Guanylyl cyclase activating protein (GCAP1) has been proposed to act as a calcium-dependent regulator of retinal photoreceptor guanylyl cyclase (GC) activity. Using immunocytochemical and biochemical methods, we show here that GCAP1 is present in rod and cone photoreceptor outer segments where phototransduction occurs. Recombinant and native GCAP1 activate recombinant human retGC (outer segment-specific GC) and endogenous GCs in rod outer segment (ROS) membranes at low calcium. In addition, we isolate and clone a retinal homolog, termed GCAP2, that shows ~50% identity with GCAP1. Like GCAP1, GCAP2 activates photoreceptor GC in a calcium-dependent manner. Both GCAP1 and GCAP2 presumably act on GCs by a similar mechanism; however, GCAP1 specifically localizes to photoreceptor outer segments, while in these experiments GCAP2 was isolated from extracts of retina but not ROS. These results demonstrate that GCAP1 is an activator of ROS GC, while the finding of a second activator, GCAP2, suggests that a similar mechanism of GC regulation may be present in outer segments, other subcellular compartments of the photoreceptor, or other cell types.

In vertebrate photoreceptor cells, the synthesis and hydrolysis of cyclic GMP (cGMP) are critical steps in phototransduction. In response to light, a cascade of reactions in the photoreceptor outer segment leads to the hydrolysis of cGMP and the closure of cGMP-gated cation channels in the outer segment plasma membrane. As a consequence, there is a reduction in the amount of calcium entering the cell. Calcium efflux owing to the Na⁺:K⁺, Ca²⁺ exchanger, however, is unaffected by light, resulting in a net decrease in the concentration of internal free calcium. This decrease in the calcium concentration leads to the activation of guanylyl cyclase (GC), which in part restores the dark conditions of the photoreceptor cell (reviewed by Lagnado and Baylor (1992)).

Photoreceptor GC, a member of the particulate GC family (Koch, 1991; Shyjan et al., 1992; Goraczniak et al., 1994; Umbarger et al., 1992; Dizhoor et al., 1994; Liu et al., 1994), responds to an activator that senses changes in the calcium concentration (Lolley and Racz, 1982; Koch and Stryer, 1988). Recently, we proposed that a calcium binding protein isolated from rod outer segments (ROS), guanylyl cyclase activating protein (GCAP, termed here GCAP1), mediates this process (Gorczyca et al., 1994a), and its molecular properties were described (Palczewski et al., 1994). GCAP1 restores the calcium sensitivity of GC in a reconstituted system, and it decreases the sensitivity, time-to-peak, and recovery time of the light response following its introduction into intact ROS (Gorczyca et al., 1994a). The molecular cloning of GCAP1 from bovine, human, mouse, frog (Palczewski et al., 1994), and chicken retina and the genomic organization of mouse and human GCAP1 (Subbaraya et al., 1994) demonstrate strong sequence conservation between species, conservation of three putative calcium binding loops, and relatedness to other neuronal calcium-binding proteins of the calmodulin superfamily. Transcripts encoding GCAP1 were localized to photoreceptor cells by in situ hybridization (Palczewski et al., 1994; Subbaraya et al., 1994), but the precise localization of the protein is not known. Independently, Dizhoor et al. (1994) proposed that another protein, p24, was responsible for the calcium sensitivity of photoreceptor GC.

In this paper, we describe the cellular localization of bovine GCAP1 by immunocytochemical and biochemical methods and provide further evidence that GCAP1 is a key element in the activation of photoreceptor GC. In addition, we show that the retina contains a second GC activator, GCAP2, that is identical with p24 and closely related to GCAP1.

MATERIALS AND METHODS

Purification of ROS and GCAP1—Fresh bovine eyes were obtained from a local slaughterhouse, and the retinas were dissected under dim red light. ROS were prepared according to Papermaster (1982). GCAP1 was purified as described previously (Gorczyca et al., 1994a).

Affinity Chromatography—A soluble extract containing GCAP1 was prepared from either bovine ROS (equivalent to 50 bovine retinas) or from 25 bovine retinas with 25 ml of water, containing 1 mM benzamidine. The extract was separated from ROS or retinal particulates by centrifugation (48,000 × g for 30 min) and loaded onto an antibody-Sepharose column (mAb G-2; 6 mg of antibody per 1 ml of the CNBr-activated Sepharose; 1 × 2 cm) equilibrated with 10 mM 1,3-bis(2-hydroxyethyl)imidazoline-propane; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; mAb, monoclonal antibody; CTP-S, guanosine 5'-O-(thiotriphosphate).

1 The abbreviations used are: ROS, rod outer segment; BTP, 1,3-bis(2-hydroxyethyl)imidazoline-propane; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; mAb, monoclonal antibody; CTP-S, guanosine 5'-O-(thiotriphosphate).

2 S. Semple-Rowland and W. Baehr, unpublished results.
mixed with incomplete adjuvant. Affinity-purified antibodies or Amplification was performed in 25 µl, using a 1-µl phage library (10⁷ plaque-forming units) as described previously (Palczewski et al., 1994a). The digest was performed at 30 °C for 24 h. Peptides were extracted from the polyvinylidene difluoride membrane, and purified by reverse-phase HPLC using a Hewlett-Packard HPLC, a 5-µm Vydac C18 column (2.1 x 250 mm), and aqueous trifluoroacetic acid/acetonitrile solvents. Peptide sequence analysis was performed with a gas protein Sequencer (Applied Biosystems, Inc. Model 470/120/900) using approximately 40 pmol of peptide.

The standard GC assay was performed using [α-35S]GTPγS, as described previously (Gorczyca et al., 1994b). Briefly, washed ROS membranes or fractions containing GCAP were incubated for 15 min at 30 °C with 0.65-1.3 µM [α-35S]GTPγS (19,000-22,000 dpm/nmol; DuPont NEN), 50 mM Heps, pH 7.8, 60 µM KCl, 20 mM NaCl, 10 mM MgCl₂, 0.4 mM EUGTA, and 0.16 mM CaCl₂ (45 mM free calcium). Free calcium was calculated using the computer program "Chelator 1.0" (Schoenmakers et al., 1995) and adjusted to higher concentrations in some assays by increasing the amount of added CaCl₂. retGC was assayed in the same condition; however, thio-GTP was replaced by [α-32P]GTP.

**Bacterial Expression of GCAP1** — A truncated version of GCAP1 (GCAP180) was amplified with forward primer 5'-GTA CAG AAA GA G TAG GCA TAT GCA AGA A and reverse primer 5'-GTA CAG AAA GA G TAG GCA GT and cloned into pGEX vector after excision with NdeI and EcoRI. The expressed fusion protein was isolated as inclusion bodies by centrifugation of bacterial lysates as described previously (Qin and Baehr, 1993).

**Expression of Bovine GCAP1 in Insect Cells** — The transfer vector pVLgcap was constructed by subcloning full length DNA fragments encoding bovine GCAP1 into the pVL941 vector (Invitrogen). High-Five cells (2-3 x 10⁶) derived from the cabbage looper (Pharmintron) were co-transfected with 0.5 µg of BaculoGold™ DNA and 5 µg of pVLgcap in a T25 tissue culture flasks as described previously (Qin and Baehr, 1994). Five days post-transfection, the supernatant containing recombinant protein was collected and amplified twice. High-Five cells were infected with amplified virus and at 48 h postinfection were harvested and suspended in Grace’s medium.

Cloning of GCAP2 — A degenerate primer (W285, 5'-GTICCGAY-AAYGAPGAPGCGACICAPFA) corresponding to an N-terminal peptide of GCAP2 was synthesized and used for PCR amplification. As a template we used an external bovine retina cDNA library in λUniZap XR was used. Amplification was performed in 25 µl, using a 1-µl phage library (10⁷ plaque-forming units), as described previously (Pittler and Baehr, 1991). The C-terminal and N-terminal portions were amplified with W285/T7, and W296/T3 (GACAGAATAAGGGGAACACATTGAC), respectively. The amplified fragments, G3 and G5 (Fig. 5A), respectively, were cloned into pGEM® vector (TA cloning kit, Invitrogen) and sequenced using the double-stranded procedure and Sequenase (USB Biochemicals/Amersham) with universal or sequence-specific primers, or using linear amplification (femtomole sequencing, Promega) with 10 pmol of end-labeled primers. The sequence derived from G3 and G5 was verified after isolation of a full length bovine GCAP2 clone from λUni-Zap library (not shown).

Preparation of Anti-GCAP Monoclonal and Polyclonal Antibodies — BALB/c mice were immunized with purified GST-GCAP fusion protein, and mAb G-2 was obtained according to the procedures of Campbell (1984), as described by Palczewski et al. (1994). Rabbit polyclonal antibodies were prepared with purified GCAP180. Antisera were raised in New Zealand White rabbits by subcutaneous immunization with approximately 50 µg of the GCAP180 solution (approximately 100 µl) mixed with an equal volume of complete Freund’s adjuvant. Animals were boosted at 1-2 week intervals with 25 µg of the GCAP180 solution mixed with incomplete adjuvant. Affinity-purified antibodies or mAb G-2 were coupled to CNBr-activated Sepharose (Palczewski et al., 1993).

Preparation of Anti-peptide Polyclonal Antibodies — Antisera were prepared with peptides derived from the known amino acid sequence of bovine GCAP1 according to the procedure described by Palczewski et al. (1993). The following peptides, conjugated to keyhole limpet hemocyanin, were used for immunization: a 27-mer of GCAP1, and the sample was loaded on a C-4 column (W-Porex 5 C4, 4.6 x 150 mm, Phenomenex) equilibrated with 30% acetonitrile in 5 mM BTP, pH 7.5 (Gorczyca et al., 1994a). GCAP180 was eluted with a linear gradient of acetonitrile (30-60% during 20 min) in 5 mM BTP, pH 7.5, at a flow rate of 1.5 ml/min, and 0.75-ml fractions were collected. GCAP180 was eluted at ~40% acetonitrile. SDS-PAGE analysis is presented on the combined fraction eluted at ~10 min.

**Preparation of Antibodies for Western Blotting** — A 30 µg sample was loaded on a 7.5% SDS-PAGE gel, run at 200 V for 2 h and dried. The proteins were transferred to nitrocellulose filter for 2 h, and then the filter was incubated with 5% (w/v) nonfat dry milk, 0.05% Tween-20 (v/v) in Tris-buffered saline (100 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20), for 1 h at room temperature. The filter was then incubated with a 1:1000 dilution of the primary antibody (bovine GCAP180) overnight at 4 °C, then washed with Tris-buffered saline containing 0.05% Tween-20 (v/v) for 30 min at room temperature. It was then incubated with a secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature. The bands were visualized with an in-house chemiluminescent detection kit (Hybridec, Perkin-Elmer) and exposed to Hyperfilm (Amersham) for 4-8 h. The bands on the film were quantitated with a laser densitometer and a phosphor screen reader (Bio-Rad). The amount of GCAP180 was normalized to the amount of β-actin.

**Protein Determinations and SDS-PAGE** — The concentration of protein was determined by the method of Bradford (1976), and SDS-PAGE was performed according to Laemmli (1970). The electrophoretic protein onto Immobilon was carried out using a Hoefer mini-gel system. For Western staining, membranes were blocked with 3% (w/v) gelatin in 20 mM Tris/HCl buffer, pH 7.5, containing 500 mM NaCl and incubated for 1 h with primary antibody at dilutions between 2,000 and 100,000. A secondary antibody conjugated with alkaline phosphatase was used as recommended by the manufacturer (Bio-Rad), and antibody binding was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Immunocytochemistry** — Human donor eyes were obtained from the Lions Eye Bank of Oregon, Legacy-Good Samaritan Hospital, Portland, OR. Monkey eyes were obtained from the Oregon Regional Primate Center, Beaverton, OR. Bovine eyes were obtained from Schank Pack ing Co. (Stanwood, WA). Bovine eyes were allowed to dark-adapt for 1-2 h prior to dissection under dim red illumination. Once the cornea and lens were removed, eyecups were fixed for 4 h at 4 °C in 4% paraformaldehyde in a buffer consisting of 140 mM NaCl, 10 mM NaHCO₃, 2 mM CaCl₂, 10 mM glucose, 2.5 mM KCl, 2 mM MgCl₂, and 10 mM Hepes, pH 7.5. In some experiments, bovine eyecups were fixed with 0.1% glutaraldehyde, 4% paraformaldehyde in the same buffer. Eyecups were cryoprotected by sucrose infiltration (Lerea et al., 1989) or by acrylamide embedding (Polans and Burton, 1988). Other species were processed similarly using 0.1 M cacodylate buffer during fixation.

Ten-µm cryosections were blocked with 1% (w/v) bovine serum albumin, 1% (v/v) normal goat serum, 0.2% Triton X-100 in 0.1 M phosphate buffer.

**Guanylyl Cyclase Activating Protein**

FIG. 1. Reverse phase chromatography of truncated GST-GCAP1 fusion protein (GST-GCAP180). The expressed GST-GCAP180 was purified from bovine lens bodies and solubilized in 5 M urea (Palczewski et al., 1994). The protein was dialyzed extensively against 10 mM BTP buffer, pH 7.5, and treated with thrombin (Qin and Baehr, 1999). Acetonitrile was then added to yield a final concentration of 15%, and the sample was loaded on a C-4 column (W-Porex 5 C4, 4.6 x 150 mm, Phenomenex) equilibrated with 30% acetonitrile in 5 mM BTP, pH 7.5 (Gorczyca et al., 1994a). GCAP180 was eluted with a linear gradient of acetonitrile (30-60% during 20 min) in 5 mM BTP, pH 7.5, at a flow rate of 1.5 ml/min, and 0.75 ml fractions were collected. GCAP180 was eluted at ~40% acetonitrile. SDS-PAGE analysis is presented on the combined fraction eluted at ~10 min.
buffer, pH 7.4, for 1 h at room temperature. Sections were incubated with anti-GCAP antibodies at 0.8 mg/ml total IgG either for 2 h or overnight. After several washes, sections were incubated for 1 h with a biotinyl-galot anti-rabbit IgG at a concentration of 3 μg/ml. Antibody binding was detected using streptavidin-Texas red at 1 μg/ml. (Antibodies and streptavidin-Texas red were diluted with blocking solution.) Sections were rinsed, dehydrated with ethanol, cleared with xylene, coverslipped, and viewed with a Zeiss Universal fluorescence microscope using a ×25 neofluar objective.

RESULTS

Immunocytochemical and Biochemical Localization of Bovine GCAP1—If GCAP1 is essential for the calcium-sensitive regulation of GC during the recovery phase of phototransduction, it should be present in rod and cone photoreceptor outer segments. We have localized GCAP1 mRNA to the myoid region of rod and cone photoreceptors by in situ hybridization (Palczewski et al., 1994); however, we have not determined the precise localization of the protein within these highly differentiated cells. Polyclonal antibody (pAb) UW-14 raised against bacterially expressed truncated GCAP180 (Fig. 1) was used to localize GCAP1 in cryosections of paraformaldehyde-fixed and sucrose-protected bovine eyes. Fluorescence light microscopy revealed that both rod and cone photoreceptor outer segments were the primary sites of staining (Fig. 2A). Specificity was shown by blocking the immunoreactivity with purified GCAP1 (Fig. 2B), and staining was not observed using preimmune sera (data not shown). In some experiments, cone inner segments were stained intensely (Fig. 2C). Variability of staining was not due to fixation or embedding procedures, since similar results were obtained with glutaraldehyde fixatives and acrylamide embedding, although the intensity of staining was reduced for both rod and cone cells. Light-adapted bovine, human, and monkey retinas gave comparable results (data not shown).

We further investigated the distribution of GCAP1 in purified bovine ROS and retinas using monoclonal antibody (mAb) G-2 coupled to CNBr-activated Sepharose. When a ROS extract was applied to the mAb G-2 column, all GC-stimulating activity was absorbed. At low pH, the activity was almost quantitatively eluted as a single peak (Fig. 3A, top panel). The GC-stimulating activity correlated with the elution of GCAP1, as identified by immunoblotting (inset in Fig. 3A, top panel) and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Fig. 3B, top panel).

Isolation of GCAP2—The experiment was repeated using a retinal extract. The flow-through from the retinal extract contained enzymatic activities that were due in part to a soluble GC, other enzymes that utilize nucleotides, and potentially other GC activators (Fig. 3A, bottom panel). The low pH elution yielded two fractions of GC-stimulating activity (Fractions 21–22) that showed calcium dependence (Fig. 3A, bottom panel). SDS-PAGE analysis of the fractions revealed a several thousandfold purification of GCAP1 (20 kDa) contaminated in part by arrestin (48 kDa, identified by immunoblotting), an unknown 30-kDa protein, and an additional protein with a mobility of 18 kDa in the presence of calcium, or 24 kDa in the presence of EGTA, termed here GCAP2 (Fig. 3B, middle panel). The most active fraction (Fraction 21) was subjected to Western blot analysis using four different anti-GCAP1 antibodies (Fig. 3C). mAb G-2 recognized GCAP1 and cross-reacted with GCAP2 (Fig. 3C, panel I), suggesting that the two proteins are related. Polyclonal antibodies obtained by immunization of rabbits with bacterially expressed GCAP1 (UW-14), N-terminal (GS-35), and C-terminal (GS-31) peptides from the GCAP1 sequence, were immunoactive only with GCAP1 (Fig. 3C, panels II-IV).

Thus, biochemical analyses revealed that GCAP1 is associated with photoreceptor outer segments, consistent with immunolocalization studies, while in these experiments GCAP2 was detected in retinal but not ROS preparations.

Molecular Cloning and Biochemical Characterization of GCAP2—To isolate GCAP2, a retinal extract was applied to a pAb GS-31-Sepharose column. Only GCAP1 bound to the column, and it was eluted by competition with the C-terminal peptide against which the antibodies were raised (Fig. 4, lane 1 of gel inset) or by low pH (data not shown). This procedure was repeated four times to absorb GCAP1 completely, while GCAP2 passed through the column. The flow-through was then applied to a mAb G-2 column which bound GCAP2. Elution at low pH yielded GCAP2 (Fig. 4, lane 2 of gel inset). Both GCAP1 and GCAP2 could activate GC in ROS membranes (Fig. 4). The potency of both proteins is comparable, and, based on staining after SDS-PAGE, the relative abundance of GCAP1 to GCAP2 in the retina is ~3:1. As assayed by GC stimulation, GCAP2 appeared to be more stable than GCAP1 in storage and during heat and acid treatments.

Polyclonal antibody pAb UW-14 and monoclonal antibody mAb G-2 were generated against truncated, bacterially expressed GCAP1 (Met10-Pro31); polyclonal antibodies pAb GS-35 and pAb GS-31 were raised against N-terminal myristoylated Gly2-Glu26 and C-terminal Asp93Asp94Lyse peptide encompassing portions of the GCAP1 sequence, respectively. pAbs were affinity-purified, while mAb G-2 was purified from ascites fluid by ammonium sulfate precipitation and ion exchange chromatography.
circles) was washed ROS membranes in the presence of either 45 nM Ca$^{2+}$ were eluted with 0.1 M glycine, pH 2.5 (fractions 20-27). Eluted fractions were immediately neutralized with 1 M Tris/HCl, pH 8.4. The flow-through from the pAb GS-31 column (containing GCAP2) was loaded on mAb G-2 column, and GCAP2 was eluted at low pH as described in Fig. 2. The activity profile is shown as the solid lines, and the bar represents 2 nmol/min/mg GC activity. Inset, SDS-PAGE of the eluted fractions from pAb GS-31 (lane 1) and mAb G-2 (lane 2). S denotes molecular weight standards, p18 indicates the electrophoretic mobility of GCAP2.

To clone GCAP2, peptides were generated by in situ proteolysis with Lys-C endopeptidase and purified by reverse phase HPLC. One GCAP2 peptide was blocked and not suitable for Edman degradation, while a second peptide yielded amino acid sequence (VPDNEEATQYVEAMFRAFDTNGDNTIDFL) homologous to GCAP1 (Fig. 5A). Importantly, the sequence obtained for the fragment of GCAP2 is identical with the corresponding portion of p24, a protein purified by Dizhoor et al. (1994). A degenerate oligonucleotide primer reversely transcribed to the portion of the peptide that was most different from GCAP1 (underlined) was used to clone GCAP2 after PCR amplification. The results show that the molecular mass, based on the predicted amino acid composition (Fig. 5A) (204 residues), is 23,759 daltons, and that the amino acid sequence is homologous to GCAP1 (~53%) (Fig. 5B). The amino acid sequence of

![Diagram](image_url)

**Fig. 4.** Separation of GCAP1 and GCAP2 by immunoaffinity chromatography of the retinal extract on pAb GS-31 and mAb G-2. The retinal extract was loaded on pAb GS-31 as described in the legend to Fig. 2. GCAP1 was eluted with a 1 mM concentration of the C-terminal peptide, and the GC-stimulating activity was determined (solid line, top). The column was washed with 0.1 M glycine (pH 2.5) and re-equilibrated with 10 mM BTP, pH 7.5. The procedure was repeated 4 times. The flow-through from the pAb GS-31 column (containing GCAP2) was loaded on mAb G-2 column, and GCAP2 was eluted at low pH as described in Fig. 2. The activity profile is shown as the solid lines, and the bar represents 2 nmol/min/mg GC activity. Inset, SDS-PAGE of the eluted fractions from pAb GS-31 (lane 1) and mAb G-2 (lane 2). S denotes molecular weight standards, p18 indicates the electrophoretic mobility of GCAP2.

Using 12% acrylamide gels and stained with Coomassie Brilliant Blue R-250. Lanes L, extracts before chromatography; lanes S, standard proteins of known molecular weights (from the top: 92-, 67-, 43-, 30-, 21-, and 14-kDa standards; Pharmacia Biotech Inc.). Open arrow indicates position of GCAP2. In control experiments, a mouse monoclonal antibody raised against purified pig retina (concentration of antibody and purification identical to mAb G-2 and the size of the column comparable to the mAb G-2 column) was coupled to CNBr-activated Sepharose, and ROS and retinal extracts were passed through the column. Only proteins that interact with immunoglobulins and Sepharose bound (like transducin from ROS extract) and were subsequently eluted, while GCAP1 and GCAP2 passed through. Note the high density of mAb G-2 (6 mg/ml) that resulted in a complete adsorption of GCAP1 or GCAP2; however, it also yielded nonspecific adsorption of other contaminating proteins.

**Fig. 3.** Immunoaffinity chromatography of ROS and retinal extracts. GCAP1 was extracted from 25 retinas or from ROS prepared from 50 retinas, as described under "Materials and Methods." The extracts were loaded onto an immunoaffinity column containing mAb G-2 coupled to CNBr-Sepharose. The fractions which passed through the column during loading were collected (fractions 1–6), the column was washed with 10 mM BTP buffer, pH 7.5, containing 200 mM NaCl, and then with 10 mM BTP, pH 7.5 (fractions 7–19), and bound proteins were eluted with 0.1 M glycine, pH 2.5 (fractions 20–27). Eluted fractions were immediately neutralized with 1 M Tris/HCl, pH 8.4. A, GC stimulating activity in ROS and retinal extracts. 5-µl aliquots of the fractions were tested to determine their effect on GC activity using washed ROS membranes in the presence of either 45 mM Ca$^{2+}$ (open circles) or 1 mM Ca$^{2+}$ (closed circles). Normalized GC activity was calculated by assuming that the total stimulatory activity present in each fraction was recovered in the volume of 1 ml. Insets: 1-µl aliquots from the indicated fractions were separated by SDS-PAGE, transferred to Immobilon P, and immunostained with pAb UW-14 (lane L, loaded extract). B, SDS-PAGE analysis. 20 µl of the indicated fractions from ROS (upper panel) or a retinal extract (middle panel) were separated using 12% acrylamide gels and stained with Coomassie Brilliant Blue R-250. Lanes L, extracts before chromatography; lanes S, standard proteins of known molecular weights (from the top: 92-, 67-, 43-, 30-, 21-, and 14-kDa standards; Pharmacia Biotech Inc.). Open arrow indicates position of GCAP2. In control experiments, a mouse monoclonal antibody raised against purified pig retina (concentration of antibody and purification identical to mAb G-2 and the size of the column comparable to the mAb G-2 column) was coupled to CNBr-activated Sepharose, and ROS and retinal extracts were passed through the column. Only proteins that interact with immunoglobulins and Sepharose bound (like transducin from ROS extract) and were subsequently eluted, while GCAP1 and GCAP2 passed through. Note the high density of mAb G-2 (6 mg/ml) that resulted in a complete adsorption of GCAP1 or GCAP2; however, it also yielded nonspecific adsorption of other contaminating proteins. C, reactivity of purified ROS and retinal extracts with anti-GCAP1 antibodies. 1-µl aliquots from fraction 21 after immunoaffinity chromatography of ROS (a) or a retinal extract (b) were separated by SDS-PAGE, transferred to Immobilon, and immunostained with antibodies: I, mAb G-2 (monoclonal against expressed GCAP1); II, pAb UW-14 (polyclonal against expressed GCAP1); III, pAb GS-31 (polyclonal against C-terminal peptide from GCAP1); and IV, pAb GS-35 (polyclonal against N-terminal peptide from GCAP1). Open arrow indicates GCAP2 staining with mAb G-2 only.
Guanylyl Cyclase Activating Protein

**FIG. 5.** cDNA sequence of bovine GCAP2 and amino acid sequence alignment of bovine GCAP2 and GCAP1. A, composite cDNA sequence derived from PCR clones G3 and G5. The degenerate sense primer W285 (I = inosine, Y = pyrimidines, P = purines) and the proteolytic GCAP2 peptide are highlighted. The three putative EF-hand calcium binding sites (EF2-4) are shaded. The amino acid and nucleotide numbering is shown on the right. B, amino acid sequence alignment of GCAP1 and GCAP2. The deduced GCAP2 sequence was aligned with bovine GCAP1, and, for optimal alignment, several gaps (hyphens) were introduced. Putative calcium binding loops are shaded in gray, while amino acids illustrated in white on black background represent identity or conservative replacement (L = I = V = M; Y = F; K = R; S = T = A). The bar above the GCAP2 sequence represents the sequence obtained by peptide microsequencing.

GCAP2 showed that it is also related to other Ca\(^{2+}\)-binding proteins of the EF-hand superfamily, including calmodulin and troponin C. Members of this family contain 2–4 canonical calcium binding motifs composed of "loops" of 12 contiguous residues with oxygen atoms involved in calcium coordination and two flanking α-helices that stabilize this complex (Kretsinger, 1980; Strynadka and James, 1989). GCAPs are most closely related to recoverin (Fig. 6); however, they differ in the number of functional calcium binding loops (three versus two) and they are more acidic (pK\(_{a}\) = 4.1–4.4 versus 5.1). GCAP2 also has Gly\(^2\) that is a putative site of heterogeneous tissue-specific acylation (Johnson et al., 1994), as well as a consensus sequence for protein kinase A phosphorylation at residue Ser\(^{201}\).

The discrepancy in the mobility of GCAP2 noted in the literature is likely a result of addition of EGTA to the sample buffer during SDS-PAGE (Dizhoor et al., 1994) and absence of the chelator in our samples as shown in Fig. 7.

**GC Activation by GCAP1 and GCAP2—**We next asked whether GCAP1 and GCAP2: (i) saturate GC activity individually, (ii) act synergistically in GC stimulation; and (iii) have different calcium specificity. GCAP1 and GCAP2 stimulated ROS GC in a saturable manner (Fig. 8A). GCAP1 fully stimulated ROS GC, and the addition of GCAP2 had only a minimal inhibitory effect (Fig. 8B). Similarly, the addition of GCAP1 to ROS GC that was maximally stimulated by GCAP2 only minimally enhanced GC activity. At nonsaturating concentrations, GCAP1 and GCAP2 did not act synergistically, rather the effect of both proteins was additive. GCAP1 and GCAP2 have almost identical calcium sensitivity in stimulating ROS GC (Fig. 8C). These results suggest that most likely GCAP1 and GCAP2 bind to the same sites in ROS membranes. Washed ROS were incubated with various amounts of puri-
fied GCAP1, the binding of GCAP1 then was determined by immunoblotting, and the GC activity was assessed in aliquots from the same samples. GCAP1 bound to membranes in the presence of moderate ionic strength buffer, and the binding of GCAP1 correlated with increased GC activity in ROS membranes (Fig. 9). The binding was independent of the calcium concentration. These results suggest that at physiological conditions, GCAP1 remains in a complex with GC/membranes despite changes in the free calcium concentration.

**Recombinant GCAP1 Stimulates ROS GC and Recombinant retGC**—Bovine GCAP1 was expressed in insect cells to 3–6% of the soluble protein, as determined by Western blot analysis and SDS-PAGE. Extracts containing GCAP1 were obtained from transfected cells and stimulated GC activity in low calcium in a dose-dependent manner (Fig. 10), while extracts from nontransfected cells lacking GCAP1 were ineffective. The stimulatory effect was noted for both native ROS GC and recombinant photoreceptor GC (Shyjan et al., 1992; Dizhoor et al., 1994). These results suggest that GCAP1 is a calcium-sensitive modulator of phototransduction, most likely interacting directly with ROS GC. This model is supported further by preliminary cross-linking studies with bifunctional reagents that demonstrate the proximity of retGC and GCAP1 (data not shown).

**DISCUSSION**

Several independent lines of evidence suggest that GCAPs are involved in the calcium-sensitive regulation of GC. (i) A combination of chromatographic procedures, independent of the calcium binding properties of the proteins, led to the purification of GCAP1 and GCAP2, which are homologous proteins. Chromatographic profiles of protein purification and GC stimulating activity are consistent with GCAP1 and GCAP2 activating GC in reconstitution experiments using washed ROS membranes (Palczewski et al., 1994; Gorczyca et al., 1994a, 1994b; Dizhoor et al., 1994) and expressed retinal GC (Fig. 10). Furthermore, expressed GCAP1 activates GC in a calcium- and dose-dependent manner, similar to the native protein (Fig. 10). (ii) mAb G-2, raised against bacterially expressed GCAP1, inhibits the calcium regulation of GC (Palczewski et al., 1994), and selectively purifies GCAPs from retinal extracts. (iii) GCAP1 is present in rod and cone outer segments, sites of calcium-sensitive regulation of GC. The occasional staining of cone processes may correlate with the presence of cGMP-regulated calcium channels (Cook et al., 1989; Rieke and Schwartz, 1994). Alternatively, the variability in cone labeling may reflect the adaptational state of the tissues. The bovine, human, and monkey retinas used in the immunohistochemical experiments are at least partially light-adapted, and placement of these...
retGC-2 is activated by purified GCAP2 (Lowe et al., 1995; Lowe et al., 1992; Koch, 1991; Dizhoor et al., 1994; Liu et al., 1992; Goraczniak et al., 1993) and has been identified recently (Yang et al., 1994). A second retina-specific GC (GC F; retGC-2) has been described under "Materials and Methods." Note that the GC activity is measured with GTP for retGC and GTP-γS for GC in ROS membranes, and the figure represents qualitatively similar activation of both GCs at low free calcium concentration.

Fig. 9. Binding of GCAP1 to ROS membranes. Washed ROS (30 µm rhodopsin in 64 µl) in 50 mM Hepes, pH 7.8, containing 60 mM KCl, 20 mM NaCl, 10 mM MgCl₂ at 45 nM free calcium were incubated with increasing amounts of purified GCAP. The membranes were collected by centrifugation, washed with 100 µl of the same buffer, and finally suspended in 50 µl of 10 mM Hepes, pH 7.5. A portion of each sample was assayed for GC activity, while GCAP was identified by Western blot analysis using mAb G-2 (inset).

Fig. 10. Stimulation of GC in ROS membranes or recombinant retGC with expressed GCAP1. GCAP1 (0.25 µg) was assayed for GC-stimulating activity in low (−; 30 nM) and high (+; 1 µM) calcium using GC in washed ROS membranes (open bars, 80 µg of rhodopsin) or expressed retGC (shaded bars, 120 µg of total protein). retGC was expressed in human embryonic kidney 293 cells that were produced as described by Shyjan et al. (1992). Expression of GCAP1 in High-Five insect cells was described under "Materials and Methods." Note that the GC activity is measured with GTP for retGC and GTP-γS for GC in ROS membranes, and the figure represents qualitatively similar activation of both GCs at low free calcium concentration.

Fig. 11. Autoradiogram of an in situ hybridization with [35S]UTP-labeled complementary RNA probes in autopsy tissues in the dark before fixation may not reproducibly reset the mechanisms governing the distribution of GCAP1 in cone cells. pAb UW-14 used for immunohistochemical staining did not react with mouse retinas, which would normally be used to explore differences between light- and dark-adapted conditions.

The major GC activity in ROS membranes can be attributed to retGC (Shyjan et al., 1992; Goraczniak et al., 1994; Umbarger et al., 1992; Koch, 1991; Dizhoor et al., 1994; Liu et al., 1994). A second retina-specific GC (GC F; retGC-2) has been identified recently (Yang et al., 1995; Lowe et al., 1995); however, the subcellular localization of this cyclase remains to be established. Considering the present data, one intriguing hypothesis would propose that both cyclases, retGC-1 (retGC) and retGC-2, are regulated in a calcium-dependent manner by their respective calcium sensors, GCAP1 and GCAP2. Indeed, retGC-2 is activated by purified GCAP2 (Lowe et al., 1995) in the same calcium-dependent manner as shown in this report (Fig. 8). The availability of recombinant GC(s) and GCAPs will assist in further deciphering their interactions, their subcellular localization, kinetics, and calcium binding properties.

The localization of GCAP2 is not yet known; however, it was not detected in our ROS extracts (Fig. 3). The amount of GCAP2 in ROS compared to retinal extracts suggests that this protein may be localized in the photoreceptor inner segments or in other parts of the retina. If correct, these observations suggest that regulation of particulate GCs by a small, acidic, calcium-binding protein might be a general phenomenon occurring in other retinal cells and possibly other sensory transduction systems. Alternatively, GCAP2, in our experiments, may be selectively lost during the purification of ROS and, like GCAP1, therefore would be involved in the regulation of retGC.

While more than 200 calcium-binding proteins of the EF-hand superfamily have been identified, the function is known for relatively few. Owing to the elucidation of the biochemical steps involved in vertebrate phototransduction, the function of several calcium-binding proteins has been assigned. Calmodulin regulates the sensitivity of the cyclic GMP-gated channel in response to light-induced changes in the levels of ligand (Hsu and Molday, 1993). Recoverin (Dizhoor et al., 1991; Polans et al., 1991; Kawamura and Murakami, 1991; Lambertre and Koch, 1991) alters the kinetics of photoreceptor GC(s). Unlike other calcium-binding proteins, GCAPs activate their target at low calcium concentrations. Of general interest, calcium and cyclic nucleotides act as second messengers to mediate a variety of physiological responses; GCAPs provide an intersection between these two small molecules and may offer the cell additional stages of regulation. It will be interesting to see whether molecules similar to GCAPs are associated with other transduction pathways involving cyclic nucleotides and calcium.

Acknowledgments—We thank Drs. J. Hurley and A. Dizhoor for comparing the GCAP2 sequence with the unpublished sequence of p24 and verification of their identity and for many suggestions during this study. We are grateful to J. Preston Van Hoose, Claudia Bues, and Tammie L. Haley for technical expertise. We would like to thank Dr. David Lowe (Genentech Inc.) for the human 293 cell line expressing retGC.

REFERENCES
Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
Campbell, A. M. (1984) Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology (Burdon, R. H., and van Knippenberg, P. H., eds) Vol. 13, Elsevier Science Publishing Co., New York
Cook, N. J., Meldal, L. L., Reid, D., Kuopp, U. B., and Molday, R. S. (1989) J. Biol. Chem. 264, 6996–6999
Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philippov, P. P., Hurley, J. B., and Stryer, L. (1991) Science 251, 915–918
Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) Neuron 12, 1345–1352
Fernandez, J., Andrews, L., and Mischo, S. M. (1994) Anal. Biochem. 216, 112–117
Garbers, D. L., and Lowe, D. G. (1994) J. Biol. Chem. 269, 218, 112–117
Garbers, D. L., and Molday, R. S. (1989) J. Biol. Chem. 264, 6996–6999
Goracyza, W. A., Gray-Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994a) Proc. Natl. Acad. Sci. U.S.A. 91, 4014–4018
Goracyza, W. A., Van Hoose, J. P., and Palczewski, K. (1994b) Biochemistry 33, 3217–3222
Gorodovikova, E. N., and Philippov, P. P. (1993) FEBS Lett. 335, 277–279
Gorodovikova, E. N., Gimelbrant, A. A., Senin, I. I., and Philippov, P. P. (1994a) FEBS Lett. 349, 187–190
Gorodovikova, E. N., Senin, I. I., and Philippov, P. P. (1994b) FEBS Lett. 353, 171–172
Gray-Keller, M. P., Polans, A. S., Palczewski, K., and Detwiler, P. P. (1993) Neuron 10, 523–531
Hsu, Y. T., and Molday, R. S. (1993) Nature 361, 76–79
Johnson, R. S., Ohguro, H., Palczewski, K., Hurley, J. B., Walsh, K. A., and
Guanylyl Cyclase Activating Protein

Neubert, T. A. (1994) *J. Biol. Chem.* **269**, 21067-21071

Kawamura, S. (1993) *Nature* **362**, 865-867

Kawamura, S., and Murakami, M. (1991) *Nature* **349**, 420-423

Koch, K.-W. (1991) *J. Biol. Chem.* **266**, 8634-8637

Koch, K.-W, and Stryer, L. (1988) *Nature* **334**, 64-66

Kretsinger, R. H. (1986) *CRC Crit. Rev. Biochem.* **8**, 119-174

Leenmili, U. K. (1970) *Nature* **227**, 680-685

Lagnado, L., and Baylor, D. (1992) *Neuron* **8**, 995-1002

Lambrecht, H. G., and Koch, K. W. (1991) *EMBO J.* **10**, 793-798

Lerea, C. L., Bunt-Milam, A. H., and Hurley, J. B. (1989) *Neuron* **3**, 367-376

Liu, X., Soneo, K., Nishizawa, Y., Hayashi, F., Yamazaki, A., Matsumoto, H., Wakebayashi, T., and Usukura, J. (1994) *Exp. Eye Res.* **59**, 761-768

Lolley, R. N., and Racz, E. (1982) *Vision Res.* **22**, 1481-1486

Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L., and Hurley, J. B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5535-5539

McGinnis, J. F., Stepanski, P. L., Baehr, W., Subbaraya, I., and Lerious, V. (1992) *FEBS Lett.* **309**, 172-176

Murakami, A., Yajima, T., and Inana, G. (1992) *Biochem. Biophys. Res. Commun.* **187**, 234-244

Palczewski, K., Buczylik, J., Lebiola, L., Crabb, J. W., and Polans, A. S. (1993) *J. Biol. Chem.* **268**, 6004-6013

Palczewski, K., Subbaraya, I., Gerczyca, W. A., Helekzer, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., Johnson, B. S., Walsh, K. A., Gray-Keller, M. P., Detwiller, P. B., and Baehr, W. (1994) *Neuron* **13**, 395-404

Papermaster, D. S. (1989) *Methods Enzymol.* **181**, 48-52

Pitler, S. J., and Baehr, W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8322-8326

Polans, A. S., and Burton, M. D. (1988) *Invest. Ophthalmol. Vis. Sci.* **29**, 1523-1532

Polans, A. S., Buczylik, J., Crabb, J., and Palczewski, K. (1991) *J. Cell Biol.* **111**, 981-989

Qin, N., and Baehr, W. (1995) *FEBS Lett.* **321**, 6-10

Qin, N., and Baehr, W. (1994) *J. Biol. Chem.* **269**, 3365-3371

Rieke, F., and Schwartz, E. A. (1994) *Neuron* **12**, 868-873

Schoenmakers, T. J., Visser, G. J., Flik, G., and Theuvenet, A. P. (1992) *BioTechniques* **12**, 870-874

Shyjan, A. W., de Sauvage, F. J., Gillet, N. A., Goeddel, D. V., and Lowe, D. G. (1992) *Neuron* **9**, 727-737

Strynnaita, N. C. J., and James, M. N. G. (1999) *Annu. Rev. Biochem.* **58**, 951-998

Subbaraya, I., Ruiz, C. C., Helekzer, B. S., Zhao, X., Gerczyca, W. A., Pettenati, M. J., Rao, P. N., Palczewski, K., and Baehr, W. (1994) *J. Biol. Chem.* **269**, 31080-31089

Yang, R.-B., Foster, D. C., Garbers, D. L., and Füll, H.-J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 602-606