Functional Characterization of a Guanylyl Cyclase-activating Protein from Vertebrate Rods

CLONING, HETEROLOGOUS EXPRESSION, AND LOCALIZATION*

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The membrane-bound guanylyl cyclase in vertebrate photoreceptor cells is one of the key enzymes in visual transduction. It is highly sensitive to the free calcium concentration ([Ca$^{2+}$]). The activation process is cooperative and mediated by a novel calcium-binding protein named GCAP (guanylyl cyclase-activating protein). We isolated GCAP from bovine rod outer segments, determined amino acid sequences of proteolytically obtained peptides, and cloned its gene. The Ca$^{2+}$-bound form of native GCAP has an apparent molecular mass of 20.5 kDa and the Ca$^{2+}$-free form of 25 kDa as determined by SDS-polycrylamide gel electrophoresis. Recombinant GCAP was functionally expressed in Escherichia coli. Activation of guanylyl cyclase in vertebrate photoreceptor cells by native acylated GCAP was half-maximal at 100 nM free [Ca$^{2+}$] with a Hill coefficient of 2.5. Activation by recombinant nonacylated GCAP showed a lower degree of cooperativity (n = 2.0), and half-maximal activation was shifted to 261 nM free [Ca$^{2+}$]. Immunocytochemically we localized GCAP only in rod and cone cells of a bovine retina.

Illumination of vertebrate photoreceptor cells triggers the hydrolysis of guanosine 3',5'-cyclic monophosphate (cGMP) by an amplifying transduction cascade. Decrease of cytoplasmic cGMP leads to the closure of cGMP-gated channels and results in the suppression of the circulating dark current. This generates the electrical signal for further processing in the retina. Restoration of the dark current depends on effective shut-off mechanisms of all excitation steps in the transduction cascade and on an efficient resynthesis of cGMP (1-4). A calcium feedback controls some of these events and is thought to be critical for both recovery and light adaptation (5, 6). Calcium homeostasis in photoreceptor cells is maintained by the balance between Ca$^{2+}$ influx through the cGMP-gated channel and Ca$^{2+}$ efflux via the Na:Ca,K exchanger. Closure of the cGMP-gated channels prevents Ca$^{2+}$ from entering the cell. Because Ca$^{2+}$ is continuously extruded by the exchanger, a net decrease of the cytoplasmic calcium concentration below the dark concentration of 400–500 nM accompanies the light response (7-10).

One of the key events triggered by the decrease of cytoplasmic [Ca$^{2+}$] is a reinforced synthesis of cGMP catalyzed by a 112-kDa guanylyl cyclase (GC) that represents a novel subtype among the membrane bound GCs (11-15). A characteristic feature of this photoreceptor-specific GC (retGC) is its strong dependence on the free [Ca$^{2+}$]. At low [Ca$^{2+}$] the enzyme is activated by a soluble calcium-binding protein (16). Recent progress has been made in the search for this protein. A calcium-binding protein named guanylyl cyclase-activating protein (GCAP) with an apparent molecular mass of 20 kDa was shown to activate the retGC in a Ca$^{2+}$-dependent manner (17). Dialyzing GCAP into lizard rod outer segments decreases the sensitivity, time to peak and recovery time of the light response. Molecular cloning and sequence analysis showed the presence of three Ca$^{2+}$-binding motifs (EF-hands) in GCAP (18, 19). Additionally, a 24-kDa calcium-binding protein was isolated from retina extracts that was able to stimulate retGC (20). The relationship of these two activator proteins and their cellular distribution in the retina is not clear at present. An unexpected complexity has also emerged from the cloning of a second photoreceptor-specific membrane guanylyl cyclase, retGC-2 (21). (We simply refer to retGC activity, because we cannot distinguish between retGC-1 and retGC-2 activities in our rod outer segment (ROS) membrane preparations.) In order to gain further insight into the control of retGC activity, we screened cytoplasmic extracts of bovine ROS for guanylyl cyclase activating factors, isolated and cloned a protein (GCAP), studied its distribution in the vertebrate retina, and compared some of the molecular properties of two recombinant forms with the native form.

EXPERIMENTAL PROCEDURES

Purification of GCAP—ROSs were prepared from freshly obtained bovine eyes as described previously (22). 12 ml of ROS (7-11 mg rhodopsin/ml) were adjusted to 100 mM NaCl, 1 mM MgCl$_2$, 3 mM ATP, 1 mM dithiothreitol and bleached for 15 min at 37 °C. The suspension was adjusted to 10 mM KH$_2$PO$_4$, incubated at 4 °C for 20 min, and centrifuged for 40 min (40,000 rpm, Ti70 rotor Beckman). The pellet was discarded, the supernatant ("light extract") was again centrifuged for 20 min (80,000 rpm, TLA 100.3 Beckman). The clear supernatant was concentrated to a final volume of maximum 1.4 ml using Centricon 10 filtration units (Amicon). The concentrated extract was applied onto a MonoQ fast protein liquid chromatography column (Pharmacia). The flow rate was 1 ml min$^{-1}$. Fractions of 2 ml were collected. The fractions were screened for Ca$^{2+}$-dependent stimulation of retGC in a reconstitution assay. The positive fractions were applied on a MonoQ fast protein liquid chromatography column (Pharmacia).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank$^{19}$/EMBL Data Bank with accession number(s) X95352.

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1 The abbreviations used are: GC, guanylyl cyclase; retGC, vertebrate photoreceptor membrane-bound guanylyl cyclase; GCAP, guanylyl cyclase-activating protein; ROS, rod outer segments; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; PB, phosphate buffer.
equilibrated with buffer A. After elution of the fraction of nonbound proteins, retained proteins were eluted with a linear NaCl gradient (buffer B, 500 mM NaCl, 20 mM Tris, pH 8.0, 1 mM dithiothreitol) going from 50 mM to 500 mM in a total volume of 20 ml. Finally the column was washed with 2 ml of 500 mM NaCl. Fractions of 0.5 ml were collected. The flow rate was 0.5 ml min\(^{-1}\). GCAP eluted at 400 mM NaCl in a 3-4 ml fraction.

Amino Acid Sequencing of Peptides—N-terminal sequencing of the undigested protein was performed after electrophoretotyping on a poly(vinylidene difluoride) membrane and excising the Amido black-stained band. For internal sequencing four protein bands (3 5 \(\mu\)g) were excised from SDS-PAGE after Coomassie Blue staining. After washing with water, 70% ethanol and H2O, the gels pieces were rehydrated in 100 \(\mu\)l of 100 mM NH4HCO3, 0.5 mM CaCl2, pH 8.1, containing 1 \(\mu\)g cytomysprin. Digestion was achieved at 37 °C overnight, and the resulting peptide fragments were extracted from the gel pieces with 2 100 \(\mu\)l of trifluoroacetic acid/water (7:3, v/v) and 2 100 \(\mu\)l of trifluoroacetic acid/acetonitrile (1:1, v/v). The combined fractions were concentrated and subjected to reversed phase HPLC using Vydac 218TP (1.6 250 mm) (23). Peptide fractions were loaded onto a polyboreen-coated glass fiber filter and sequenced by automated Edman degradation using a model 477A sequenator connected to a model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems, Weiterstadt, Germany).

Isolation and Characterization of cDNA Clones—Degenerate primers 1 (TATGACGANGRTGYCC) and 2 (TAYTCCATRAARTCDATRTA) based on sequences of peptide 2 and 5, respectively, were used to amplify a fragment of GCAP transcript via PCR on a bovine retina-specific first strand cDNA. Forty cycles were performed according to the dideoxy nucleotide chain termination technique (24) using T7 DNA polymerase (Pharmacia). These sequence of the coding region and of parts of nontranslated regions was confirmed by sequencing of three independent recombinants.

Expression and Isolation of Recombinant Protein—In order to express recombinant GCAP, two different subclones (pG-PCAPX and pG-GCAPT) were constructed using pGEX-2T (Pharmacia) as vector. Press recombinant GCAP, two different subclones (pG-GCAPX and pG-GCAPT) were constructed using pGEX-2T (Pharmacia) as vector.

GC Assay and Reconstitution Experiments—Activity of retGC was assayed by the HPLC method as described previously (12, 22). PDE inhibitor Zaprinast was present at 440 \(\mu\)M in all reaction samples. Final concentration of Mg2+ was 10 mM. The GC assay was performed under very different conditions. Light for reconstitution experiments, ROS membranes were diluted 5-fold with 5 mM Tris-HCl, pH 8.0 and centrifuged for 15 min (40,000 rpm, TLA45, 4 °C, Beckman). The resulting pellets were suspended in the same buffer, and the washing procedure was repeated. The pel let was resuspended in one half of the original ROS volume in 500 mM KCl, 20 mM NaCl, 50 mM Hepes-KOH, pH 7.4, 1 mM dithiothreitol. Fractions from column chromatography were added to washed ROS membranes and incubated at high and low Ca2+ for 5 min at 30 °C in a total volume of 50 \(\mu\)l. Native and recombinant GCAP preparations were added to washed ROS membranes at ratios of 1:800 to 1:5 GCAP per rhodopsin. Incubation was done at 30 °C for 5 min and stopped by adding 50 \(\mu\)l of ice-cold 100 mM EDTA and boiled for 5 min. HPLC analysis of the membrane samples was done as described (12, 22).

RESULTS

Purification and Cloning of GCAP—An extract of illuminated bovine ROS contained a guanylyl cyclase-stimulating activity that could be further fractionated by column chromatography (Fig. 1A). When proteins were separated on a Superdex 75 16/60 gel filtration column, this activity coeluted in an elution volume between 26 and 36 ml. The main protein (about 80%) in this fraction was the calcium-binding protein recoverin (22, 25). Subsequent fractionation on an anion exchange column (MonoQ) separated GCAP activity from recoverin. The best separation of these two calcium-binding proteins was achieved by using a NaCl gradient of 0–500 mM in 20 ml. This gradient had a different steepness from the one we have previously described for the purification of recoverin (22). Fractions with activity eluted at 400 mM NaCl and were analyzed by SDS-PAGE (Fig. 1B). Samples contained a faintly stained protein band of 20.5 kDa. Complexing Ca2+ with EGTA in the protein sample caused a striking gel shift to 25 kDa.
Activity of retGC at high \([\text{Ca}^{2+}]\) was 2–3 nmol cGMP min\(^{-1}\) mg\(^{-1}\) rhod (Fig. 1C). When free \([\text{Ca}^{2+}]\) was lowered to less than 100 nM native GCAP activated retGC in washed ROS membranes to 5–7 nmol cGMP min\(^{-1}\) mg\(^{-1}\) rhod (Fig. 1C). The addition of 100 \(\mu\text{M}\) ATP enhanced retGC activity independent of the free \([\text{Ca}^{2+}]\) about 2-fold (Fig. 1C, control + ATP). Maximal activity of retGC was obtained at low free \([\text{Ca}^{2+}]\) with native GCAP and 100 \(\mu\text{M}\) ATP resulting in 12–14 nmol cGMP min\(^{-1}\) mg\(^{-1}\) rhod (Figs. 1C and 4B). Low concentrations of ATP (0.1–0.2 mM) were reported to enhance retGC activity in whole ROS preparations (26–28) and in a reconstituted system with purified GCAP (27). Contrary, Sitaramayya et al. reported an inhibitory effect of ATP on retGC activity (29). We only observed inhibition of retGC at higher ATP concentrations (1–2 mM). The inhibition by ATP probably reflects competition with the GTP binding site as also suggested by Gorczyca et al. (27). Occasionally we observed retGC activities at low free \([\text{Ca}^{2+}]\) as high as 23 nmol cGMP min\(^{-1}\) mg\(^{-1}\) rhod in whole ROS and also with purified native GCAP.

Microsequencing of proteolytically obtained peptides of the 20.5-kDa protein revealed six partially overlapping peptides (Fig. 2). N-terminal sequencing failed due to N-terminal modification (covalent acylation; see “Discussion”). Based on the sequences of peptides 2 and 5 degenerate oligonucleotide primers were designed for PCR on bovine retinal cDNA. PCR products were subcloned and sequenced. A fragment that was found to harbor the sequence coding for peptides 2 and 5 as well as for peptide 4 was used to screen a bovine retina-specific library. The longest isolated recombinant consisted of 945 base pairs. One long open reading frame was identified. The translation initiation site was assigned to the first ATG codon (nucleotides 238–240) that appears downstream of a nonsense sequence for eukaryotic initiation sites (CC(A/G)CCATGG; Ref. 30). An in-frame TGA translation termination codon is found at positions 616–618. The deduced polypeptide sequence consisted of 205 amino acids with a calculated molecular mass of 23,510 daltons. All microsequenced peptides of the native protein were found in the full-length clone. While this work was in progress, the cDNA derived amino acid sequence of GCAP from several vertebrate species was published representing a novel subfamily of \(\text{Ca}^{2+}\)-binding proteins (18, 19). Our sequence is identical to the bovine homologue. Three canonical \(\text{Ca}^{2+}\)-binding motifs (EF-hands) were present in the sequence (see shaded parts in Fig. 2).

Functional Comparison of Native and Recombinant GCAP—We obtained 3–4 mg of recombinant GCAP from 1 liter of culture medium after purification on glutathione S-transferase-agarose and a MonoQ anion exchange column (Fig. 3). We compared the \(\text{Ca}^{2+}\)/EGTA-dependent electrophoretic mobility shift of our two recombinant GCAP forms with the native one.
All proteins showed the same relative shift of about 5 kDa. The two recombinant forms GCAPT and GCAPX run slightly higher than the native form (about 1 kDa) in both conditions (Ca\(^{2+}\) and EGTA) probably due to the lack of acylation. Difference of mobility between the two recombinant forms was less pronounced but still visible (GCAP with six additional amino acids run at a 0.5–1 kDa higher molecular mass).

The calcium-dependent activation of retGC by the native and the two recombinant GCAP forms was tested at different free [Ca\(^{2+}\)] from 1 nM to 150 μM. Native GCAP stimulated GC activity 5–10-fold with high cooperativity in the physiologically significant range from 50 to 400 nM (EC\(_{50}\) = 100 nM; Hill coefficient n = 2.5). The ratio of GCAP to rhodopsin in Fig. 4B was 1:800 (open circles). Molar ratios of recombinant GCAPT to rhodopsin was 1:20 (open squares). All incubations were done in the presence of 0.1 mM ATP. No GCAP was added in the control (filled diamonds). The data represent triplicates.
minus (GCAPX) activated retGC in a similar fashion.

The effect of recombinant GCAP was most efficient at rather high concentrations of GCAP. In order to exclude any nonspecific protein-protein interactions, samples were incubated in the presence of 0.03 mg of bovine serum albumin. Incubation with bovine serum albumin alone did only result in basal nonstimulated retGC activity. The addition of anti-GCAP antibody inhibited the Ca\(^{2+}\)-sensitive regulation of retGC.

**Immunocytochemistry—Polyclonal antibodies produced against purified recombinant GCAP recognized the native GCAP in ROS preparations on Western blots and were used for immunocytochemical studies (Fig. 5).**

The anti-GCAP antibody was used to localize GCAP in vertical sections of the bovine retina. GCAP-like immunoreactivity was found in the photoreceptor layer. Staining was prominent in outer segments of both rods and cones but was also present in other compartments of the photoreceptor like soma, axon, and axon terminal. In addition to rods, cones were heavily labeled. Cone outer segments, somata, axons, and pedicles could be clearly identified (Fig. 5). In the inner retina, only occasionally small somata in the inner nuclear layer and the ganglion cell layer were stained (not shown). Except for this extremely rare amacrine cell population, no other cell types were found to be immunoreactive. No staining was observed without the primary antibody and after preadsorption with recombinant GCAP (not shown).

**DISCUSSION**

The decrease of cytoplasmic [Ca\(^{2+}\)] in a vertebrate photoreceptor cell is considered to be a principal control step that modulates phototransduction and is critical for light adaptation. One of the key events for recovery from a light pulse and for light adaptation is the increase of retGC activity at low [Ca\(^{2+}\)]. We have demonstrated that a calcium-binding protein with a calculated molecular mass of 23.51 kDa serves as a cytoplasmic activator of retGC. Following a different strategy in purification and cloning we identified the same activator protein that Palczewski et al. have described as GCAP (18). Our retGC activities were comparable with values in the literature for whole ROS (16, 22, 25, 28). Activation of retGC in ROS membranes by purified native GCAP was identical to the activation measured in whole ROS regarding Ca\(^{2+}\) sensitivity, cooperativity, and maximal stimulation. Using the physiological substrate GTP we measured higher activities than Gorczyca et al. obtained with a phosphorothioate analogue of GTP (17, 27).

Functional expression of recombinant GCAP has not been reported so far and allowed us a functional comparison with the native protein. Furthermore we could address specific questions concerning the molar ratios (i.e. cellular concentrations) of retGC and GCAP and the role of fatty acid modification on GCAP. Native and recombinant GCAP exhibited different properties with respect to activation of retGC. The maximal activation at low [Ca\(^{2+}\)] with native GCAP was exactly the same as it is observed in whole ROS preparations. It was half-maximal at 100 nm and showed a high cooperativity (Fig. 4B). Efficient activation with recombinant GCAP was achieved at higher concentrations of GCAP (Fig. 4B). We observed a change in the EC\(_{50}\) value and a lower degree of cooperativity when recombinant GCAP was assayed. Several reasons for the observed differences are conceivable: 1) Because heterologous expression in E. coli yields recombinant proteins without posttranslational modification, a lack of N-terminal acylation in recombinant GCAP could cause the change in the EC\(_{50}\) value and in cooperativity. The amino acid sequence of GCAP contains the consensus sequence for N-terminal myristoylation at Gly\(^{1}\). Heterogeneous N-terminal acylation of native GCAP was demonstrated by mass spectrometry (18). In addition, the N-terminal part of GCAP seems to be critical for the activation process as investigated by peptide competition experiments (18). (2) Our recombinant GCAP preparation could have an impaired Ca\(^{2+}\) binding or could be partially denatured or misfolded due to sonification during the lysis step (see “Experimental Procedures”). However, the Ca\(^{2+}\)-induced conformational change monitored by the mobility shift assay (Fig. 4A) works to the same extent in native and recombinant GCAP forms.

Preliminary cross-linking experiments (data not shown) indicated that GCAP interacts with retGC and that retGC exists in native ROS membranes as an oligomer. An oligomeric form of retGC was also suggested from previous gel filtration studies that showed elution of GC activity in association with a high molecular weight complex (11, 31, 32). We speculate that GCAP could act on an oligomeric form of retGC.

We cannot exclude at the moment that additional factors play a significant role in retGC activation. Dizhoo et al. reported that human retGC could be activated at low [Ca\(^{2+}\)] by an extract partially purified from whole retinas (20). Enrichment of a 24-kDa protein correlated with retGC stimulating activity. Starting from fresh bovine ROS preparations, retGC stimulating activity correlated in our chromatographic fractions only with GCAP. As far as we can judge from Coomassie Blue- or silver-stained gels, no other protein of similar molecular mass (16–26 kDa) was detectable.

Low concentrations of ATP (100 \(\mu\) M, below the concentration that causes a competitive interference with the substrate) enhanced retGC activity independent of the free [Ca\(^{2+}\)]. We used 100 \(\mu\) M ATP in all retGC assays to maximize the response. Wolbring and Schnetkamp recently reported that retGC activi-
ity can be increased by ATP (28). This increase was inhibited by specific protein kinase C inhibitors, suggesting that a phosphorylation step plays an additional modulatory function.

The polyclonal anti-GCAP antibody was an effective tool in immunocytochemical and Western blotting studies. Strong labeling of the photoreceptor layer in the bovine retina is in agreement with a specialized function of GCAP in controlling retGC activity. Labeling of cone cells also indicates that the same or a very similar form of GCAP exists in both rods and cones.

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