Functional Reconstitution of Photoreceptor Guanylate Cyclase with Native and Mutant Forms of Guanylate Cyclase-Activating Protein 1[†]

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ABSTRACT: In rod and cone photoreceptor cells, activation of particulate guanylate cyclase (retGC1) is mediated by a Ca^{2+}-binding protein termed GCAP1, that detects changes in $[Ca^{2+}]_{free}$. In this study, we show that N-acylated GCAP1 restored Ca^{2+} sensitivity of native and recombinant photoreceptor retGC1. ATP increased the affinity of retGC1 for GCAP1 and accelerated catalysis. Using peptides derived from the GCAP1 sequence, we found that at least three regions, encompassing the N-terminus, the EF-1 motif, and the EF-3 motif, were likely involved in the interaction with retGC1. Mutation of ^{2}Gly to Ala (GCAP1-G2A), which abolished myristoylation and a 25 amino acid truncation at the N-terminus (Δ25-GCAP1) reduced retGC1-stimulating activity dramatically, while deletion of 10 amino acids (Δ10-GCAP1) reduced the specific activity by only ∼60% and modified the Ca^{2+} sensitivity. At 10^{-6} M $[Ca^{2+}]_{free}$, in conditions that inactivated native GCAP1, retGC1 showed significant activity in the presence of Δ10-GCAP1. Native and all three mutant forms of GCAP1 had similar affinities for Ca^{2+} as demonstrated by gel filtration and the changes in tryptophan fluorescence. All mutants bound to ROS membranes in a Ca^{2+}-independent manner, except Δ25-GCAP1, which was mostly soluble. These findings suggest that the N-terminal region is important in tethering of GCAP1 to the ROS membranes.

Guanylate cyclase (GC)[1] catalyzes the conversion of GTP to 3′,5′-cyclic-GMP (cGMP) and pyrophosphate. Soluble forms of GC are heterodimers bearing an iron atom coordinated by the heme prosthetic group that is the site of regulation by NO or CO (Koesling *et al.*, 1991; Schmidt, 1992). Particulate forms of GCs are proteins composed of an extracellular domain connected by a single-transmembrane segment to an intracellular domain that includes a protein kinase-like region, a dimerization domain, and a catalytic site (Garbers *et al.*, 1994). Regulation of particulate GCs is complex and may involve extracellular peptide ligands, oligomerization, phosphorylation, nucleotides, and Ca^{2+}-binding proteins (Garbers & Lowe, 1994).

During phototransduction in vertebrate photoreceptor cells, particulate GC replenishes cGMP that has been hydrolyzed by phosphodiesterase as a consequence of a light-dependent cascade of reactions in rod and cone cell outer segments. cGMP is produced at accelerated rates under low $[Ca^{2+}]_{free}$, which in turn is regulated by influx via cGMP-gated channels and light-insensitive efflux via Na^+/K^+-Ca^{2+} exchanger [for review see Koutalos and Yau (1996) and Polans *et al.* (1996)]. This Ca^{2+}-sensitive stimulation of photoreceptor GC is mediated by Ca^{2+}-binding proteins, GCAPs, that belong to the calmodulin family (Palczewski *et al.*, 1994; Gorczyca *et al.*, 1995; Dizhoor *et al.*, 1995). GCAP1 contains three putative Ca^{2+}-binding EF-hand motifs and is acylated at the N-terminus by fatty acids (Palczewski *et al.*, 1994). In contrast to other particulate GCs, the photoreceptor enzyme is stimulated intracellularly (Laura *et al.*, 1996; Duda *et al.*, 1996), most likely via the kinase-like domain (Duda *et al.*, 1996).

Two photoreceptor-specific GCs, retGC1 and retGC2 (Goraczniak *et al.*, 1994; Lowe *et al.*, 1995; Yang *et al.*, 1995), were cloned. However, only retGC1 (termed also ROS-GC and GC-E) and GCAP1 were isolated from ROS (Margulis *et al.*, 1993; Gorczyca *et al.*, 1995; Frins *et al.*, 1996; Aparicio & Applebury, 1995). Immunocytochemical localizations of retGC1 (Dizhoor *et al.*, 1994; Liu *et al.*, 1994) and GCAP1 (Gorczyca *et al.*, 1995; Frins *et al.*, 1996) to rod and cone outer segments are consistent with their function in phototransduction. The retGC2 was found mostly in the photoreceptor cell layer by *in situ* hybridization (Lowe

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[1] Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; GC, guanylate cyclase; GCAP, guanylate cyclase-activating protein; mAb, monoclonal antibody; pAb, polyclonal antibodies; ROS, rod outer segments.

et al., 1995), but the precise subcellular localization within these highly differentiated cells has not been reported. The most intense GCAP2 immunoreactivity was found in the inner segments, somata, and synapses of monkey and frog cones (Otto-Bruc *et al.*, 1997).

The difficulty of purifying GCAP1 from native tissue was recently overcome by expressing GCAP1 in an insect cell system (Gorczyca *et al.*, 1995). However, this heterologously expressed GCAP1 has not been extensively characterized and compared to the native protein. This is particularly important because GCAP1 readily undergoes oligomerization and proteolysis to several 14−20 kDa forms and becomes ineffective in retGC1 stimulation (Gorczyca *et al.*, 1994a; this study). The function of the N-terminal region of GCAP1 is not known, but it is highly conserved among GCAP1s from different species and only weakly related to the N-terminus of GCAP2. Peptide inhibition studies suggest that the N-terminus might be a part of an interacting domain with retGC1 (Palczewski *et al.*, 1994). However, the inhibition could be explained by detergent-like property of the N-myristoylated peptide. In addition, which form of GCAP1, Ca^{2+}-free or with one or two Ca^{2+}-bound, is the most effective in the stimulation of retGC1? Are Ca^{2+} and ATP involved in promoting the association of GCAP1 with membranes?

The present study used biochemical and biophysical methods to address many of these questions. We report that retGC1 stimulation by GCAP1 involves several regions of GCAP1, including the N-terminal region, and propose that GCAP1 stimulates GC via its intracellular domain in an interaction enhanced by ATP.

MATERIALS AND METHODS

Preparation of ROS. ROS were prepared from fresh bovine retinas (Schenk Packing Co., Inc., Stanwood, WA) according to Papermaster (1982). ROS were suspended in 50 mM Hepes, pH 7.8, containing 60 mM KCl and 20 mM NaCl (final concentration, 8−12 mg/mL of rhodopsin). Washed ROS were prepared from ROS by removing soluble proteins, including GCAP1, as described previously (Gorczyca *et al.*, 1994a).

Expression of Bovine GCAP1 in High Five 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 [$(2-3) \times 10^6$] derived from the cabbage looper (Pharmingen) were cotransfected with 0.5 μg of BaculoGold DNA and 5 μg of pVLgcap in a 25 cm^2 tissue culture flask as described previously (Gorczyca *et al.*, 1995).

Preparation of GCAP1. Gorczyca *et al.* (1995) developed a combination of affinity chromatography methods for isolation of GCAP1 and GCAP2 from retinal or GCAP1 from ROS extracts and from an insect cell expression system. However, these methods produced GCAPs contaminated by several high molecular weight proteins. Subsequently, we found that the interaction between GCAP1 and UW14 pAb or G2 mAb was strong and withstood up to 6 M urea and 1 M NaCl, while GCAP2 eluted under these conditions. The chromatography, for example, of a retinal extract on UW14 pAb showed that after washing with urea, GCAP1 was eluted with glycine/HCl (pH 2.5) as a pure protein. The chromatography was specific for GCAP1, as GCAP2 was eluted in flow-through fractions. Similar results were obtained using G2 mAb coupled to CNBr-Sepharose.
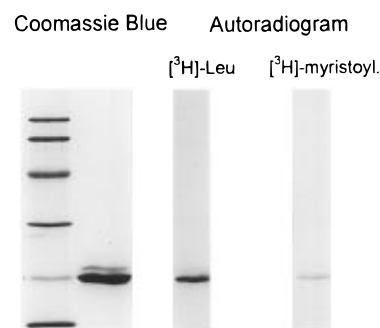

FIGURE 1: Purification of radiolabeled GCAP1 expressed in insect cells. An extract of High Five cells that had been grown for 1 day in the presence of 0.25 mCi of [^{3}H]Leu or 9,10-[^{3}H]myristic acid and then transfected for 3 days with pVL941GCAP was purified on the mAb G2 column as described in Materials and Methods. An aliquot of GCAP1 was analyzed by SDS−PAGE [molecular markers (in kDa): 92, 67, 43, 30, 20, and 14; from Pharmacia] and stained with Coomassie Blue. SDS−PAGE gels containing [^{3}H]-Leu- or 9,10-[^{3}H]myristoyl-GCAP1 were stained with Coomassie Blue or incubated with 2,5-diphenyloxazole (Coligan *et al.*, 1995), dried, and the radioactive band was visualized by fluorography.

A soluble extract containing GCAP1 was prepared from either bovine ROS (equivalent to 50 bovine retinas), from 25 bovine retinas, or from 10 plates (15 cm diameter) of High Five insect cells expressing GCAP1 with 25 mL of water, containing 1 mM benzamidine. The extract was separated from membrane particulates by centrifugation (48000g for 30 min) and loaded onto an antibody-Sepharose column (UW14 pAb; 6 mg of antibody per 1 mL of the CNBr-activated Sepharose; 1 × 2 cm; Gorczyca *et al.*, 1995) equilibrated with 10 mM BTP buffer, pH 7.5, at a flow rate of 15 mL/h. The column was washed with 10 mM BTP buffer, pH 7.5, containing 200 mM NaCl, with 10 mM BTP, pH 7.5, containing 4 M urea, followed by 10 mM BTP, pH 7.5. The elution was performed with 0.1 M of glycine, pH 2.5. Fractions (0.5 mL) were collected, immediately neutralized with 1 M Tris/HCl, pH 8.4, and analyzed for retGC1-stimulating activity.

Site-Directed Mutagenesis. GCAP1 was mutated employing site-directed mutagenesis using a Transformer Kit (Clontech Laboratories, Palo Alto, CA). The mutagenic primer for GCAP1-G2A was 5′-GCC TGA GCG ATG GCG AAC ATT in which the codon for Gly (GGG) was replaced by the codon for Ala (GCG, underlined). The mutation was introduced directly into the pVL941 insect cell vector construct expressing GCAP1 (Gorczyca *et al.*, 1995). Deletions were introduced by specific primers and PCR. To delete residues 2−11 of GCAP1 (Δ10-GCAP1), a sense primer 11M (5′-CAG GCC TGA GCG ATG GAG CTG AGC AGC) and an antisense primer W233 (Palczewski *et al.*, 1994) located ten bp downstream of the stop codon were used. To delete residues 2−25 of GCAP1 (Δ25-GCAP1), 11M was replaced by 26M (5′- CAG GCC TGA GCG ATG ACA GAG TGC CCC). 11M and 26M primers retain 9 bp of upstream sequence including an in-frame stop codon (TGA), and ATG of GCAP1. The amplified products were cloned into PCRII vector (Invitrogen), excised with *Bam*HI and *Hin*dIII, and recloned into the corresponding sites of pVL941. All insect expression vector constructs were completely sequenced on both strands with tagged pVL941-
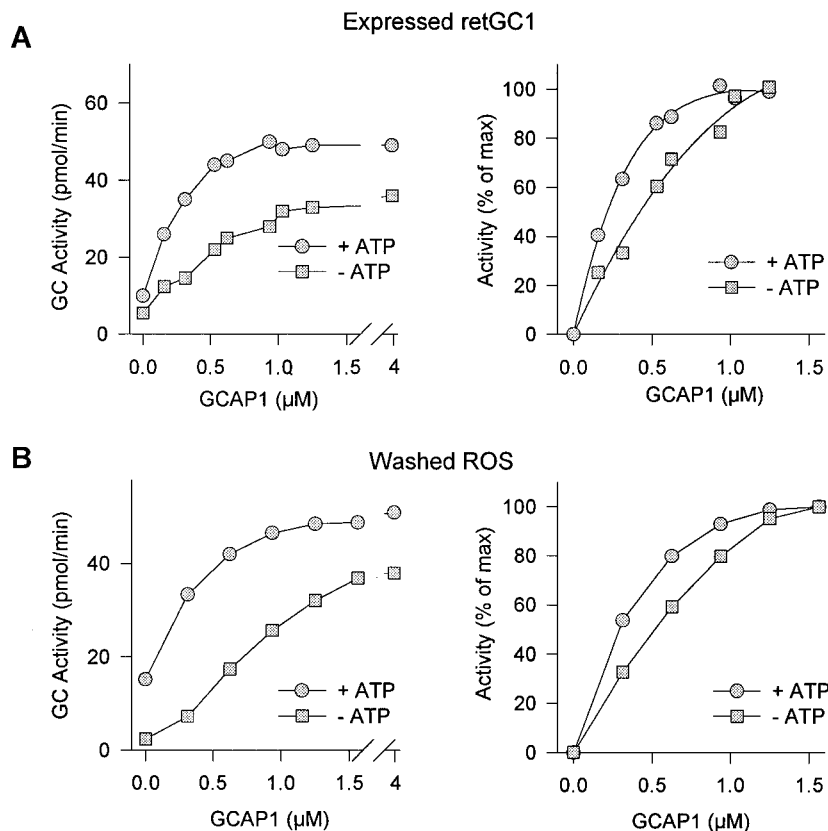
FIGURE 2: Effect of increasing amounts of GCAP1 on stimulation of retGC1 in washed ROS and retGC1 expressed in insect cells in the presence of ATP. (Left Panels) Activity is expressed in pmol/min. (Right Panels) Activity is given as a percent of maximal stimulation at 4 μM of GCAP1. ATP concentration was 0.4 mM. The GC assays using retGC1 expressed in insect cells (**A**) or washed ROS (**B**) were performed as described in Materials and Methods.

specific primers using a LI-COR L4000 automatic sequencer. All mutants were expressed in the High Five cells and purified using G2 affinity chromatography as described for recombinant GCAP1. To produce radioactive-labeled GCAP1 and its mutants, the cell culture was carried out in the presence of 1 mCi of [^{3}H]Leu or 0.5 mCi of [^{35}S]Met. GCAP1 was also radioactively labeled when the tissue culture medium was supplemented with 1 mCi of [^{3}H]myristic acid ([9,10-^{3}H(N)]tetradecanoic acid; NEN DuPont). No radioactivity was found associated with GCAP1-G2A, Δ10-GCAP1, or Δ25-GCAP1 mutants, when expressed in the presence of [^{3}H]myristic acid.

Expression of GC. Human photoreceptor GC (retGC1) was produced in a kidney 293 cell line (a gift from D. Lowe; Genentech, Inc.) as described previously (Shyjan *et al.*, 1992). Bovine retGC1 (obtained from Dr. R. Sharma) was cloned into pVL941 and expressed in High Five insect cells as described previously (Gorczyca *et al.*, 1995).

Peptide Synthesis. Peptides were synthesized by the solid phase method using an automated synthesizer (model 431A, Applied Biosystems, Foster City, CA). Synthesis was performed at the 0.25 mmol level using the Fmoc protocol. Half of the *p*-alkoxybenzyl resin containing peptide 2−28 was acylated with myristoyl chloride (Sigma) in pyridine. Following deprotection, peptides were purified by preparative reverse phase liquid chromatography on a 2.5 × 25 cm Partisil-10 ODS-3 column (Whatman), using a 0−40% gradient of acetonitrile in 0.05% acetic acid. Peptides had the expected composition by amino acid analysis and were single components when examined by analytical HPLC (0−40% gradient of acetonitrile in 0.01% phosphoric acid on a 4.6 × 150 mm column of Partisil-5 ODS-3).

GC Assays. The GC assays were performed using GTP-α-^{35}S(S$_p$) and washed ROS or GTP-α-^{32}P using expressed retGC1 as described previously (Gorczyca *et al.*, 1995). When indicated, the concentration of the substrate was changed and ATP was included (typically 0.4 mM). [Ca^{2+}]$_{free}$ was calculated using the computer program Chelator 1.00 (Schoenmakers *et al.*, 1992) and adjusted to higher concentrations in some assays by increasing the amount of added CaCl$_2$. In addition, [Ca^{2+}]$_{free}$ was measured from fluorescence changes of Fura-2 in the presence of Ca^{2+} according to Grynkiewicz *et al.* (1985). Ca^{2+} solutions were also used from calibrated [^{45}Ca^{2+}] stock solution. All solutions used for the Ca^{2+} titration were depleted of Ca^{2+} by passing through a Chelex-Sepharose gel.

Although some of the data are without standard deviations, they are the averages of two determinations. Similar results were obtained from at least two different sets of experiments performed in duplicate. Due to high sensitivity of the GC system [for details see Gorczyca *et al.* (1994a,b)], the absolute values of one series occasionally varied from another by 10−15% but with preservation of the ratio between activity of two different preparations (for example, mutants of GCAP1) since 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 or high [Ca^{2+}]$_{free}$ and ± GCAP1).

Protein Determinations and SDS−PAGE. GCAP1 concentration was determined spectrophotometrically (1 mg/mL of GCAP1 yielded A_{280nm} = 2.58) and assuming the molecular mass 23.5 kDa. This value was obtained directly from amino acid analysis of a GCAP1 solution of known absorption. The concentrations of other proteins were determined
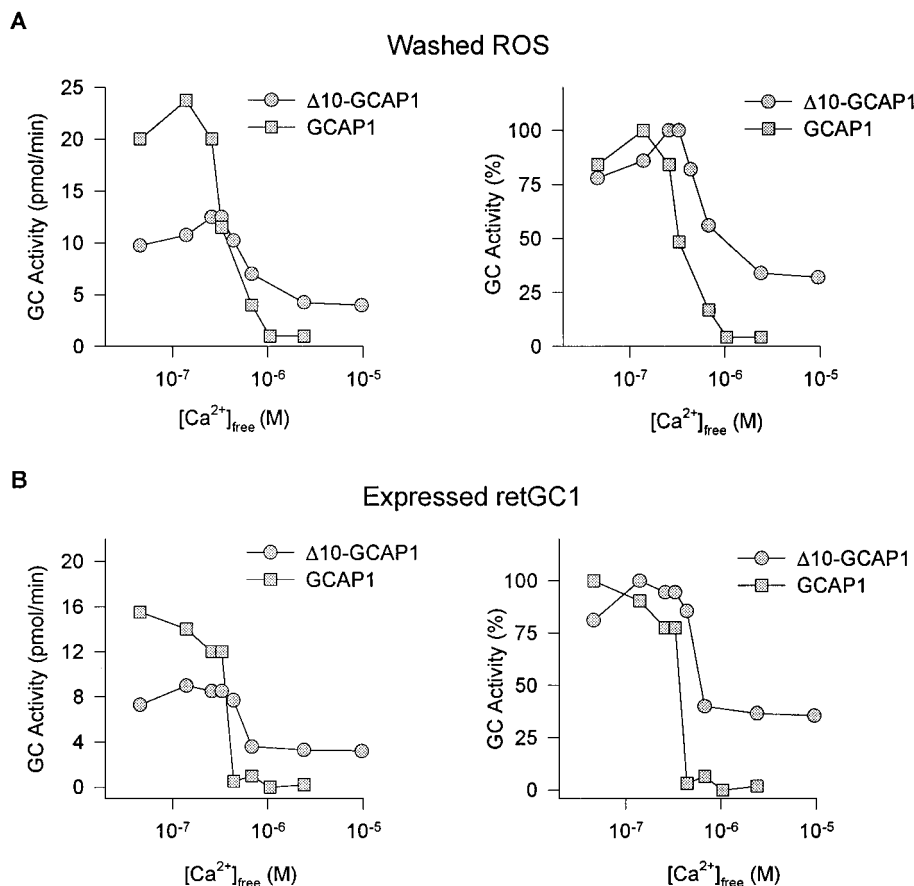
A

Inhibition: □ <30%; ▨ 30-60%; ■ >60%

B

GCAP1	^{1}MGNIMDGKSV	^{11}EELSSTECHQ	^{21}WYKKFMTECP	^{31}SGQLTLY...
GCAP1-G2A	M[A]NIMDGKSV	EELSSTECHQ	WYKKFMTECP	SGQLTLY...
Δ10-GCAP1	M - - - - - - - -	- ELSSTECHQ	WYKKFMTECP	SGQLTLY...
Δ25-GCAP1	M - - - - - - - -	- - - - - - - - - -	- - - - - - TECP	SGQLTLY...

FIGURE 3: (A) Inhibition of GC stimulation by peptides encompassing the whole sequence of GCAP1. Numbers represent the length of the peptide fragments and the position in the sequence of GCAP1; the differential shading shows inhibition by peptides at 100 μM for which the inhibition was <30%, 30−60%, or >60% in standard GC assay using washed ROS. IC_{50} represents 50% inhibition of GC stimulation by GCAP1 for the indicated peptide. (B) Amino acid sequence of GCAP1 and mutants at the N-terminal region. Three mutant GCAP1 forms were produced: GCAP1-G2A, in which Gly^2 was mutated to Ala (black background); Δ10-GCAP1 and Δ25-GCAP1 deletion mutants ("-" indicates deleted amino acids) were made retaining ^{1}Met. Note that ^{21}Trp is deleted in Δ25-GCAP1.

FIGURE 4: Stimulation of GC activity in washed ROS membranes and retGC1 by GCAP1 and its mutants. (A) Stimulation of GC activity in washed ROS (left panel). (B) Expressed retGC1 (right panel) by native and mutant forms of GCAP1.

by the method of Bradford (1976). SDS−PAGE was performed according to Laemmli (1970).

Fluorescence Measurements. Intrinsic fluorescence of GCAP1 was measured using a Perkin-Elmer LS 50B fluorimeter and a 1 × 1 cm quartz cuvette. The sample was stirred continuously and thermostated at 25 °C. The buffer contained 50 mM Hepes, pH 7.8, 60 mM KCl, 20 mM NaCl, 1 mM EGTA, and 1 mM DTT. The excitation wavelength was at 290 nm, and the emission scan speed was 100 nm/min. The excitation and emission slit widths were 5 nm. A concentrated solution of $CaCl_2$ (5−50 mM) was added several times in order to raise $[Ca^{2+}]_{free}$ from 10^{-8} to 3 × 10^{-5} M. Spectra were corrected by the dilution due to the addition of $CaCl_2$.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF). The samples of native and mutant forms of GCAP1 (20−100 pmol/μL) were placed on a stainless steel target plate that contained 100 mM

sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) dried from acetone. The protein was dried and overlaid with 1 μL of matrix (25 mM sinapinic acid in 30% acetonitrile). The mass spectra were recorded on a MALDI Voyager Elite in the linear and positive ion modes by summing an average of 100 laser pulses. The voltage acceleration was 30 kV, and the laser wavelength 337 nm. The instrument is housed in the Department of Biochemistry (Dr. K. A. Walsh and Dr. L. H. Ericsson).

RESULTS

Expression of GCAP1 in Insect Cells. Most of GCAP1 expressed in High Five insect cells was myristoylated as shown by a combination of the mAb G2 chromatography and autoradiography of [^{3}H]myristoylated GCAP1 and [^{3}H]Leu-labeled GCAP1 (Figure 1). The autoradiogram of SDS−PAGE gels showed that two forms were produced: one (93% on the basis of the radioactivity) that is identical

FIGURE 5: Ca^{2+} titration of GC activity in washed ROS and retG1 expressed in insect cells. (Left Panels) Activity is expressed in pmol/min. (Right Panels) Activity is expressed as a percent of maximal activity at low Ca^{2+} and with 1 μg of GCAP1. The GC assay using washed ROS (A) or retGC1 expressed in insect cells (B) was carried out as described in Materials and Methods. $[Ca^{2+}]_{free}$ was adjusted by Ca^{2+}/EGTA buffer as described in Materials and Methods.

to native GCAP1 (myristoylated with a molecular mass similar to native protein as determined by MALDI TOF) and a second unmyristoylated form with reduced mobility. Myristoylated GCAP1 could be separated from the unmyristoylated form on a CN-HPLC column (Duda *et al.,* 1996), although this step was typically omitted since we observed no difference in retGC1 stimulation between HPLC purified (myristoylated GCAP1) and mAb G2 purified GCAP1 (myristoylated GCAP1 + unmyristoylated GCAP1). These results demonstrate that active GCAP1 was expressed to a high level in insect cells and could be radiolabeled for further biochemical and structural characterization.

Effect of Ca^{2+} and ATP on retGC1 Stimulation by GCAP1. GCAP1 increased the V_{max} of photoreceptor retGC1 without affecting the K_M for its substrate GTP or α-thio-GTP. For example, when $[Ca^{2+}]_{free} = 10^{-6}$ M, the V_{max} was 0.3 nmol/min/mg for washed ROS membranes; while when the $[Ca^{2+}]_{free} = 2.2 \times 10^{-7}$ M and $[Ca^{2+}]_{free} = 4.3 \times 10^{-8}$ M, the V_{max} was 0.6 and 3 nmol/min/mg, respectively. These data suggest that GCAP1 increases the efficiency of the catalysis, but does not influence the affinity for the nucleotide substrate.

Another important factor in GC regulation is ATP. RetGC1 is activated by adenosine nucleotides in the presence of GCAPs. The nucleotides cause an increase in the V_{max} of retGC1 and slightly affect the K_M (Gorczyca *et al.,* 1994b). Similar levels of stimulation were observed for retGC1 and GCAP2 (Laura *et al.,* 1996). In addition, Ca^{2+}-sensitivity of retGC1 is modulated by protein kinase A phosphorylation (Wolbring & Schnetkamp, 1996), and retGC1 has been

reported to possess kinase-like activity and undergoes autophosphorylation in the presence of ATP (Aparicio & Applebury, 1996). Here, we found that ATP doubled the affinity of retGC1 for GCAP1 in reconstituted systems composed of purified GCAP1 and washed ROS or photoreceptor retGC1 expressed in insect cells (Figure 2A and 2B). Similar results were obtained using a combination of photoreceptor retGC1, expressed in a human kidney embryonic cell line (Shyjan *et al.,* 1992), with expressed or native GCAP1 (data not shown). These data suggest that ATP is a key component of the retGC1 regulation that influences the rate of cGMP production by increasing the rate of catalysis and affinity for GCAP1.

Regions of GCAP1 That Affect retGC1 Stimulation. The N-terminal region of GCAP1 was suggested to be involved in the regulation of retGC1, since a myristoylated peptide corresponding to the N-terminal 20 amino acids, strongly inhibited the effect of GCAP1 (Palczewski *et al.,* 1994). In addition to the N-terminal region, peptides encompassing the EF-1 and EF-3 motifs strongly inhibited retGC1 stimulation (Figure 3A). A "scrambled" peptide that contained the same amino acids as the N-terminal peptide, which was also myristoylated, had 20-fold reduced inhibitory properties as compared with the native peptide (data not shown). The peptide encompassing the C-terminus, the most divergent region among GCAP1 from different species, did not affect retGC1 stimulation (Figure 3A). These data suggest that the interaction between GCAP1 and photoreceptor retGC1 on disc membranes is provided by a multipoint attachment that
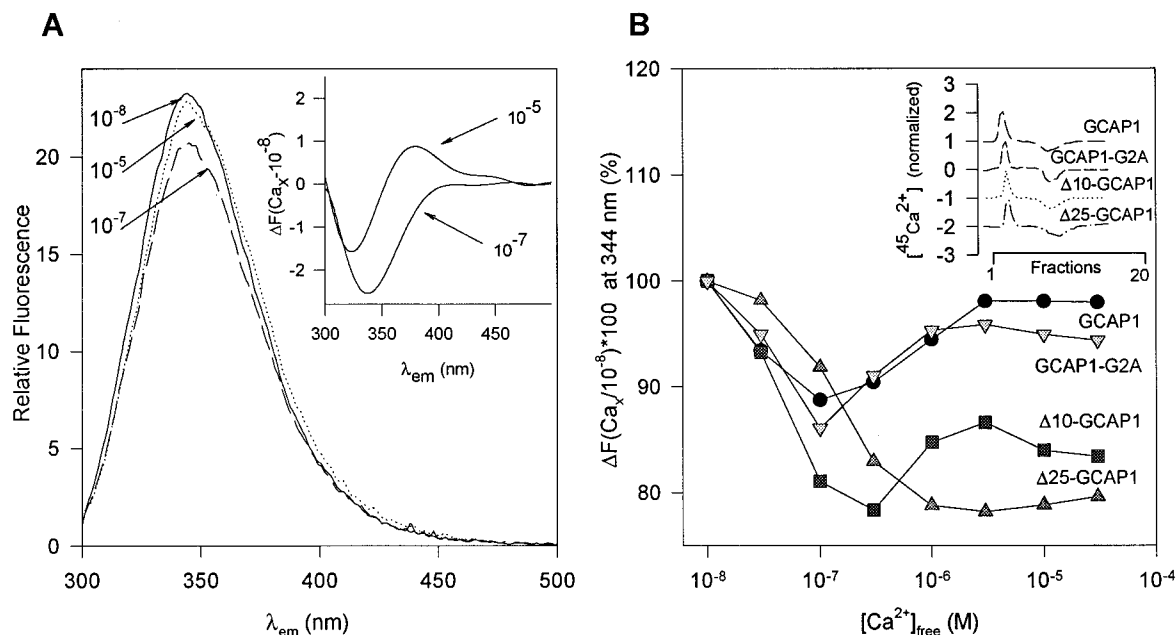
A

B

FIGURE 6: Fluorescence emission spectra of native and mutant forms of GCAP1 as a function of $[Ca^{2+}]_{free}$. (A) Three selected fluorescence spectra of GCAP1 (0.15 μM) with excitation at 290 nm in 50 mM Hepes, pH 7.8, 60 mM KCl, 20 mM NaCl, 1 mM EGTA, and 1 mM DTT, containing 10^{-8}, 10^{-7}, and 10^{-5} M $[Ca^{2+}]_{free}$. Inset: Difference fluorescence spectra of GCAP1 measured at 10^{-7} and 10^{-5} M $[Ca^{2+}]_{free}$ minus values at 10^{-8} M $[Ca^{2+}]_{free}$. (B) Changes in the maximum fluorescence intensity at 344 nm in $[Ca^{2+}]_{free}$ from 10^{-8} to 3×10^{-5} M for GCAP1, GCAP1-G2A, Δ10-GCAP1, and Δ25-GCAP1. Inset: Gel filtrations (Hummel & Dreyer, 1962) of native and mutant forms of GCAP1 were carried out using G-25 Sephadex (1.6 × 2.5 cm) at a flow rate of 0.2 mL/min. The buffer (10 mM BTP, pH 7.5, 100 mM NaCl) contained 1 μM [^{45}Ca]CaCl$_2$ and 1.6–3.8 μM proteins. Other concentrations of CaCl$_2$ used were 0.25, 0.5, and 2 μM (not shown).

most likely involves the N-terminal region EF-1 and EF-3 motifs of GCAP1.

Mutant Forms of GCAP1 with Modified N-Terminal Region. To explore the importance of the conserved N-terminus of GCAP1 (only two divergent residues in the first 35 amino acids of vertebrate GCAP1s) (Semple-Rowland et al., 1996; Palczewski et al., 1994), we generated mutants lacking 10 (Δ10-GCAP1) or 25 amino acids (Δ25-GCAP1) at the N-terminus, and a mutant in which ^{2}Gly was replaced with Ala, GCAP1-G2A (Figure 3B). All mutants lacked a site for N-terminal myristoylation. The N-termini of these mutants were inaccessible for Edman degradation, suggesting that ^{1}Met was not removed and acetylated, but not myristoylated, as observed for GCAP1-G2A and Δ10-GCAP1 which lacked the incorporation of [^{3}H]myristic acid (by analogy to the experiment presented in Figure 1). The molecular mass for GCAP1, as estimated by MALDI TOF mass spectrometry, was found at the center of a peak to be 23 729 ± 360 at half-height (for myristoylated GCAP1 the calculated mass = 23 587.4 Da); for GCAP1-G2A, the peak was at 23 636 ± 345 (calculated for acetylated mutant = 23 564.5); for Δ10-GCAP1, the peak was at 22 479 ± 270 (calculated for acetylated mutant = 22 519.5); and for Δ25-GCAP1, the peak was at 20 486 ± 140 (calculated for acetylated mutant = 20 619.4). These data are consistent with acetylation of N-terminal Met in all GCAP1 mutants. Similar to GCAP1, all mutants exhibited characteristic shifts in SDS–PAGE in the presence of EDTA (data not shown).

Mutation of ^{2}Gly to Ala (Figure 3B) significantly reduced the ability of the mutant to stimulate retGC1 (Figure 4A). Surprisingly, the removal of 10 amino acids in Δ10-GCAP1, restores stimulation to ~30–45% of the original GCAP1 level. Further truncation of the N-terminus in Δ25-GCAP1 nearly abolished the retGC1 stimulation. Similar results were observed when ROS membranes were replaced by photoreceptor retGC1 expressed in insect cells (Figure 4A) or

retGC1 expressed in human kidney cells (data not shown). GCAP1-G2A did not compete with GCAP1 for binding to retGC1 and did not affect the retGC1 stimulation by GCAP1 significantly (data not shown).

Δ10-GCAP1 displayed distinct Ca^{2+} sensitivity (Figure 5A and 5B). The retGC1 activity was reduced by ~60% at high Ca^{2+}, as assayed using washed ROS membranes or expressed retGC1. These results suggest that the N-terminal 10 amino acids, although ~70 amino acids distant from the nearest EF motif, contribute to Ca^{2+}-sensitive modulation of retGC1. The retGC1 activity was stimulated additively in the presence of GCAP1 and Δ10-GCAP1. GCAP1-G2A has lower specific activity but its Ca^{2+} titration was similar to that of native GCAP1 (data not shown).

Ca^{2+}-Binding Properties of Native and Mutant Forms of GCAP1. GCAP1 exhibits Ca^{2+}-sensitive changes in its intrinsic fluorescence (Palczewski et al., 1994). We have noticed that the prolonged incubation of GCAP1 at room temperature in the presence of Ca^{2+}, or unbuffered EGTA, leads to aggregation or complex formation with the chelator (Gorczyca et al., 1994a), respectively. Thus, all of our measurements were completed in 30–40 min and with a concentration no lower than 10 nM [Ca^{2+}]$_{free}$ and no higher than 50 μM [Ca^{2+}]$_{free}$. These changes in the fluorescence, as a function of [Ca^{2+}]$_{free}$, resulted from changes in emission of fluorescence from one or more of the three Trp residues (at positions 21, 51, and 94). At low [Ca^{2+}]$_{free}$, GCAP1 had an emission maximum at 344 nm. The changes of GCAP1 fluorescence, as a function of [Ca^{2+}]$_{free}$, were biphasic; initially, as [Ca^{2+}]$_{free}$ increased, the fluorescence decreased at $\lambda_{max\ em}$ at 10^{-7} M [Ca^{2+}]$_{free}$. After addition of extra CaCl$_2$, the fluorescence gradually increased from the lowest value, but did not reach the value at lowest Ca^{2+} and the $\lambda_{max,em}$ shifted to 346 nm (Figure 6). The fluorescence measurements revealed two processes: one which occurred at 10^{-8}– 10^{-7} M [Ca^{2+}]$_{free}$ and led to Ca^{2+}-induced changes in the
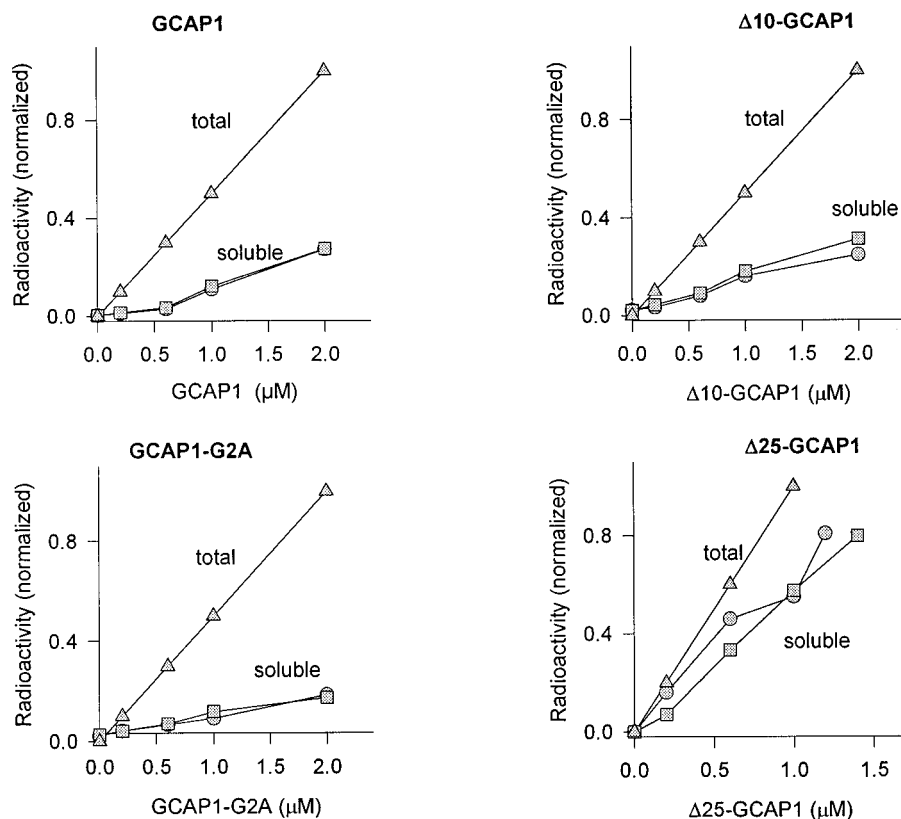
FIGURE 7: Binding of native and mutant forms of GCAP1 to ROS membranes. (A) Radiolabeled GCAP1 was incubated with washed ROS membranes in the standard GC assay conditions at 45 nM (circles) and 10^{-4} M (squares) $[Ca^{2+}]_{free}$. The complex between GCAP1 and membranes was collected by ultracentrifugation (15 min; 86000g) and the amount of unbound protein determined by measuring radioactivity (normalized, 1 or 2 μM = 1; triangles) left in the supernatant. (B−D) Results of similar experiments are shown for GCAP1 mutants.

GCAP1 conformation, and a second process that correlates with the Ca^{2+}-dependent inactivation. Similar results were obtained using native GCAP1 (data not shown). These fluorescence changes agree with the Ca^{2+}-dependent changes in GCAP1 stimulation of retGC1, which is highest at $\sim$$10^{-7}$ M $[Ca^{2+}]_{free}$ (Figure 5; Frins et al., 1996). More importantly, GCAP1-G2A exhibited qualitatively similar fluorescence changes, suggesting that its integrity and the tertiary structure were not dramatically altered due to the mutation and lack of myristoylation.

Truncation near ^{21}Trp in Δ10-GCAP1 modified fluorescence properties only at higher $[Ca^{2+}]_{free}$. The second phase of the fluorescence changes recovered only to $\sim$40%, as compared with GCAP1 (Figure 6). These results correlate with the partial activity of this mutant at high $[Ca^{2+}]$. Δ25-GCAP1, which lacks ^{21}Trp, also lacked this second phase of Ca^{2+}-induced fluorescence changes and, as shown before, had almost no stimulating activity. Using gel filtration (Hummel & Dreyer, 1962), we tested if these mutants were qualitatively abnormal in the binding at 0.25, 0.5, 1, and 2 μM $[Ca^{2+}]_{free}$. In all conditions tested, no differences were found in Ca^{2+} binding between GCAP1 and any of the mutants (Figure 6B, inset). These results suggest that the N-terminal region, which includes ^{21}Trp, is part of a domain sensitive to Ca^{2+} binding and is probably involved in retGC1 regulation or membrane association.

Membrane Association of Native and Mutant Forms of GCAP1. The differences in retGC1 stimulation of native and mutant forms of GCAP1 could result from impairment of membrane association. We tested, therefore, the ability of radiolabeled GCAP1s to associate with ROS membranes (Figure 7). Except for Δ25-GCAP1, which does not associate with membranes, the mutants showed Ca^{2+}-independent

binding to membranes (GC) with similar affinities. The GCAP1-G2A mutant, with the unprocessed N-terminus (acetyl-M-A-Q-...) lacking myristoylation, showed even slightly increased membrane association (Figure 7C). The inability of Δ25-GCAP1 to associate with membranes is not due to misfolding, since Ca^{2+} binding, which requires correct folding of the protein, is not impaired (Figure 6B, inset). These results suggest that GCAP1 is membrane-associated at low and high $[Ca^{2+}]$, as proposed earlier (Gorczyca et al., 1995; Koutalos et al., 1995). In addition, it appears that the myristoyl group is not critical for the membrane binding.

DISCUSSION

In this study, we developed a significantly improved method to isolate GCAP1 from the retina and from an insect cell expression system. We found that the expressed protein is myristoylated ($>$90%) and able to stimulate photoreceptor retGC1 indistinguishably from native protein. On the basis of peptide competition experiments, the interaction between GCAP1 and retGC1 may involve several regions of GCAP1 including the N-terminal region and EF-1 and EF-3 motifs (Figure 3). The N-terminal lipid modification is not essential for activity since Δ10-GCAP1 stimulates retGC1, although with reduced specific activity. At μM levels of $[Ca^{2+}]_{free}$, retGC1 remains active in the presence of Δ10-GCAP1 while it is inactivated in the presence of GCAP1. Interestingly, the GCAP1-G2A mutation, which lacks myristoylation and retains the acetyl-Met, has low specific activity, although its Ca^{2+} sensitivity was similar to that of native GCAP1. The N-terminal modification in this mutant may prevent the correct orientation in the complex with GC, resulting in low specific activity. Complete removal of the N-terminal domain (Δ25-GCAP1) largely abolished the ability to bind

to membranes and stimulate retGC1. These properties of the Δ25-GCAP1 mutant did not result from instability or misfolding as tested by several independent methods (SDS−PAGE, Ca^{2+} binding by fluorescence and gel filtration methods).

Our results are reminiscent of a model originally proposed by Zozulya and Stryer (1992) and Dizhoor *et al.* (1993) for recoverin. The binding of Ca^{2+} to recoverin induces the exposure of a hydrophobic surface, and the myristoyl group is extruded enabling it to insert into a lipid bilayer membrane. In a Ca^{2+}-free form, this hydrophobic surface may interact with the hydrophobic tail. This Ca^{2+}-myristoyl switch could be responsible for the Ca^{2+}-dependent inactivation of GCAP1 that prevents stimulation of retGC1. Removal of part of the GCAP N-terminus (Δ10-GCAP1) produced a molecule that preserved retGC activity even at μM levels of $[Ca^{2+}]_{free}$. Consistent with our results, experiments described by Frins *et al.* (1996) showed that a recombinant GCAP1 fusion protein had lower specific activity (1:40) and that Ca^{2+} sensitivity was shifted to higher values.

On the basis of biochemical evidence (Gorczyca *et al.*, 1995; Duda *et al.*, 1996; this study) and under physiological conditions (Koutalos *et al.*, 1995), it is likely that GCAP1 stays in a complex with retGC1 irrespective of $[Ca^{2+}]_{free}$ or is sequestered in proximity to the cyclase. The affinity of GCAP1 for retGC1, however, is low, and the complex in ROS can be disrupted in hypotonic buffer or in the presence of detergent. The stimulation of photoreceptor retGC1 occurs intracellularly, with GCAP1 sensing changes in $[Ca^{2+}]_{free}$ levels during phototransduction (Duda *et al.*, 1996; Laura *et al.*, 1996). This interaction may be further weakened when ATP is exhausted (or significantly reduced), resulting in slower production of cGMP and affecting the rate of recovery from the illumination.

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