Gene Array and Expression of Mouse Retina Guanylate Cyclase Activating Proteins 1 and 2

Kim Howes,1,6 J. Darin Bronson,1,6 Yan Li Dang,2 Ning Li,1 Kai Zhang,1 Claudia Ruiz,2 Bharati Helekar,2 Muriel Lee,3 Iswari Subbaraya,2 Helga Kolb,1 Jeannie Chen,4,3 and Kim Howes,1,6 J. Darin Bronson,1,6 Yan Li Dang,2 Ning Li,1 Kai Zhang,1 Claudia Ruiz,2 Bharati Helekar,2 Muriel Lee,3 Iswari Subbaraya,2 Helga Kolb,1 Jeannie Chen,4,3 and Wolfgang Baehr1

PURPOSE. To identify gene arrangement, chromosomal localization, and expression pattern of mouse guanylate cyclase activating proteins GCAP1 and GCAP2, retina-specific Ca2+-binding proteins, and photoreceptor guanylate cyclase activators.

METHODS. The GCAP1 and GCAP2 genes were cloned from genomic libraries and sequenced. The chromosomal localization of the GCAP array was determined using fluorescent in situ hybridization. The expression of GCAP1 and GCAP2 in mouse retinal tissue was determined by immunocytochemistry.

RESULTS. In this study, the mouse GCAP1 and GCAP2 gene array, its chromosomal localization, RNA transcripts, and immunolocalization of the gene products were fully characterized. The GCAP tail-to-tail array is located at the D band of chromosome 17. Each gene is transcribed into a single transcript of 0.8 kb (GCAP1) and 2 kb (GCAP2). Immunocytochemistry showed that both GCAP genes are expressed in retinal photoreceptor cells, but GCAP2 was nearly undetectable in cones. GCAP2 was also found in amacrine and ganglion cells of the inner retina. Light-adapted and dark-adapted retinas showed no significant difference in the distribution of the most intense GCAP2 staining within the outer segment and outer plexiform layers.

CONCLUSIONS. Identical GCAP gene structures and the existence of the tail-to-tail gene array in mouse and human suggest an ancient gene duplication-inversion event preceding mammalian diversification. Identification of both GCAPs in synaptic regions, and of GCAP2 in the inner retina suggest roles of these Ca-binding proteins in addition to regulation of phototransduction. (Invest Ophthalmol Vis Sci. 1998;39:867-875)

Guanylate cyclase activating proteins (GCAPs) are Ca2+-binding proteins of the calmodulin gene family1 that contain four EF hand Ca2+-binding motifs. To date, two GCAPs (GCAP1 and GCAP2) have been identified in mammalian retina.2-6 Both GCAPs activate photoreceptor guanylate cyclase in the presence of low [Ca2+], a regulatory mechanism that promotes accelerated synthesis of cGMP, the internal messenger of phototransduction, after photobleaching. The human GCAP genes are arranged in a compact tail-to-tail gene array in which both genes are transcribed from opposite strands.7 Biochemical and immunologic studies to determine the cellular and subcellular distribution of GCAPs in the retina have produced conflicting results. RNA expression studies show nearly identical expression patterns of GCAP1 and GCAP2 mRNAs in rod and cone photoreceptors.8,9,10 Bovine GCAP1 was isolated from rod outer segment membranes,3,10 but GCAP2 could3 or could not be isolated9,10 from this source. Immunocytochemistry using GCAP1-specific antibodies has revealed that GCAP1 is present in rod and cone outer segments and in synaptic regions.5 GCAP2 was seen in outer and inner segments of rods but not of cones by one group of investigators.5 It was seen in the inner segments of rods and cones but not in outer segments by another group.2 The reasons for these discrepancies in GCAP2 distribution are unknown.

In this study, we characterized the GCAP genes and their expression patterns in the retina of the mouse, an animal amenable to genetic manipulation. The ultimate goal of this research was to prevent expression of GCAP genes by targeted gene replacement (gene knockout) and to analyze the consequences of this manipulation on the physiology and morphology of photoreceptor cells. As a first step toward this goal, we describe the GCAP...
gene arrangement and chromosomal localization in mouse, and
discuss expression patterns of both GCAPs in the retina.

**MATERIALS AND METHODS**

**Cloning of Mouse GCAP2 cDNA and DNA Sequencing**

Mouse GCAP1 cDNA clones were described previously. To isolate mouse GCAP2 cDNA clones, a mouse retina cDNA library was screened with a bovine GCAP2 cDNA insert. Two clones were isolated, mG2-6 and mG2-7. The inserts of λazapII bacteriophage (Invitrogen, San Diego, CA) clones were excised according to the manufacturer's protocol. Supercoiled plasmid DNA was sequenced using the double-stranded procedure described previously or using an automatic sequencer (LI-COR Model 4000L) and universal primers labeled with an infrared fluorescent tag. The coding portions of all clones were completely sequenced on both strands. mG2-6 was 5'-truncated in exon 1. Clone mG2-7 contained the complete coding sequence from the gene sequence.

**Northern Blot Analysis**

Mouse retina mRNA was isolated (Fasttrack; Invitrogen), was separated on 0.43 M formaldehyde agarose gels, and was transferred to maximum strength nylon filters (Nytran; Schleicher and Schuell), as described previously. The GCAP1 probe was mG1-4, the GCAP2 probe mG2-6 (Fig. 1). The mouse opsin cDNA probe used as a control has been described previously. The GCAP1 probe was hybridized to normal adult mouse retina mRNA and reprobed with mouse GCAP1. Lane 1, the blot was stripped and reprobed with mouse GCAP2. Lane 2, the blot was stripped and reprobed with mouse opsin (mOPS) cDNA as a control. The mOPS gene is transcribed into five mRNA species differing in polyadenylation sites. Known size standards are indicated on the right in kilobases.

**Mouse GCAP Genomic Clones**

A nick-translated GCAP1 fragment was used to screen a mouse genomic library (mouse 129SVJ strain, prepared from liver of a 4–8-week-old female; Stratagene, La Jolla, CA). Three genomic clones (AMG1, AMG2, and AMG3) with inserts of 15 to 18 kb were identified and characterized by subcloning. EcoRII fragments of AMG2 corresponded to the two major GCAP1 genomic fragments seen on Southern blots, and were subcloned into pUC13 to yield pUC15 and pUC-top (Fig. 2). Puc-bot and pUC-top contained introns b and c and most of intron a of the GCAP1 gene. The remainder of intron a was amplified with sequence-specific primers. Introns of the GCAP2 gene were amplified from genomic DNA with exon-specific primers (Fig. 2), using either Taq polymerase according to the Cetus (Berkeley, CA)/Perkin-Elmer (Norwalk, CT) protocol, or Taq/Fwo polymerases (Expand Long PCR system, Boehringer–Mannheim, Indianapolis, IN), and cloned into PCRII (Invitrogen).

**Antibodies**

Polyclonal antibody UW14 was raised against bacterially expressed, truncated bovine GCAP1. Polyclonal UW50 was raised against bovine GCAP2 expressed in bacteria. The anti-human growth hormone antibody (anti-hGH) was characterized previously.

**Chromosomal Localization by Fluorescent In Situ Hybridization**

DNA from clone AMG1 was labeled with bio-16-dUTP (Boehringer–Mannheim) by nick translation and hybridized to mouse embryonic stem cells and to mouse embryonic liver cells as described. Hybridization signals were detected with successive layers of avidin Texas red (Vector Laboratories, Burlingame CA), biotinylated antiavidin (Vector), and avidin Texas Red. The initial localization was confirmed by using a mouse chromosome 17 paint (Cambio, La Jolla, CA).

**Immunocytochemistry**

Normal C57BL/6 and transgenic mice of both sexes were killed under normal illumination. For dark-adapted experiments, three females of one litter were killed at night (12 midnight) under red light after 6 hours of dark adaptation. Once the eyes were removed, an incision was made through the anterior chamber to facilitate fixation in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After fixation for 6 hours at 4°C, the lenses were removed, and the eyes were rinsed and cryoprotected in 30% buffered sucrose overnight at 4°C. Eight-micrometer-thick cryosections were incubated in 10% normal goat serum for 30 minutes to inhibit nonspecific binding of the antibodies. In some cases, the tissue sections were also permeabilized with 0.1 mg/ml proteinase K for 2 minutes before blocking with normal goat serum. Primary and secondary antibodies were diluted with 0.1 M PB and 0.3% Triton X-100 for all reactions. Sections were rinsed with 0.1 M PB after each incubation. Sections were incubated overnight at 4°C with 1:2000 UW50 and 1:3000 UW14 antibodies. Fluorescein isothiocyanate (FITC) or Texas red–conjugated goat antirabbit IgG (Vector) was used at a dilution of 1:100 for 1 hour at room

![Figure 1. Northern blot of normal adult mouse retina mRNA probed with GCAP (guanylate cyclase activating protein) 1 and GCAP2. Two micrograms of retina mRNA were loaded. Lane 1, the blot was probed with mouse GCAP1. Lane 2, the blot was stripped and reprobed with mouse GCAP2. Lane 3, stripped and reprobed with mouse opsin (mOPS) cDNA as a control. The mOPS gene is transcribed into five mRNA species differing in polyadenylation sites. Known size standards are indicated on the right in kilobases.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933205/ on 10/17/2018)
temperature. For double-labeling experiments, sections were processed by one of two methods. In the first, sections were incubated with 10 μg/ml FITC- or Texas red-peanut agglutinin (PNA: Sigma, St. Louis, MO) and UW50 or UW14 antibodies, then by FITC-conjugated or Texas red-conjugated goat anti-rabbit IgG. In the second method, sections were sequentially

**Figure 2.** Physical map of mouse cDNA and genomic clones, and diagram of the GCAP (guanylate cyclase activating protein) 2-GCAP1 gene array. The 5'UTRs of the GCAP2 (left) and GCAP1 (right) genes are flanking the gene array, which is depicted as an 18-kb contig. Exons are shown as boxes; the coding portions are filled. Introns (length in kilobases) and flanking sequences are shown as lines. Boxes within introns depict dinucleotide repeats of various lengths. EcoRI restriction sites identified in the gene sequence and cloned fragments (pUC-bot, pUC-top) are shown. Bars underneath and above the exons indicate the extent of cloned cDNA for both genes (mGCAP1, clones mG1-4, and mG1-2; and mGCAP2, clones mG2-6 and mG2-7, respectively). Lines under the GCAP2 gene marked by a, b, and c symbolize intron clones generated by amplification with exon-specific primers. Large arrows indicate the direction of transcription. λMG1, λMG2, and λMG3 are genomic Afz11l clones. Vertical broken lines identify their starting and ending points, if known, determined by direct sequencing of ADNA (for details, see the Genbank submission).

**Figure 3.** Chromosomal localization of the GCAP (guanylate cyclase activating protein) gene array. (A) Localization of the GCAP gene array by fluorescent in situ hybridization to chromosome 17. (B) Ideogram of chromosome 17 and location of the gene array at 17D.
incubated with the hGH primary antibody, and the goat anti-rabbit secondary antibody and then the UW14 or UW50 primary antibody and fluorochrome-conjugated goat antirabbit secondary antibody. Immunofluorescence was photographed with an inverted laser scanning confocal microscope (LSM410; Carl Zeiss, Oberkochen, Germany).

RESULTS

GCAP1 and GCAP2 mRNA

All GCAP1 and GCAP2 clones were truncated at the 5′-UTR, the 3′-UTR, or both. None contained a polyA tail, and only mouse GCAP1 appeared to be alternatively spliced at very low levels. Northern blots of mouse retina mRNA revealed single transcripts of 0.8 kb (GCAP1) and 2 kb (GCAP2; Fig. 1). The open reading frames of the GCAP1 and GCAP2 mRNA predict polypeptides of nearly identical size (202 and 201 amino acids, respectively) and identical domain structure (see Fig. 7). The predicted polypeptide sequences are very similar (90%) to those of corresponding human and bovine GCAPs (Fig. 7 and Discussion section).

Tail-to-Tail GCAP Gene Array

To elucidate the gene arrangement in the mouse, we isolated three overlapping genomic clones AMG1, λMG2, and AMG3 (Fig. 2). Subcloning of genomic EcoRI fragments, polymerase chain reaction amplification of the intergenic region, and direct sequencing showed that the tail-to-tail gene array is preserved in the mouse and that AMG3 contained the entire GCAP1 gene, whereas AMG1 and λMG2 contained the GCAP2 gene and only portions of the GCAP1 gene (Fig. 2). The GCAP1 and GCAP2 coding regions were each interrupted by three introns whose respective positions were identical (Figs. 2, 7). The sizes of the introns varied, ranging from 3.5 kb to 280 bp, and no sequence similarity was found among corresponding introns of the two genes. Transcription start points of the GCAP genes have not been determined, but the size of the mRNA for GCAP1 (800 bp) would predict transcription start and polyadenylation sites within 100 bp of the borders of the coding region of this gene. No consensus polyad-
FIGURE 5. Immunolocalization of GCAP (guanylate cyclase activating protein) 2 in retinal sections from normal and transgenic human growth hormone (hGH) mice. (a) GCAP2 staining is detectable in outer segments, inner segments, and soma of photoreceptors; the outer plexiform layer; amacrine cells (a) of the inner nuclear layer; and ganglion cells. (b) Proteinase K treatment produces a staining pattern identical with that shown in (a) for all layers, with an increase in intensity of staining of the signal within the inner segments of photoreceptors. (c) GCAP2 immunostaining is indicated by fluorescein isothiocyanate (FITC) staining of the outer and inner segments of rods, rod soma, and synaptic termini. Weak double-staining of cones with Texas red-conjugated peanut agglutinin indicates the presence of GCAP2. (d) Blue cone soma and inner segments labeled with Texas Red also show a relatively weak continuation of labeling of the outer segments with GCAP2 (FITC). (e) lower magnification of c. Scale bars, 12.5 μm. BC, bipolar cell; CS, cone somata.

enylation signals could be identified close to the translation stop codons of either gene, but AATAAA signals are present farther downstream. Thus, the presence of untranslated exons in the 3'UTR cannot be excluded. The distance between the translation stop codons of the two mouse GCAP genes is 2.65 kb, approximately one half of the intergenic distance (4.5 kb) observed in human.

The GCAP Array Is Located on Mouse Chromosome 17

We predicted by synteny with human chromosome structure, that the mouse GCAP1 gene should be located on chromosome 17. To verify the location of the GCAP gene array, we used biotinylated AMG3 as a probe for fluorescent in situ hybridization (FISH) studies of mouse chromosomes. The initial localization to chromosome 17 was made on 4,6-diamidino-2-phenylindole (DAPI)-banded chromosomes and was confirmed using mouse chromosome 17 paint (Fig. 3). No significant labeling was observed on other chromosomes, indicating absence of pseudogenes or other closely related GCAP genes, a result consistent with those obtained by fluorescent in situ hybridization in human chromosomes.

Immunolocalization of GCAP1

We performed immunocytochemical analyses using monospecific antibodies for GCAP1 and GCAP2 in sections of normal mouse retina, in combination with fluorochrome-conjugated PNA to identify cones. To distinguish among cone types, we also analyzed sections of transgenic mouse retinas that express hGH specifically in blue cones and bipolar cells. For GCAP1 immunolocalization, we used UW14, a monospecific polyclonal antibody raised against bacterially expressed GCAP. When applied to sections from normal mouse retina, the most intense response was seen in the outer segments and synaptic termini of rods and cones (Fig. 4a), a distribution consistent with that reported earlier in the bovine retina. The signal was completely abolished by
FIGURE 6. Immunocytochemical localization of GCAP (guanylate cyclase activating protein) 2 in dark-adapted (a) versus light-adapted (b) mice. Color-enhanced fluorescein-labeled section superimposed on the bright-field background. Predominant staining for GCAP2 resides within outer segments and the outer plexiform layer for light- and dark-adapted mice. A slight increase in GCAP2 signal is visible in the inner segment and outer nuclear layer of dark-adapted mouse retinas. Bar, 25 μm. IS, inner segment; OS, outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer.

Immunolocalization of GCAP2

For GCAP2 localization experiments, we used a monospecific polyclonal antibody raised against bacterially expressed GCAP2. In normal adult mouse retinas, GCAP2 responses were seen in the outer segments and, to a lesser extent, in the inner segments of rods and in the synaptic terminals (Fig. 5a). Pretreatment of sections with proteinase K resulted in a substantial increase in GCAP2 immunolabeling only in the inner segments (Fig. 5b). Proteinase K treatment did not alter GCAP1 or hGH staining (data not shown). The intensity of GCAP2 staining of the rod inner segment was much stronger than that observed in GCAP1 (Fig. 4a). In addition, immunostaining was seen in the inner retina in amacrine and ganglion cell types (but not in bipolar cells), whereas GCAP1 staining had been limited to photoreceptors (Fig. 4a). When cones were double labeled with PNA (Texas red) and UW50 (FITC), only relatively weak GCAP2 immunostaining was detected in cone outer and inner segments (Figs. 5c, 5e). Blue cones, specifically identified with anti-hGH antibody, also only weakly costained with UW50 (Fig. 5d). In monkey and human, GCAP2 was strongly detected by immunocytochemistry in cone inner segments. In bovine, GCAP2 staining of the cone inner segments is less evident than that seen in monkey and human.

Light–Dark Dependence of GCAP2 Staining

When GCAP2 staining was performed in parallel with light- and dark-adapted retinas (Fig. 6), no significant differences were observed in regard to predominant staining for GCAP2 in the outer segments of rods and the outer plexiform layer. The only differences are a slight increase in GCAP2 staining in the inner segment region as well as in the outer nuclear layer of dark-adapted mice, possibly indicating a replenishment mechanism for GCAP2 during the scotophase. We conclude that in mouse, GCAP2 staining is strong in rods, including synaptic termini, but is nearly undetectable in cones. In contrast to GCAP1, GCAP2 can also be detected in the inner retina, particularly in amacrine and ganglion cells in which its function is unknown. There is no significant effect of light–dark adaptation on the apparent distribution of GCAP2 in the mouse retina.
FIGURE 7. Alignment of GCAP (guanylate cyclase activating protein 1 and GCAP2 amino acid sequences from various species. The sequences were divided into two subgroups, GCAP1 and GCAP2. In the GCAP1 subgroup, the mouse GCAP1 sequence was aligned with GCAP1 sequences from bovine, human, frog, and chicken. In the GCAP2 subgroup, the mouse GCAP2 sequence was aligned with the GCAP2 sequences from human, bovine, and chicken. L=I=V=M; Y=F; E=D; R=K; A=T=S are considered conservative substitutions. For best fit, several gaps were introduced (shown by hyphens). Residues conserved in all GCAP sequences are shown on black background. Residues identical in only one of the groups are lightly shaded. Predicted EF hand Ca²⁺-binding domains (EF2-EF4) are boxed, EF1 (presumably not functional for Ca²⁺-binding) is boxed by a broken line. The identical positions of introns a-c in mouse, human, and bovine GCAPs are shown by triangles above the alignments. Domains conserved in all GCAPs shown are identified by a shaded bar above the alignment (CD1-CD3), and variable domains are shown by VD1-VD4. A dendrogram (PC-GENE, IntelliGenetics; Mountain View, CA) calculated on the basis of the amino acid sequences is shown at the top left.

DISCUSSION

GCAPs are members of a subfamily of Ca²⁺-binding proteins belonging to the large superfamily of calmodulin-like Ca²⁺-binding proteins, which characteristically contain four EF hand helix-loop-helix motifs. Five GCAP1 and GCAP2 genes-cDNA have been cloned to date from various vertebrate species. A consensus is emerging concerning conserved and variable domains and concerning domains involved in membrane association and GC stimulation. Variable domains include the N- and C-terminal ends and spacer domains between EF1-EF2 and EF3-EF4 (Fig. 7). Functionally indispensable domains include regions surrounding the four EF hand motifs. The exact stoichiometry of bound Ca²⁺/GCAP has not been determined, but EF1 is thought to be nonfunctional, whereas EF2, EF3 and EF4 conform to the EF hand consensus sequence and most likely are fully functional. Inactivation of EF hand Ca²⁺-binding domains in GCAP2 by mutagenesis produces a constitutive activator of GC lacking Ca²⁺ sensitivity. Similar effects have been seen with mutant GCAP1. For GCAP1 we also showed that the N-terminal domain, although variant in the GCAPs, is indispensable for GC stimulation and membrane association. All GCAPs appear to be myristoylated at the N terminus of the processed proteins (Gly-2; Fig. 7). The fatty acid side chain, however, is not directly involved in membrane association, because a GCAP1-G2A mutant, in which the myr anchor Gly-2 was replaced by Ala, sediments with rod outer segment membranes. Membrane association of GCAP1 was only abolished when the N-terminal 25 amino acids were deleted, a domain that does not contain an obvious motif for protein-membrane interaction.

In the present study, we attempted to identify the cell types and the subcellular compartments of cells expressing GCAPs in the mouse retina. The mouse retina has no fovea, but...
it contains a substantial number of cones (3%-9% of all photoreceptors). The cones can be identified, among other techniques, by staining with peanut agglutinin. Our study shows apparent distribution of GCAP2 mostly in rods and certain cell types of the inner retina but near absence of GCAP2 in cones. In bovine retina, the GCAP2 signal is generally more intense in the inner segments of rods and is weaker in cones, but in monkey and human retinas, the GCAP2 signal is more intense in cone inner segments and is much weaker in rods. The reason for this apparent discrepancy among species is unknown. The specificity of the antibodies used in this study was examined by early experiments in which a complete loss of immunocytochemical signal in the mouse retina occurred when UW14 and UW50 antibodies were preabsorbed with bovine GCAP1 and GCAP2 proteins, respectively (data not shown). One possible reason for the discrepancy in GCAP2 staining among species could be the partial masking of the epitope recognized by the anti-GCAP2 antibody UW50. To investigate this possibility, mouse retina sections were subjected to limited proteinase K treatment before immunocytochemistry. GCAP2 staining in inner segments of rods was substantially increased (compare Fig. 5A with 5B), consistent with partial removal of masking protein antigens. Limited proteolysis, however, did not significantly improve GCAP2 staining in mouse cones. Furthermore, comparison of dark- and light-adapted mouse retinas did not reveal significant differences in GCAP2 distribution. Based on these results and reports by others on GCAP2 distribution in other animals, we conclude that there is an apparent species-dependent variation of GCAP2 levels in cones.

In contrast to most other calmodulin-like Ca\textsuperscript{2+}-binding proteins, the function of GCAPs has been unambiguously established: Both GCAPs stimulate photoreceptor GC in low free [Ca\textsuperscript{2+}]. Important questions that remain concern the specific roles that GCAPs play in regulating the function of photoreceptors and in pathways unrelated to phototransduction in photoreceptors or in other retinal cells. Common to both GCAPs is their presence in the synaptic region of photoreceptors, a region remote from phototransduction. cGMP-gated channels have been observed in these subcellular compartments, which suggests that a GC-GCAP system unrelated to phototransduction may exist in synaptic terminals of photoreceptors. A major difference is the presence of GCAP2 and absence of GCAP1 in the inner retina, particularly in amacrine and ganglion cells. The function of GCAP2 in the inner retina, or the pathways in which it participates, are unknown.

Distinct cellular or subcellular expression of GCAPs would be consistent with the tail-to-tail arrangements of their genes. Such arrangements of related genes occur in a variety of mammalian genes. Gene duplication mechanisms that lead to tail-to-tail orientations require an inversion in addition to nonhomologous breakage. The consequence of inversion is that the 5\textsuperscript{′} regulatory elements governing tissue specificity are located on opposite ends of the gene arrangement, thus allowing for divergent evolution and differential tissue or subcellular expression. The presence of GCAP tail-to-tail arrangements in human and mouse suggests that the GCAP duplication-inversion event occurred before mammalian diversification, more than 300 million years ago.

Based on the tail-to-tail gene arrangement and similar expression patterns of the GCAPs, we conclude that the mouse will provide a unique model for genetic manipulation. The gene array will allow for double-knockout constructs, and the consequence of absence of both GCAPs on retinal development and function can be examined. In addition, single knockouts will answer the question of whether the GCAPs provide redundant or unique functions in photoreceptor cells. Eventually, genetically altered mouse models will most likely resolve unanswered questions on localization and function of GCAPs in the retina.

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