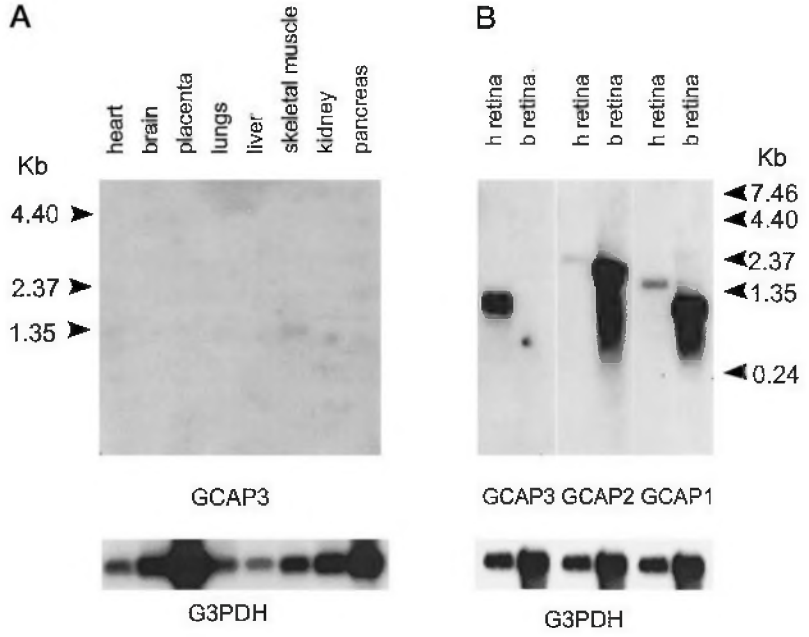


FIG. 2. **Tissue distribution of GCAP3 mRNA.** *A*, a Northern blot containing 2 $\mu$g of poly(A) RNA from human retina and from various human tissues was probed with $^{32}$P-labeled GCAP3 cDNA or with a control $^{32}$P-labeled glyceraldehyde-3-phosphate dehydrogenase probe. *B*, a Northern blot containing 2 $\mu$g of poly(A) RNA from human and bovine retina was probed with $^{32}$P-labeled GCAP1, GCAP2, or GCAP3 cDNA. The positions of size markers are shown on the *side*.

The 3′-end was amplified with primers K3 (5′-TGT GAG TCA AGA TGG GGA ATG GCA, gene-specific primer) and λgt10S (5′-AGC AAG TTC AGC CTG GTA AG). After heating at 95 °C for 5 min, the reaction was cycled 35 times through 94 °C for 30 s, 68 °C for 2 min. A secondary nested PCR amplification was carried out with primers K4 (5′-GGC AAA TCT ATA GCT GGT GAT CAGA) and λgt10S as described above. The 5′-end was amplified following the same PCR conditions with primers K6 (5′-GGC CTT CTG ATT CAG ACC TTG CAG, gene-specific primer) and λgt10S. At least two amplification products for each PCR were subcloned into pCRII-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and sequenced by dyedeoxyterminator sequencing (ABI-Prism, Perkin-Elmer).

*Northern Blot Analysis*—Total RNA was isolated from human retinal tissue, obtained from The Wisconsin Eye Bank at the University of Wisconsin, or from bovine retinas (Schenk Packing Co., Inc, Stanwood, WA) using the Ultraspec RNA isolation system (Biotecx, Inc.). mRNA was purified from total RNA using the mRNA purification kit (Amersham Pharmacia Biotech), resolved by agarose gel electrophoresis in the presence of 0.66 M formaldehyde, and transferred to nylon membranes. Hybridization with a $^{32}$P-labeled human GCAP3 cDNA (1 kb) was performed in 40% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, 35 $\mu$g/ml of herring sperm DNA in 50 mM Tris, pH 7.4. Hybridizations were washed at room temperature in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS for 30 min followed by 30 min at 50 °C in 0.015 M

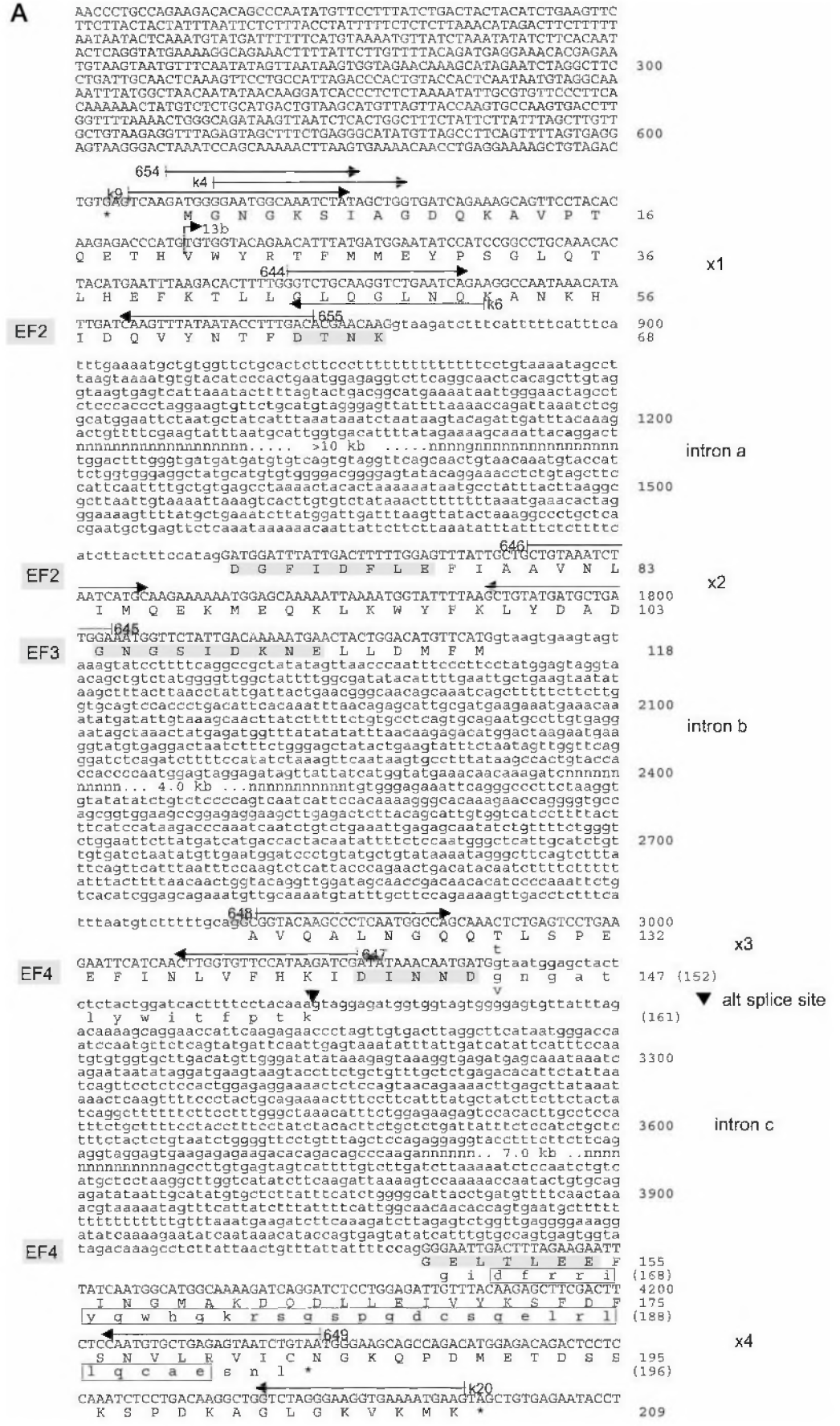


FIG. 3. **Partial gene sequence, exon/intron junctions of human GCAPs, and chromosomal localization of human GCAP3.** *A*, gene sequence. Exons (*x1–x4*) are shown in *capital letters*, introns (*a–c*) in *lowercase*. All intron sequences are shown only partially. Primers used for

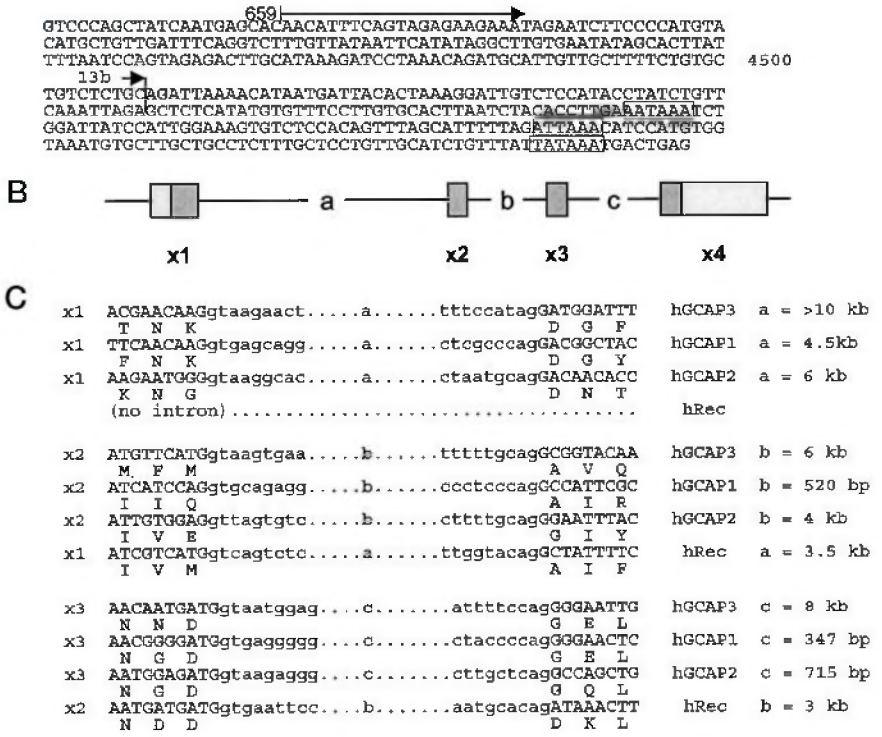


FIG. 3—*continued*

NaCl, 0.0015 M sodium citrate, and 0.1% SDS. A human multiple-tissue Northern blot containing 2 $\mu$g of poly(A)$^+$ RNA from various human tissues (CLONTECH Laboratories, Inc.) was hybridized with the $^{32}$P-labeled GCAP3 or glyceraldehyde-3-phosphate dehydrogenase cDNA according to the manufacturer's instructions.

*Expression of GCAP3 in Insect Cells*—The full coding sequence for GCAP3 was amplified from human retina cDNA by PCR with primers K9 (5′-CAT ATG GGG AAT GGC AAA TCT ATA GCTG), which placed an *Nde*I site on the ATG, and K10 (5′-CTA GTG ATG GTG ATG GTG ATG CTT CAT TTT CAC CTT CCC TAG ACC AG), which added a $His_6$ tag at the 3′-end. PCR was performed using a denaturing temperature of 94 °C for 30 s and an annealing and extension temperature of 68 °C for 2 min through 35 cycles. The PCR product was cloned in pCRII-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and sequenced by dyedeoxyterminator sequencing (ABI-Prism, Perkin-Elmer). An *Xba*I–*Hin*dIII fragment was inserted between corresponding sites of the pFastBac1 expression vector (Life Technologies, Inc.). Sf9 insect cells were transfected with the recombinant baculovirus shuttle vector using cationic liposome-mediated transfection (CellFECTIN reagent; Life Technologies) according to the manufacturer's protocol.

*Expression of GCAP3 in Escherichia coli*—The coding sequence for GCAP3 was amplified from human retina cDNA by PCR with primers K9 (5′-CAT ATG GGG AAT GGC AAA TCT ATA GCTG), which placed an *Nde*I site on the ATG, and K20 (5′-CTA CTT CAT TTT CAC CTT CCC TAGA), using a denaturing temperature of 94 °C for 30 s and an annealing and extension temperature of 68 °C for 2 min through 35 cycles. The PCR product was cloned in pCRII-TOPO vector and sequenced by dyedeoxyterminator sequencing. GCAP3 was inserted as a fragment *Nde*I–*Bam*HI in pET-3b vector (Novagen). GCAP3 was expressed in BL21 bacteria after induction with 0.2 mM isopropyl-1-thio-$\beta$-D-galactopyranoside. The bacteria were sonicated for 5 min in water, and the extract was centrifuged at 80,000 × *g* for 20 min. The pellet containing the inclusion bodies was solubilized in 8 M urea, 80 mM $\beta$-mercaptoethanol and incubated for 90 min at room temperature. The insoluble material was centrifuged for 60 min at 340,000 × *g*. The supernatant was dialyzed against water.

*Preparation of Anti-GCAP3 Antibodies*—Rabbit anti-GCAP3 polyclonal antibodies (UW84) were raised in New Zealand White rabbits by subcutaneous immunization with 100 $\mu$g of GCAP3 expressed in *E. coli* mixed with an equal volume of complete Freund's adjuvant (Cocalico Biologicals, Reanstown, PA). Animals were given booster injections at 1–2-week intervals with 50 $\mu$g of GCAP3 mixed with incomplete Freund's adjuvant. UW14 (anti-GCAP1) and UW50 (anti-GCAP2) were prepared following the same procedure (8, 23).

*Purification of UW14, UW50, UW84, and UW87*—For UW50, 2 ml of rabbit serum UW50 diluted 1:1 in 10 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane, pH 7.5, containing 50 mM NaCl, were depleted of antibodies cross-reacting with GCAP1 by filtering through a GCAP1-Sepharose column (1.5 ml; 8 mg of protein/ml of CNBr-activated Sepharose 4B). The flow-through was then applied on a GCAP3-Sepharose (1.5 ml; 8 mg of protein/ml of CNBr-activated Sepharose 4B) to remove GCAP3 cross-reacting antibodies. The anti-GCAP2 antibodies present in the flow-through were finally purified on GCAP2-Sepharose (1.5 ml; 8 mg of protein/ml of CNBr-activated Sepharose 4B) and eluted with 0.1 M glycine, pH 2.5. For UW14, rabbit serum UW14 was depleted of cross-reacting antibodies by passing through GCAP2 and GCAP3 columns and then purified on GCAP1-Sepharose. For UW84, the flow-through of successive GCAP1 and GCAP2 columns loaded with rabbit serum UW84 was purified on GCAP3-Sepharose. UW87 antiserum was prepared against a 20-amino acid peptide, amide-CETDSSKSPDKA-GLGKVKMK prepared by Quality Controlled Biochemicals Inc., conjugated to keyhole limpet hemocyanin. The sequence is derived from the C-terminal region unique to GCAP3. The peptide also contains the N-terminal Cys to facilitate conjugation to keyhole limpet hemocyanin and CNBr-activated Sepharose. UW87 antiserum was purified on GCAP3-Sepharose.

*Enzyme-linked Immunosorbent Assay*—Microtitration plates were coated for 1 h at 37 °C with purified GCAP1 or GCAP2 at about 0.8

amplification of exons and introns are indicated by *arrows* pointing to the *left* (antisense) or to the *right* (sense). The predicted amino acid sequences of the exons are shown in *single letter code*, and the residues are numbered on the *right*. *Capital letters* denote the normal GCAP3 sequence, and *lowercase letters* show the predicted sequence of the splice variant 13b. The amino acid sequence of the splice variant is *numbered* on the *right* in *parentheses*. The putative area of sequence similarity to human calbindin is *boxed*. The alternative splice site in intron c is marked by a *triangle*. Potential polyadenylation sites (37) in the 3′-untranslated region are *boxed*. The beginning and end of the 13b DNA sequence are identified by *arrows*. Functional EF-hand consensus sequences for $Ca^{2+}$ binding are *shaded*. *B*, *schematic diagram* of the gene structure that is identical for all known GCAPs. *C*, intron/exon junctions of GCAP1 to -3 and recoverin. The approximate lengths of the introns are given in the *far right column*. *bp*, base pairs. *D*, fluorescence *in situ* hybridization of a GCAP3 cDNA probe to human chromosomes. The *arrow* identifies the location of the GCAP3 gene on 3q13.1.


FIG. 3—*continued*

$\mu$g/well. After coating, plates were blocked with PBS, 3% bovine serum albumin for 1 h at room temperature. After washing with PBS, 0.05% Tween, anti-GCAP1 (UW14), anti-GCAP2 (UW50), or anti-GCAP3 (UW84), antibodies (0.45 $\mu$g) were added to the plates with increasing amounts of competitors (GCAP1, GCAP2, and GCAP3) for 1 h at room temperature. After washing with PBS, 0.05% Tween, the plates were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) for 30 min at room temperature. The plates were read at 405 nm after the addition of *p*-nitrophenyl phosphate.

*GC Assays*—The GC assays were performed using [$\alpha$-$^{32}$P]GTP and washed ROS or using recombinant GC1 and GC2 as described previously (23). GCAP1 and GCAP3 proteins used in the GC assays were purified from insect cells on $Ni^{2+}$-nitrilotriacetic acid columns in nondenaturing conditions according to the manufacturer's protocol (Qiagen). $[Ca^{2+}]_{free}$ was adjusted by EGTA/Ca buffer as described previously (8). Bovine GC1 (obtained from Dr. R. Sharma) and human GC2 (obtained from Dr. A. Dizhoor) were cloned into pVL941 and expressed in High Five insect cells as described previously (8). The cells were harvested; washed with 10 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane, pH 7.5, containing 100 mM NaCl, and resuspended at ~0.5 mg/ml for the GC assays.

The results of GC assays are an average of two determinations. Similar results were obtained from at least three different sets of experiments performed in duplicate. Due to the high sensitivity of the GC system (for details, see Ref. 8), the absolute values of one series occasionally varied from another by 10–20%, but with preservation of the ratio between activity of two different preparations (for example, the activity of GCAP1 *versus* GCAP3). Because only a limited number of the test samples could be performed in a single assay (maximally 24 samples) that always included a relevant control (low, high $[Ca^{2+}]_{free}$ and with or without GCAP1), the results are shown without S.D. values. The $IC_{50}$ and $EC_{50}$ with S.D. were calculated from these independent experiments.

*Southern Blotting*—Genomic Southern blots containing *Eco*RI-digested DNA from various species were purchased from BIOS laboratories (New Haven, CT). The blots were probed with $^{32}$P-labeled human GCAP3 cDNA insert. The hybridization and washing procedures were performed according to the manufacturer's protocol. Briefly, hybridization was carried out overnight with $10^7$ cpm/ml of labeled GCAP3 cDNA at 65 °C in 6× SSC (0.9 M NaCl, 0.09 M sodium citrate), 1% SDS, 5× Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 200 $\mu$g/ml sheared, denatured salmon sperm DNA, and 10% dextran sulfate. The washing solution contained 1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate), 0.5% SDS and was performed at room temperature and at 35 °C for 10 min each. The blots were finally exposed to Kodak XAR5 film in the presence of intensifying screens.

*Gene Characterization*—A bacterial artificial chromosome (BAC) clone containing the entire GCAP3 gene was purchased from Genome Systems, Inc. BAC DNA was prepared using the Qiagen method for low copy plasmids (Qiagen). Introns b and c of the GCAP3 gene were amplified with exon-specific primers from human genomic DNA with *Taq* (Promega) or *Expand* (Boehringer Mannheim) DNA polymerases. Amplified introns were cloned into pCR2.1 vector and sequenced with universal primers using a model LI-COR 4000L automatic DNA sequencer. The junctions of intron a were directly sequenced from the BAC clone using fluorescent chain terminators and an ABI automatic sequencer. PCR was performed with 2 $\mu$g of BAC DNA, and 20 pmol of primer. The cycle conditions (MJ cycler, MJ Research) were 95 °C for 5 min (96 °C for 20 s, cooling at 1 °C/s to 50 °C, 50 °C for 15 s, heating at 1 °C/s to 60 °C, 60 °C for 4 min). The cycle shown in parentheses was repeated 49 times. The length of intron a was estimated from *Eco*RI-digested fragments of the BAC clone, probed after Southern blotting with DNA containing exons 1 and 2 (results not shown). Genomic sequences upstream of ATG and downstream of the translational stop codon were obtained by direct BAC sequencing with sequence-specific primers.

*Chromosomal Localization*—The chromosomal location of the human GCAP3 gene was identified by PCR with primers 654 (5′-ATG GGG AAT GGC AAA TCT ATAG) and 655 (5′-CGT GTC AAA GGT ATT ATA AAC TTG) using human-hamster somatic cell hybrids (BIOS Laboratories) as templates. The PCR amplification was performed as described previously (24). For subchromosomal localization, the GCAP3 cDNA probe was labeled with biotin-14-dATP using the BioNick labeling system (Life Technologies). The probe was hybridized to prometaphase chromosomes prepared from chromosomal normal peripheral blood lymphocytes obtained by standard clinical laboratory techniques. Fluorescence *in situ* hybridization (FISH) was performed as described previously (24) with some modifications. Pretreatment was performed with 0.1 $\mu$g/ml proteinase K (Sigma) in 0.3 M NaCl, 0.03 M sodium citrate (2× SSC). The probe (200 ng) was combined with Cot-1 DNA, precipitated with ethanol, and hybridized to metaphase chromosomes for 72 h. Postwashing was done at 45 °C with a 5-min incubation in 50% formamide, 2× SSC followed by a 2-min rinse in 2× SSC and PBS. The labeled probe was detected after two layers of avidin-fluorescein (ONCOR, Gaithersburg, MD). Slides were counterstained with 4′,6-diamidino-2-phenylindole (Vysis, Downer's Grove, IL) diluted 1:1 with antifade (Vectashield, Vector, Burlingame, CA). Hybridized G band-like metaphase cells were obtained and viewed using the M-FISH version 3.012 software program in the VYSIS QUIPS Imaging system.

## RESULTS

*Cloning of GCAP3 cDNA*—An expressed sequence tag (GenBank™ accession number AA364442) with homology to GCAPs was found by searching data bases with GCAP2 sequence using the TFASTA program (GCG Package). Primers designed within this expressed sequence tag were used to amplify the 5′- and

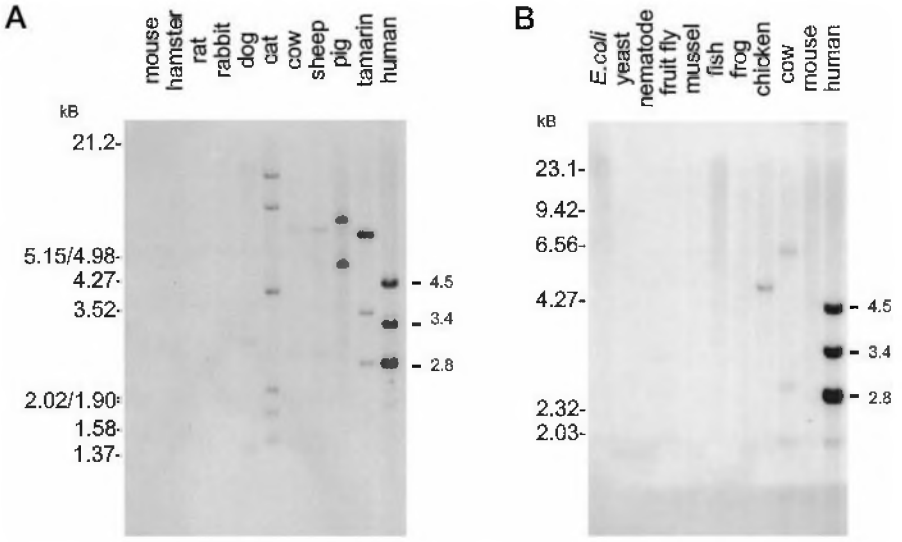


FIG. 4. **Southern blots of various vertebrates and invertebrates probed with GCAP3 cDNA.** *A*, *Eco*RI digests of genomic DNA from 11 mammalian species. *B*, *Eco*RI digests of three mammalian DNAs, three vertebrates, three invertebrates, yeast, and *E. coli*. Size markers are indicated on the *left*. Fragments containing coding sequences of the human GCAP3 gene are identified on the *right*.

3′-ends of GCAP3 in two overlapping fragments by PCR employing a human retina cDNA library. A contiguous cDNA comprising the complete coding sequence, suitable for expression, was amplified using N- and C-terminal primers. The first ATG present on the cDNA matched the Kozak consensus sequence (25) for initiation of translation with a G residue following the ATG and a purine, preferably A, three nucleotides upstream (Fig. 3). The cDNA encodes a protein of 209 amino acids with a calculated molecular mass of 23.8 kDa (Fig. 1). The sequence shows a putative site for *N*-myristoylation at $Gly^2$ and three putative EF hands involved in $Ca^{2+}$ binding. The amino acid sequence of GCAP3 shares closest homology with the human GCAP1, displaying 58% similarity and 45% sequence identity (Fig. 1, *dendrogram*). GCAP3 is an acidic protein with an isoelectric point of 4.8. This value is similar to those calculated for GCAP1 ($pK_a$ = 4.1) and GCAP2 ($pK_a$ = 4.5).

*Splice Variants*—PCR amplification of GCAP3 and screening of a human retina cDNA library yielded normal GCAP3 cDNA clones and also two distinct variants, which have retained 40 base pairs of intron c (Fig. 3). One variant carried a G → T transition at the first base of intron c, abolishing the exon/intron junction consensus sequence (Fig. 3). Conceptual translation of these variants predicts 196-residue polypeptides, which, if produced, would have a C-terminal domain distinct from other GCAPs. The fourth EF-hand motif would be in part deleted and rendered nonfunctional. The 49-amino acid-long sequence specific for the splice variants has 61% sequence similarity over a stretch of 31 residues with human calbindin, a S100-like $Ca^{2+}$-binding protein (26, 27). It is unclear whether these variants would have GC-stimulating activity, since the C-terminal region of GCAP1 is necessary for its interaction with GC (28), and the truncation of the C terminus in the GCAP3 variant may lead to inactivation. Preliminary quantitative PCR using specific primers suggested that the cDNA encoding GCAP3 may be less abundant than the splice variant (data not shown).

*Tissue Distribution of GCAP3 Expression*—The expression of GCAP3 was tested in different human tissues by Northern blot analysis (Fig. 2). A single mRNA of 1.3 kb was detected primarily in human retina. A low intensity band of the same mobility was detectable in skeletal muscle, but no attempt has been made to verify its identity. Using human GCAP3 cDNA as a probe, a GCAP3 ortholog was not detectable in bovine (Fig. 2) and mouse retina (data not shown), suggesting low levels of expression or a low degree of sequence conservation.

*Gene Structure of GCAP3*—A human BAC genomic DNA library was screened with the full-length GCAP3 cDNA probe (Genome Systems, Inc.) and two positive clones were obtained. One of the BAC clones was used for direct sequencing to determine the positions of the intron and exon splice junctions. The GCAP3 gene (Fig. 3*A*) consists of four exons and three introns localized at the same positions as those of GCAP1 and GCAP2 genes. The lengths of the GCAP3 introns are larger, and the intron sequences show no sequence similarity with corresponding introns of the GCAP1 and GCAP2 genes (Fig. 3*C*). The GCAP3 gene encompasses more than 25 kb of genomic DNA, while the GCAP1/GCAP2 gene array is contained in less than 20 kb. The identical gene structure of the GCAP genes suggests that they were generated by gene duplication from a common ancestral gene.

*Chromosomal Localization of the GCAP3 Gene*—Amplification of a portion of exon 1 of the GCAP3 gene with primers 654 and 655 (Fig. 3*A*) from human-hamster somatic hybrid panels located the gene to chromosome 3. The GCAP3 gene localization was further analyzed by FISH using a GCAP3 cDNA probe, which fine-mapped the gene to 3q13.1 on banded metaphase chromosomes (Fig. 3*D*). The locus of the GCAP3 gene on chromosome 3, at band q13.1, is different from the loci of GCAP1 and GCAP2 both arranged in a tail-to-tail cluster on chromosome 6 (p21.1). This suggests that GCAP genes not only have been duplicated but also have been translocated to another chromosome.

*Southern Blot Analysis*—The presence of the GCAP3 gene in diverse species was analyzed by genomic Southern blotting under stringent hybridization conditions. A signal, distinct from those observed with GCAP1 and GCAP2 genes, was detected in tamarin, human, pig, cat, chicken, cow, sheep, and dog, but not in rodents. Under the hybridization conditions, the GCAP3 gene was not detectable in amphibians, fish, invertebrates, and prokaryotes (Fig. 4). The results suggest that the GCAP3 gene sequence is well conserved only in some of the higher vertebrates.

*Activity of GCAP3 Expressed in Insect Cells*—GCAP3 was expressed in insect cells, purified by affinity chromatography, and assayed for stimulation of GC. GCAP3 was partially myristoylated (>75%, data not shown) as determined using [$^3$H]Leu and [$^3$H]myristic acid (28). Myristoylation of GCAP3 was not absolutely required for activity, since unmyristoylated GCAP3 expressed in *E. coli* had approximately 50% of the specific activity of GCAP3 expressed in insect cells. In contrast, GCAP1 and GCAP2 expressed in *E. coli* were only ~5 and

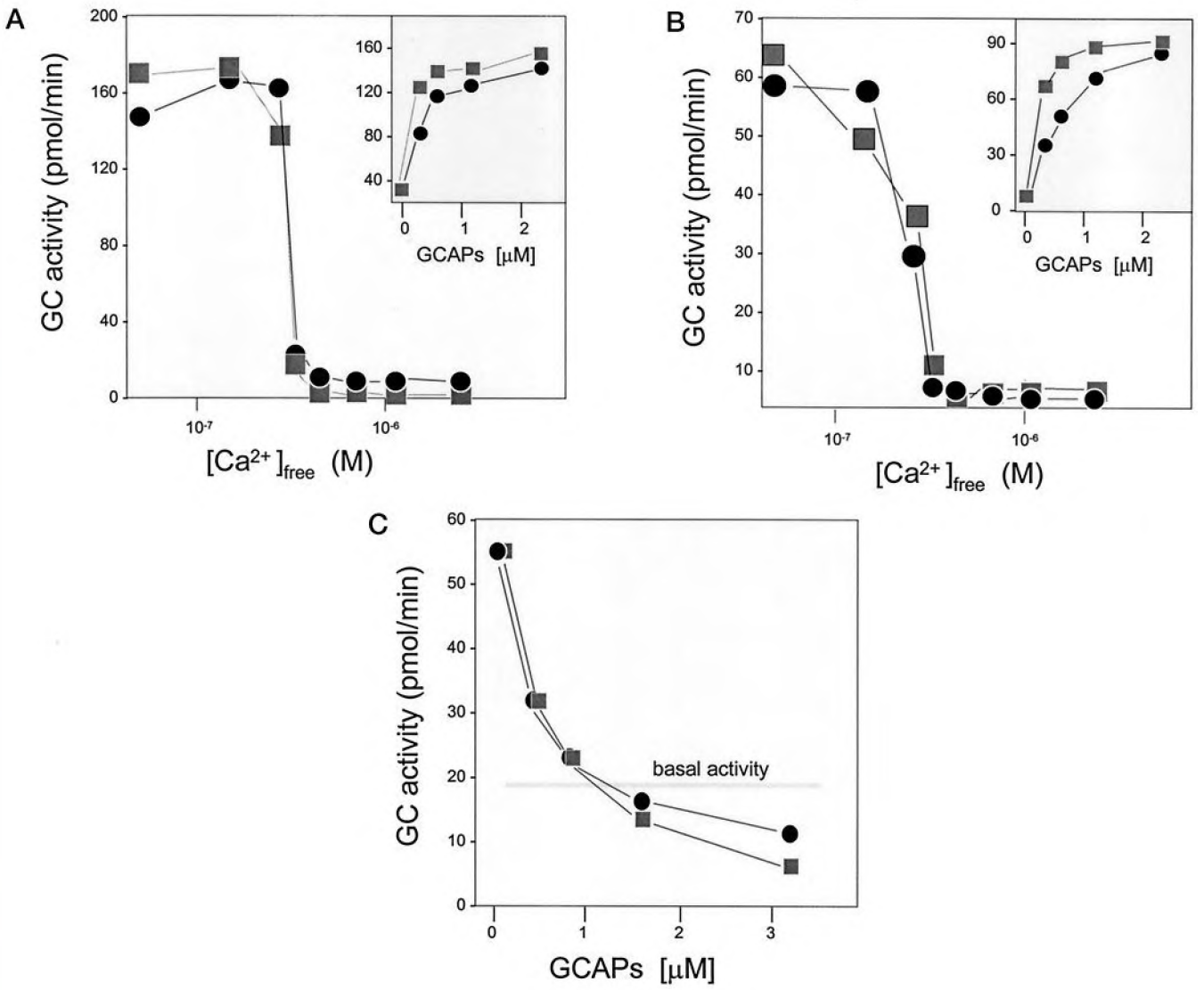


FIG. 5. **Stimulation of GC activity by GCAP3.** *A*, $Ca^{2+}$ titration of GC activity in washed ROS membranes in the presence of 4 μg of GCAP3 or GCAP1. For GCAP1, $IC_{50}$ = 240 ± 42 nM $[Ca^{2+}]_{free}$; for GCAP3, $IC_{50}$ = 254 ± 48 nM $[Ca^{2+}]_{free}$. *Inset*, dose dependence of GC activity in washed ROS by GCAP1 ($EC_{50}$ = 0.7 ± 0.2 μM) and GCAP3 ($EC_{50}$ = 0.6 ± 0.2 μM). *B*, $Ca^{2+}$ titration of GC1 activity in insect cell membranes in the presence of 4 μg of GCAP3 or GCAP1. For GCAP1, $IC_{50}$ = 180 ± 35 nM $[Ca^{2+}]_{free}$; for GCAP3, $IC_{50}$ = 204 ± 42 nM $[Ca^{2+}]_{free}$. *Inset*, dose dependence of GC1 activity in insect cells membranes by GCAP1 ($EC_{50}$ = 0.8 ± 0.1 μM) and GCAP3 ($EC_{50}$ = 1.0 ± 0.2 μM). *C*, inhibition of GC stimulation by GCAP1 triple mutant. GC in washed ROS was stimulated by 1.5 μM GCAP1(E75D,E111D,E155D) mutant at $[Ca^{2+}]_{free}$ = 2 μM in the presence of various concentrations of GCAP1 ($IC_{50}$ = 0.7 ± 0.1 μM) and GCAP3 ($IC_{50}$ = 0.6 ± 0.1 μM). ●, bovine GCAP1; ■, human GCAP3

~20% as active as the native GCAPs, respectively.

At low $[Ca^{2+}]$, GCAP3 stimulated photoreceptor GC in washed ROS and in recombinant GC1 with similar affinity and efficiency (Fig. 5, *A* and *B*, *insets*) and displayed $Ca^{2+}$ sensitivity comparable with GCAP1 (Fig. 5, *A* and *B*). To test if GCAP3 and GCAP1 interact with the same site on GC1, competition assays were done with a constitutively active GCAP1(E75D,E111D,E155D) triple mutant. This mutant does not bind $Ca^{2+}$ and thus activates GC at high $[Ca^{2+}]_{free}$ (28). Similar amounts of GCAP1 and GCAP3 competed with the triple mutant (Fig. 5*C*), suggesting that the binding sites for both proteins are at least partially overlapping. Inhibition of the basal activity of GC was also observed with GCAP3. GCAP1, GCAP2, and GCAP3 stimulated recombinant GC1 (Fig. 6, *A*, *C*, and *E*), while only GCAP2 and GCAP3 were capable of significant stimulation of GC2 (Fig. 6, *B*, *D*, and *F*).

*Specificity of Anti-GCAP Antibodies*—To avoid cross-reactivity between anti-GCAP antibodies, each anti-GCAP serum was depleted of contaminating IgGs by affinity chromatography and subsequently individually purified using the original antigen as affinity matrix. Western and enzyme-linked immunosorbent assays were used to test the specificity of purified anti-GCAP1 (UW14), anti-GCAP2 (UW50), and anti-GCAP3 (UW84) (see "Materials and Methods"). In addition, anti-peptide antibody UW87, specific to the unique C terminus of GCAP3, was obtained. On Western blot, all antibodies were monospecific (Fig. 7*A*). The binding of anti-GCAP1 antibody UW14 to GCAP1-coated plates was competed only by GCAP1 and not by GCAP2 and GCAP3 in a molar range of dilution 1:1000 (Fig. 7*B*). The two other antibodies were also completely specific, UW50 to GCAP2 and UW84 to GCAP3. These antibodies were used to verify the level of expression of GCAP1, GCAP2, and GCAP3 in human and bovine retina (Fig. 7*C*). GCAP1 and GCAP2 were readily detectable in both tissues, while GCAP3 was not detected by UW84 under Western conditions similar to those used with UW14 and UW50 (Fig. 7*C*). Longer exposure of the blot to the alkaline phosphatase substrate revealed weak staining (data not shown). In con-

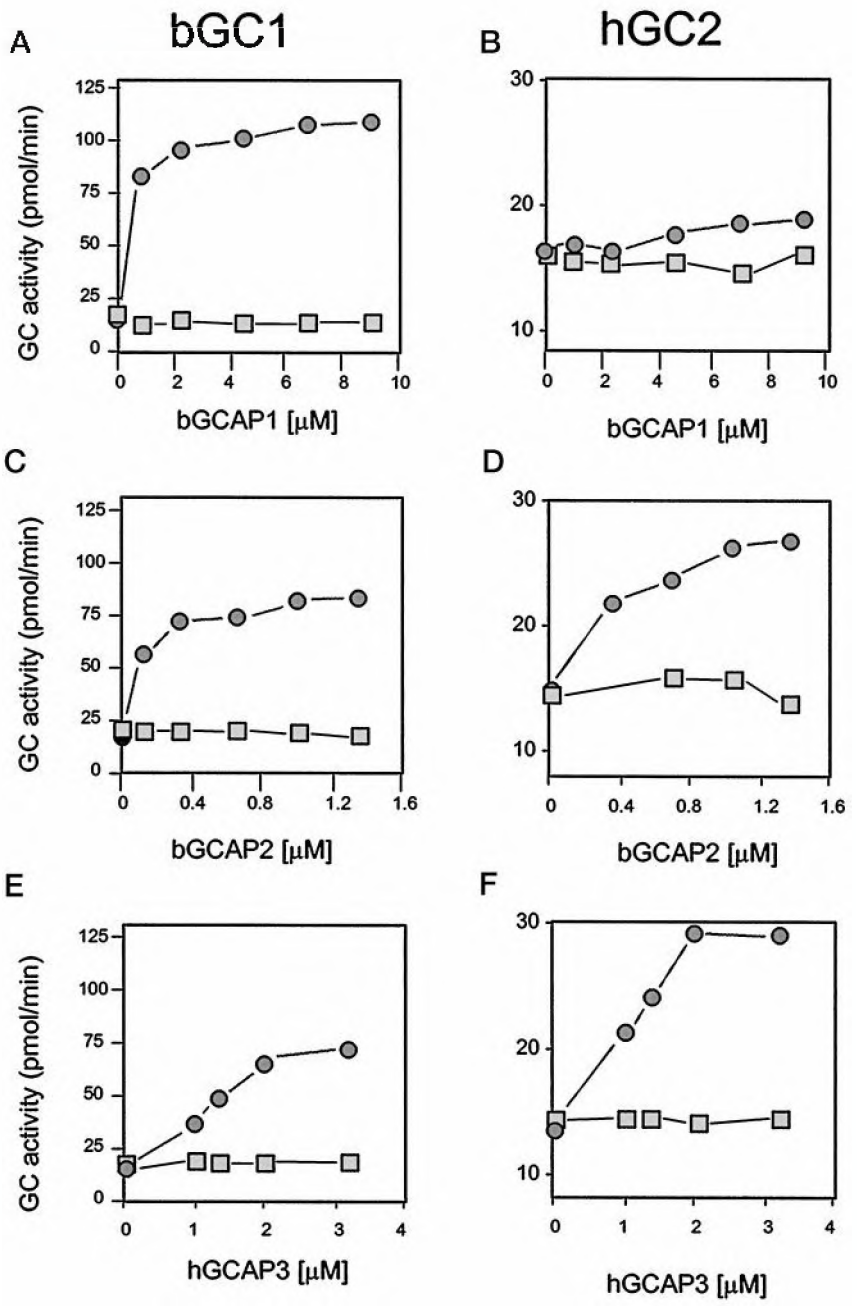


FIG. 6. **Stimulation of GC1 and GC2 by GCAP1, GCAP2, and GCAP3.** Dose dependence of bovine GC1 (*bGC1*) and human GC2 (*hGC2*) stimulation at low and high $[Ca^{2+}]_{free}$ by recombinant bovine GCAP1 (*bGCAP1*; *A* and *B*), recombinant bovine GCAP2 (*bGCAP2*; *C* and *D*), and recombinant human GCAP3 (*hGCAP3*; *E* and *F*). 50% of maximal stimulation was observed for the following pairs: GCAP1/GC1, 0.8 ± 0.1 μM; GCAP1/GC2, not measurable; GCAP2/GC1, 0.3 ± 0.1 μM; GCAP2/GC2, 0.5 ± 0.2 μM; GCAP3/GC1, 1.0 ± 0.2 μM; and GCAP3/GC2, 1.1 ± 0.3 μM. ○, 50 nM $[Ca^{2+}]_{free}$; ▨, 2 μM $[Ca^{2+}]_{free}$.

trast, UW87 readily detected GCAP3 in human tissue and not in bovine tissue when incubated overnight with the blot (Fig. 7*C*).

## DISCUSSION

In this paper, we describe the characterization of a new guanylyl cyclase-activating protein, termed GCAP3. The GCAP3 protein shows high sequence similarity to GCAP1 (57%) and to a lesser degree to GCAP2 (49%) and other $Ca^{2+}$-binding proteins (40% and 35% similarity with recoverin and calmodulin, respectively). The homology is localized mostly in and around the EF-hands able to bind $Ca^{2+}$ (Fig. 1). All GCAPs are predicted to have three functional EF-hands for binding of $Ca^{2+}$ and have acidic isoelectric points, similar molecular masses (GCAP3, 23.4 kDa; GCAP1, 22.9 kDa; GCAP2, 23.4 kDa), and a consensus sequence for *N*-myristoylation at $Gly^2$. The myristoylation of GCAP3 was confirmed by heterologous expression in insect cells in the presence of [$^3$H]myristic acid. The precise function of the myristoylation in GCAPs is unclear, although this modification is conserved among GCAPs and among related recoverins/neurocalcins (6, 29, 30).

Multiple-tissue Northern blot analysis revealed a single band of 1.3 kb abundantly detectable only in human retina. The level of GCAP3 mRNAs in human retina was found at least as high or higher than that of GCAP1 and GCAP2. This was surprising, since GCAP3 proteins appear to be less abundant than GCAP1 and GCAP2 as judged by Western blots with monospecific antibodies (Fig. 7). This discrepancy between the mRNA and protein levels may be explained by the abundant transcription of mRNA encoding a splice variant retaining part of intron c. Alternatively, the amount of GCAP3 in the retina may be regulated at a posttranscriptional level. GCAP3 mRNA was not detectable in bovine retina with human cDNA as a probe. Attempts to clone GCAP3 from bovine and mouse cDNA by PCR or library screening were so far unsuccessful. However, the bovine GCAP3 gene was detectable in Southern blot analysis. Rodent genomic DNA, in contrast, did not reveal distinct

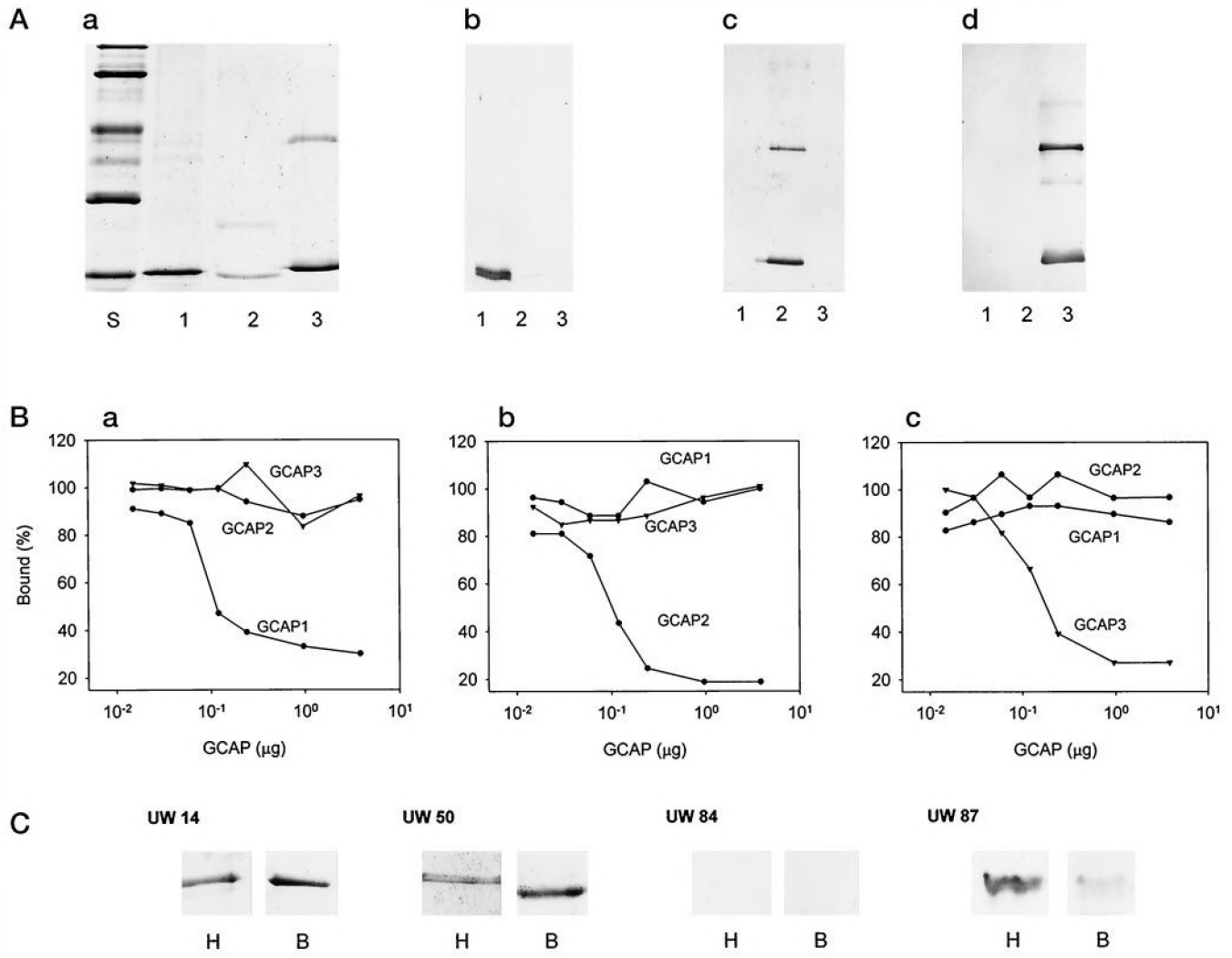


FIG. 7. **Western blot analysis of the expression of GCAPs in human and bovine retina using specific anti-GCAP antibodies.** *A*, specificity of anti-GCAP antibodies. Anti-GCAP sera were depleted from cross-reactive IgGs and purified as described under "Materials and Methods." *Lane 1*, purified GCAP1 (1 μg); *lane 2*, purified GCAP2 (1 μg); *lane 3*, purified GCAP3 (1 μg). *a*, SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R-250. *Lane S*, standard proteins (94, 67, 43, 30, 20, and 14 kDa); *b–d*, reactivity of GCAP1/GCAP2/GCAP3 by Western blot analysis (loaded as in *a*) with anti-GCAP1 (UW14), anti-GCAP2 (UW50) and anti-GCAP3 (UW84), respectively. *Lane S*, standard proteins (104, 81, 47.7, 34.6, 28.3, and 19.2 kDa). *B*, specificity of anti-GCAP antibodies tested by enzyme-linked immunosorbent assay. *a*, anti-GCAP1. UW14 antibody (0.45 μg) and increasing amounts of GCAPs were added to GCAP1-coated plates. GCAP2 and GCAP3 had no effect, and for GCAP1, $IC_{50} = 0.09 \pm 0.02$ μg; *b*, anti-GCAP2 antibodies; UW50 antibodies (0.45 μg) and increasing amounts of GCAPs were added to GCAP2-coated plates. GCAP1 and GCAP3 had no effect, and for GCAP2, $IC_{50} = 0.10 \pm 0.02$ μg; *c*, anti-GCAP3. UW84 antibodies (0.5 μg) and increasing amounts of GCAPs were added to GCAP3-coated plates. GCAP1 and GCAP2 had no effect, and for GCAP3, $IC_{50} = 0.25 \pm 0.04$ μg. *C*, Western blot analysis of the expression of GCAPs in bovine and human retina. Human (*H*) or bovine (*B*) retinal extracts (~8 μg each) were probed with anti-GCAP1 (UW14), anti-GCAP2 (UW50), anti-GCAP3 (UW84), or anti-GCAP3 peptide (UW87).

signals under the same stringency (Fig. 4), suggesting the absence of the GCAP3 gene or lower sequence conservation within these species.

Recently, localization of GCAP1 and GCAP2 has been investigated by immunocytochemical methods in various species (15, 21). Clear differences of the levels and cell distributions of GCAPs between species were observed. Since the regulation of GCs by GCAPs is a key step in light adaptation (desensitization) (3), these results were not surprising, since different animals may evolve unique desensitization mechanisms in controlling the levels of cGMP. The apparent differences in the mRNA GCAP3 levels would be consistent with varying levels of GCAP1 and GCAP2 in other species. *In situ* hybridization results indicate that in human retina, GCAP3 mRNA is present in the myoid region of photoreceptors.[2] GCAP3 monospecific antibodies suitable for immunocytochemical studies are currently not available. A separate study dedicated to cellular expression and subcellular localization of GCAP3 is currently under way.

The intron/exon arrangement of human GCAP3 is identical to that of the GCAP1/GCAP2 gene array. However, the GCAP3 gene is not arranged within the GCAP1/GCAP2 cluster but has been translocated to chromosome 3(q13.1) (Fig. 3*D*). Another cluster of genes comprises the S-100 $Ca^{2+}$-binding protein gene family on chromosome 1q21. Some of the S-100 genes, however, are found also on other chromosomes (31). Thus, the chromosomal location of GCAP1 and GCAP2 in a cluster on 6p and another homologous gene, GCAP3, on 3q has other precedents. It is interesting to note that the positions of the two introns of the human recoverin gene (located on 17q) are exactly as those of introns b and c of the GCAP genes (Fig. 3*C*). The close relationship between the GCAP and recoverin gene structure suggests that these genes were generated by gene duplication from a common ancestor. While GCAP1 on 6p21.1 is associated

[2] R. N. Farris and K. Palczewski, unpublished results.

with autosomal dominant cone dystrophy, the 3q13.1 locus has not yet been associated with any known retinal disease.

The three GCAPs interact with GC1 employing overlapping sites, in part, because they all compete with the constitutively active mutant of GCAP1. The three GCAPs stimulate recombinant GC1 with a similar affinity and efficiency. GCAP3 and GCAP2 activate both GC1 and GC2, whereas GCAP1 activates only GC1. What is particular to GCAP2 and GCAP3 that would account for the specific interaction with GC2? Only seven amino acids are identical or represent conservative substitutions ($Ser^{6}$, $His^{38}$, $Asn^{82}$, $Gln^{127}$, $Pro^{131}$, $Ile^{135}$, and $Ile^{168}$; see Fig. 1) between GCAP3 and GCAP2 on one side and GCAP1 on the other. It is known, from hybrid studies between bovine GCAP2 and neurocalcin (32), that the amino acids between $Lys^{30}$ and $Phe^{49}$, and between $Asn^{168}$ and $Asn^{189}$, are necessary for GCAP2 to activate GCs. This suggests that $His^{38}$ and/or $Ile^{168}$ of GCAP3 might be important for the interaction with GC2.

GCAP3 is the fifth $Ca^{2+}$-binding protein that interacts with GC. This list includes GCAP1, GCAP2, guanylyl cyclase-inhibitory protein, and perhaps S100$\beta$ (6, 7, 8, 13, 33). These findings do not come as a surprise, since $Ca^{2+}$ has been postulated to control the rate of recovery to dark condition upon illumination and to establish the level of desensitization during light adaptation (3). The identification of multiple proteins that sense changes in $[Ca^{2+}]$ within the cone and rod cells, including GCAP3 described in these studies, suggest complex regulatory mechanisms for controlling the activity of photoreceptor GCs.

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