Structural and Functional Characterization of Retinal Calcium-Dependent Guanylate Cyclase Activator Protein (CD-GCAP): Identity with S100β Protein†

Nikolay Pozdnyakov,‡ Rafal Goraczniak,§ Alexander Margulis,‡ Teresa Duda,§ Rameshwar K. Sharma,§ Akiko Yoshida,|| and Ari Sitaramayya*,‡

Eye Research Institute, Oakland University, Rochester, Michigan 48309, The Unit of Regulatory & Molecular Biology, Department of Cell Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084, and Department of Ophthalmology, Tohoku University School of Medicine, Sendai, Japan

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ABSTRACT: Calcium-dependent guanylate cyclase activator protein (CD-GCAP) is a low-molecular-weight retinal calcium-binding protein which activates rod outer segment guanylate cyclase (ROS-GC) in a calcium-dependent manner. This investigation was undertaken to determine the protein's structure and identity. Partial amino acid sequencing (72% of the protein), mass spectral analysis, cloning, and immunological studies revealed that CD-GCAP is identical to S100β, another low-molecular-weight calcium-binding protein whose structure was known. We had shown earlier that the latter protein, which is usually called S100b (S100$\beta\beta$ or dimer of S100β), also activates ROS-GC but that the V_{max} of activated cyclase was about 50% lower than when stimulated by CD-GCAP. S100b also required about 15 times more calcium (3.2×10^{-5} vs 1.5×10^{-6} M) for half-maximal stimulation of cyclase. To investigate the possibility that CD-GCAP is a post-translationally modified form of S100b, both proteins were treated with 1 M hydroxylamine which is known to deacylate proteins. After the treatment, CD-GCAP did not activate cyclase while S100b activation remained unaffected suggesting that CD-GCAP could not be a modified form of S100b. Hydroxylamine also broke down CD-GCAP into smaller fragments while leaving S100b intact. It therefore appeared that in spite of identical primary structures, the conformations of the two proteins were different. We then investigated the possibility that the purification procedures of the two proteins, which were quite different, could have contributed to such conformational differences: CD-GCAP purification included a step of heating at 75 °C in 5 mM Ca, while S100b purification included zinc affinity chromatography. To test the influence of these treatments on the properties of the proteins, CD-GCAP was subjected to zinc affinity chromatography and purified as S100b (CD-GCAP→S100b) and S100b was heated in Ca and purified as CD-GCAP (S100b→CD-GCAP). Cyclase activation, calcium-sensitivity, and hydroxylamine-lability measurements revealed that CD-GCAP→S100b is identical to S100b and that S100b→CD-GCAP is identical to CD-GCAP. Taken together the results demonstrate that CD-GCAP and S100b are one and the same protein and that their functional differences are due to different interconvertible conformational states.

Membrane guanylate cyclases whose mechanisms of regulation are understood can be broadly classified into two groups. The first consists of enzymes referred to as receptor guanylate cyclases which respond to specific extracellular signaling peptides such as atrial natriuretic peptide, guanylin, and speract (*1*−*3*). In the second group the cyclases have no known extracellular ligands and are typically regulated by intracellular calcium-binding proteins. Calmodulin-activated guanylate cyclases in Tetrahymena and Paramecium (*4*, *5*) and the vertebrate brain (*6*) were the earliest recognized members of this group. Also belonging to this group are the photoreceptor guanylate cyclases in vertebrate retina. Until recently the photoreceptor cyclases were considered to be stimulated solely in response to a decrease in calcium concentration (*7*). The stimulation is mediated by calcium-binding proteins referred to as guanylate cyclase-activating proteins (GCAPs) which dissociate from calcium at concentrations below 500 nM and bind to and stimulate the cyclase (*8*, *9*). This is an uncommon mechanism because calcium signals are usually mediated by proteins which are inactive in the calcium-free form and recognize effectors upon binding calcium. Indeed, we have recently discovered that the bovine rod outer segment guanylate cyclase (ROS-GC) is actually a dual-regulated enzyme: it is stimulated by GCAP at nanomolar calcium concentrations and by another class of proteins at micromolar (above 0.25 μM) calcium concentrations. Two proteins of the latter class were recently identified. One is a novel protein isolated from bovine retina and referred to as calcium-dependent guanylate cyclase activator protein (CD-GCAP) (*10*) and the other, the well-known S100 protein. All three forms of the S100 protein ($\alpha\alpha$ or S100a_o, $\alpha\beta$ or S100a, and $\beta\beta$ or S100b) stimulate ROS-GC, with S100b being the most potent (*11*).

In this study, we have characterized the retinal CD-GCAP by physical, molecular biological, immunological, and biochemical methods. The results show that the retinal CD-GCAP is identical to S100β in molecular weight and primary structure but stimulates cyclase more efficiently. The proteins also differed in their sensitivity to hydroxylamine,

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* Corresponding author. Tel: 248-370-2399. Fax: 248-370-2006. E-mail: ari@oakland.edu.

‡ Oakland University.

§ University of Medicine and Dentistry of New Jersey.

|| Tohoku University School of Medicine.

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a deacylating agent. This report shows that the differences between the proteins are not due to post-translational modifications but to different conformational states which are interconvertible.

MATERIALS AND METHODS

Preparation of Proteins

CD-GCAP. CD-GCAP was purified as described earlier (*10*). Briefly, 100 retinas were extracted with 50 mL of 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, and 5 mM $MgCl_2$ containing protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, and 50 μg/mL benzamidine); the extract was made 5 mM in $CaCl_2$ and 50 mM in Tris (pH 8.0) and heated for 3 min at 75 °C. After centrifugation, the supernatant was dialyzed and chromatographed on DEAE-sepharose, on phenylsepharose, and finally on an HPLC gel filtration column. Approximately 150−200 μg of pure CD-GCAP was isolated from 100 retinas. The protein was concentrated to about 2 mg of protein/mL and stored at 4 °C.

S100b. The procedure employed was a combination of the methods described by Isobe et al. (*12*) and Baudier et al. (*13*). Briefly, 100 fresh bovine retinas were homogenized in 200 mL of 0.1 M phosphate buffer, pH 7.1, 2.66 M ammonium sulfate, 1 mM EDTA, 5 mM mercaptoethanol, and protease inhibitors (as in the isolation of CD-GCAP), and the homogenate was centrifuged at 12000*g* for 30 min. To the supernatant was added ammonium sulfate to 85% saturation, and pH was adjusted to 4.2. After 20 h at 4 °C, the suspension was centrifuged, and the pellet was dissolved in 0.1 M phosphate buffer, pH 7.1, containing 1 mM EDTA and 0.05 M NaCl and dialyzed against 100 volumes of the same buffer. The material was clarified by centrifugation and chromatographed on DEAE-sepharose as described by Isobe et al. (*12*). The fractions which contained cyclase stimulatory activity were pooled and chromatographed on phenylsepharose according to Baudier et al. (*13*). The material was dialyzed against column buffer (50 mM Tris, pH 7.5, and 2 mM mercaptoethanol), clarified by centrifugation, supplemented with 1 mM $ZnSO_4$, and loaded on a 6- × 1-cm column of phenylsepharose equilibrated in column buffer containing 0.3 M NaCl and 0.25 mM $ZnSO_4$. The column was washed with 2.5 bed volumes of equilibration buffer, washed with 2.5 bed volumes of the same buffer without NaCl, and then eluted with the same buffer which contained 2 mM EDTA instead of 0.25 mM $ZnSO_4$. S100b in the eluate was concentrated by filtration through Centricon-10, washed to remove Zn and EDTA, and stored at 4 °C. Approximately 230 μg of S100b was obtained from 100 retinas. The yield from 100 g of bovine brain was about 1.6 mg. Proteins prepared by this method from either retina or brain had properties identical to those of bovine brain S100b preparations purchased from Sigma Chemical Co. (catalog #S-8390) and Calbiochem (catalog #559290).

For routine calculation of the concentrations of CD-GCAP and S100b, a molecular weight of 21 000 Da was used.

Preparation of S100β Monomer and Dimer. The proteins were prepared essentially as described by Barger et al. (*14*). Briefly, 1 mg of S100b was incubated for 21 days at −20 °C in 20 mM Tris, pH 7.5, containing 0.5 M NaCl and 3 mM Ca. The protein was then thawed and electrophoresed in Tris-tricine SDS−PAGE in the absence of reducing agents as described below. The monomer and dimer bands were excised from the gel, and the proteins were extracted into 10 volumes of 10 mM Tris, pH 7.5, by three 12-h incubations with agitation at 4 °C. The extracted proteins were concentrated on Centricon-10, washed to reduce SDS concentration, and analyzed for their ability to activate ROS-GC. Concentrations of monomer and dimer were calculated using molecular weights of 10 500 and 21 000 Da, respectively.

Photoreceptor Guanylate Cyclase. As a source of guanylate cyclase, we used dark-adapted rod outer segment membranes which were prepared as follows: Fresh bovine eyes were purchased from a local slaughter house (Wolverine Packing Co., Detroit, MI) and dark-adapted for 3 h at room temperature. Retinas were isolated and processed under infrared illumination. Intact rod outer segments were isolated according to the protocol of Schnetkamp et al. (*15*), subjected to one cycle of freezing and thawing, and washed three times in 10 mM Tris, pH 7.5, to remove soluble and peripheral proteins. Washed membranes were suspended in 5 mM isobutylmethylxanthin (IBMX) containing 5 mM dithiothreitol, aliquoted, and stored in the dark at −80 °C. In experiments where the dimer of S100b was tested, the membranes were prepared and suspended in IBMX alone without reducing agents.

Washed rod outer segment membranes were assayed for guanylate cyclase activity in the dark. Only those preparations which had no measurable cyclic GMP phosphodiesterase activity and whose cyclase activity was not influenced by 1 mM $CaCl_2$ or 2 mM EGTA were used in the experiments described here. Basal guanylate cyclase activity in washed ROS membranes varied between preparations and was usually in the range of 0.7−1.2 nmol/min/mg of protein at 37 °C. The actual activity of preparations and the assay temperature are reported in the figure legends where appropriate.

Recombinant ROS-GC and its mutants (ext$^-$, deleted extracellular domain, aa 8-409, and kin$^-$, deleted kinase-like domain, aa 447-730) were expressed in COS cells (*16*). At 60 h post-transfection, cells were washed with 50 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$ buffer, scraped into 1 mL of the buffer, homogenized, centrifuged at 5000*g*, and washed three times with the same buffer. The pellets reresented the crude membranes.

Guanylate Cyclase Assay. Cyclase activity of washed ROS membranes was measured under infrared light. The assay mixture contained 40 mM Hepes, pH 7.4, 1.0 mM GTP, 6 μCi of [α-^{32}P]GTP, 2 mM cyclic GMP, 0.2 μCi of [^{3}H]cyclic GMP, 15 mM $MgCl_2$, 1.25 mM IBMX, 10 mM dithiothreitol, and the desired concentration of calcium. Usually 20 μg of washed ROS protein was used per assay. The reactions were carried out at 37 °C (25 °C) for 10 min, and the cyclic GMP formed was measured by thin layer chromatography as described earlier (*17*).

In experiments with recombinant ROS-GC and its deletion mutants, guanylate cyclase activity was assayed in crude membranes of COS cells exposed to various concentrations of CD-GCAP or S100b, and the cyclic GMP formed was measured by radioimmunoassay as described earlier (*16*).

To measure the stimulation of cyclase, ROS-GC activity in washed ROS membranes was measured in the absence (control) and presence of the activator fraction in reaction mixtures containing the desired free calcium concentration. In routine assays where a protein fraction was tested for

stimulatory activity, calcium concentration in the assays was 1 mM. To measure calcium dependence of the stimulation, assays were conducted at 25 °C for 20 min and dibromo-BAPTA-buffered calcium solutions were used. Free calcium concentrations were calculated using Maxchelator (*18*) and verified by Corning calcium electrode.

SDS-Polyacrylamide Gel Electrophoresis (SDS−PAGE). Proteins were electrophoresed in Tris-tricine 16.5% polyacrylamide gels using the procedure of Schagger and von Jagow (*19*). Electrophoresis was stopped when the marker dye was within 1 cm of the anodic end of the gel. Protein bands were visualized by Coomassie blue staining.

Nondenaturing Gel Electrophoresis. Proteins were electrophoresed in 10−20% gradient polyacrylamide gels (*20*) in the absence of SDS and stained with Coomassie blue.

Western Blotting. S100 proteins and CD-GCAP were subjected to SDS−PAGE in the absence of reducing agent, transferred to nitrocellulose membranes, fixed according to Van Eldik and Wolchok (*21*), blocked with 5% dry milk in TTBS (0.5 M NaCl in 20 mM Tris, pH 7.5, and 0.05% Tween-20), and incubated for 60 min at room temperature with primary antibody diluted 500-fold in 1% bovine serum albumin dissolved in TTBS. After washing, the nitrocellulose membrane was incubated for 30 min with biotinylated secondary antibody followed by incubation for 30 min with avidin−horseradish peroxidase complex. Blots were developed with 4-chloro-1-naphthol.

Cloning of CD-GCAP. A cDNA library from bovine retina was constructed as described in Goraczniak et al. (*22*). The library was screened with a partially degenerated oligonucleotide probe, 5′-GA(AG) GAA ATC AAA GAG CA(AG) GA(AG) GT-3′, designed based on the amino acid sequence, EEIKEQE, of CD-GCAP (and S100β). Approximately 2.1×10^5 bacterial colonies were screened by hybridization to the ^{32}P-labeled oligonucleotide. The hybridization was performed at 50 °C by using 1×10^6 cpm of labeled oligonucleotide/mL in 3 × SSC (SSC: 0.15 M NaCl and 15 mM sodium citrate)/5 × Denhardt's solution containing 0.1% SDS and 0.1 mg/mL denatured salmon sperm DNA. The stringency washing was done in SSC at 37 °C for 1 h. The full length cDNA was isolated and sequenced by using Sequenase 2.0 (United States Biochemicals) according to the manufacturer's protocol. Nucleic acid and amino acid sequences were analyzed with DNASTAR software (DNASTAR, Madison, WI).

NH_2OH Treatment of CD-GCAP and S100b. The proteins were incubated at 30 °C in freshly prepared 1 M NH_2OH, pH 7.9. After a desired period of incubation, hydroxylamine in the preparations was removed by dilution and filtration using Centricon-3, and the proteins were assayed for their ability to stimulate ROS-GC.

Protein Chemistry. Electron spray mass spectrometric analysis of CD-GCAP and S100β was done by Princeton Biomolecules, Columbus, OH. Tryptic digestion of CD-GCAP, purification of tryptic fragments, and sequencing of fragments were done at the Wistar Protein Microsequencing Facility, Philadelphia, PA (director: Dr. David W. Speicher).

Materials. [α-^{32}P]GTP and [8-^{3}H]cyclic GMP were purchased from ICN Biomedicals. S100a$_o$ and S100a proteins and anti-S100α and anti-S100β monoclonal antibodies were from Sigma. S100b protein was from Calbiochem. Commercially obtained S100 proteins were washed in Centricon-10 with 50 mM Tris, pH 7.5, to remove traces of EDTA. Biotinylated anti-mouse IgG and avidin−peroxidase were from Vector Laboratories. Dibromo-BAPTA was purchased from Molecular Probes. Other reagents were from Sigma.

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 61 D S D G D G E C D F Q E F M A F V A M I

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FIGURE 1: Primary structure of CD-GCAP fragments compared to S100β. The amino terminus of CD-GCAP was found to be blocked. The protein was digested with trypsin, the tryptic fragments were separated by C-18 reverse-phase chromatography, and two of them were sequenced. These sequences, shown in bold, were identical to the corresponding ones in S100β. Treatment of CD-GCAP with hydroxylamine produced fragments, some of which were also sequenced. These, shown in italics, were also identical to the corresponding sequences in S100β. The regions encompassing the calcium-binding loops in the protein are underlined.

RESULTS AND DISCUSSION

Calcium-dependent guanylate cyclase activator protein was isolated from bovine retina and shown to stimulate the activity of ROS-GC both in the native membranes as well as in the membranes of COS cells in which its cDNA clone was expressed (*10*). The first indication that CD-GCAP might be related to S100 proteins came from the observation that it appeared as a 6−7-kDa calcium-binding protein on SDS−PAGE (*10*). S100 proteins are known to run anomalously as 6−7-kDa proteins in SDS−PAGE though their subunit molecular weight, calculated from amino acid sequence, is about 10.5 kDa (*23*). Further evidence for the similarity between CD-GCAP and S100 proteins emerged from mass spectrometric analysis which showed that the molecular masses of retinal CD-GCAP subunit and brain S100β were 10 580 and 10 582 Da, respectively, suggesting that the two proteins were identical. Analysis of CD-GCAP for amino acid sequence revealed that the N-terminus was blocked, as in the case of S100β (*24*). The protein was digested with trypsin, and two tryptic peptides were isolated and partially sequenced. These sequences, shown in bold in Figure 1, were found to have identical matches in the amino acid sequence of bovine brain S100β: one peptide had a sequence identical to the A6−Y17 region of S100β and the other with the E34−E67 region (Figure 1). Considering that the two sequenced peptides constituted 50% of the length of S100β and that no primary structure differences were found between S100β proteins in different tissues of the same species (*25, 26*), the complete identity of the two fragments of bovine retinal CD-GCAP with bovine brain S100β suggested that the two proteins were either identical or very closely related in primary structure. As shown later, it was found that hydroxylamine destroyed the activity of

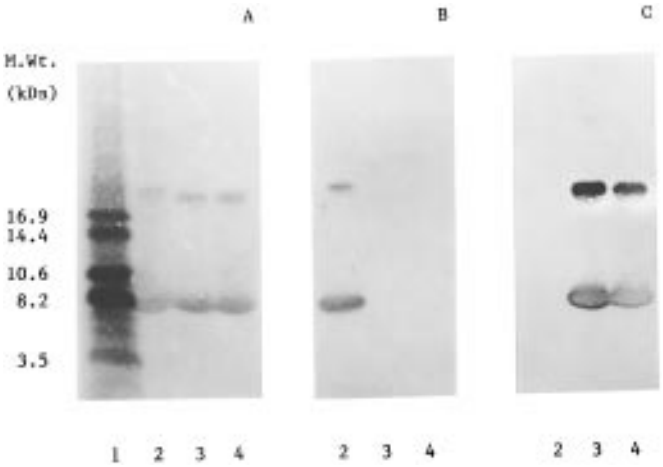

FIGURE 2: Immunoreactivity of CD-GCAP. Proteins were electrophoresed in the absence of reducing agents and transferred to nitrocellulose membrane. One set of lanes was stained with Amidoblack (A), one was tested with antibodies against S100α (B), and one was tested with antibodies against S100β (C). Lanes: 1, molecular weight markers; 2, S100a_o (αα); 3, S100b ($\beta\beta$); 4, CD-GCAP.

CD-GCAP. Hydroxylamine treatment also broke the protein into several fragments. Amino acid sequences of three of them, KLKKSEL, DFQUFMA, and HEFFHE, shown in italics in Figure 1, provided additional evidence for the identity of the primary sequence of CD-GCAP with that of S100β.

Cloning Studies. Duda et al. (*16*) amplified the bovine retinal cDNA using primers corresponding to amino acids 8−15 from the N-terminal region and amino acids 67−74 in the C-terminal region of bovine brain S100β. They purified and sequenced the amplified fragment and found that the deduced amino acid sequence matched that of bovine brain S100β suggesting that brain and retinal proteins were identical. That still left out the possibility that there could be differences between retinal and brain proteins in the regions corresponding to amino acids 1−7 and 75−91. Peptide sequencing already showed that amino acids 6, 7, 75, and 85−91 were identical with those of S100β thus leaving the regions 1−5 and 76−84 to be investigated. Using a partially degenerated oligonucleotide probe constructed based on the amino acid sequence of the peptide EEIKEQE found in both S100β and CD-GCAP, we isolated a single cDNA clone of 0.7 kb from the bovine retinal cDNA library. The amino acid sequence deduced from the nucleotide sequence of this cDNA was identical to that chemically determined by Isobe et al. (*24*). The lack of multiple clones in the retinal library with variant sequences suggested that CD-GCAP and S100β are unlikely to be coded for by different genes.

Immunoreactivity of CD-GCAP with Anti-S100 Antibodies. Monoclonal antibodies specific to S100α and S100β were tested for cross-reactivity with CD-GCAP. As shown in Figure 2, CD-GCAP reacted with S100β antibodies but not with those for S100α. Two immunoreactive bands were seen for both S100β and CD-GCAP. Of the two, the lower band corresponded to the 6−7-kDa monomer and the upper band to a 21-kDa protein, apparently a dimer of the 6−7-kDa band. The amount of dimer was drastically reduced when electrophoresis was done in the presence of reducing agents (data not shown). The reaction of CD-GCAP with the antibodies of S100β but not of S100α was in agreement with the protein's primary structure.

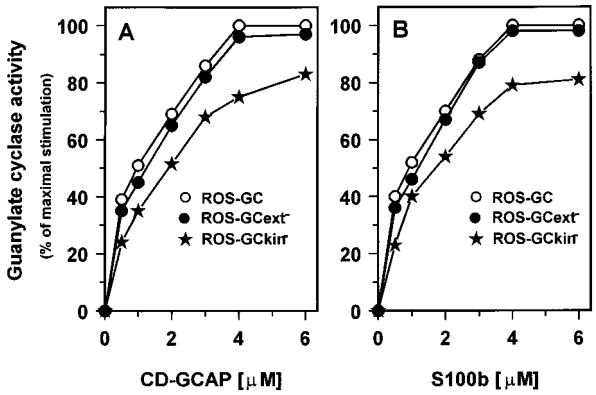

FIGURE 3: Identification of cyclase domain involved in activation by CD-GCAP and S100b. Wild-type and ext⁻ and kin⁻ deletion mutants of ROS-GC were incubated with incremental concentrations of CD-GCAP or S100b, and the cyclase activity was measured as described in Materials and Methods. For each activator, the maximum activity obtained with the wild-type cyclase was treated as 100% stimulation.

Cyclase Domain Involved in Stimulation by CD-GCAP and S100b. Duda et al. (*16*) reported that while GCAP stimulates ROS-GC by interacting with the kinase-like domain of the enzyme, S100a activates by interacting with the C-terminal domain. Deletion of extracellular or kinase-like domain did not substantially affect activation by S100a suggesting that the other domain—C-terminal to the kinase-like domain—is involved in the activation. In the present study, we tested the ability of CD-GCAP and S100b to activate wild-type and ext⁻ and kin⁻ deletion mutants of ROS-GC. Figure 3 shows that both proteins were essentially similar in activating cyclase and its mutants, that they stimulate cyclase in the same manner as S100a, and that the domain of interaction on cyclase, by inference, is in the C-terminal end.

Calcium Sensitivity and Amplitude of Cyclase Activation. In spite of the similarities in structural properties noted above, CD-GCAP and S100b differed prominently in functional properties. As shown in Figure 4, CD-GCAP stimulation of ROS-GC was half-maximal at 1.55 μM calcium concentration (1.5 and 1.6 μM in two experiments), while the stimulation by S100b was half-maximal at 32 μM (31 and 33 μM in two experiments). Also the maximum activation achieved by CD-GCAP was about 2-fold higher than that by S100b.

Effect of Hydroxylamine on the Activity of CD-GCAP. We speculated that post-translational modifications such as attachment of acyl groups could have rendered CD-GCAP more active and more calcium-sensitive than S100b. In order to test this possibility, the effects of removing acyl groups on the activity of CD-GCAP were investigated. Acyl groups attached to proteins via thioester bonds are known to be labile to hydroxylamine (*27*, *28*). We therefore tested the effect of incubation with hydroxylamine on the activities of CD-GCAP and S100b. Figure 5 shows that S100b was unaffected by the treatment, but CD-GCAP was labile losing about 96% of its activity in 1 h. If CD-GCAP was derived from S100b via a post-translational modification, then hydroxylamine should have reduced its activity to that of S100b and not completely eliminated it. It therefore appeared that hydroxylamine-labile post-translational modifica-

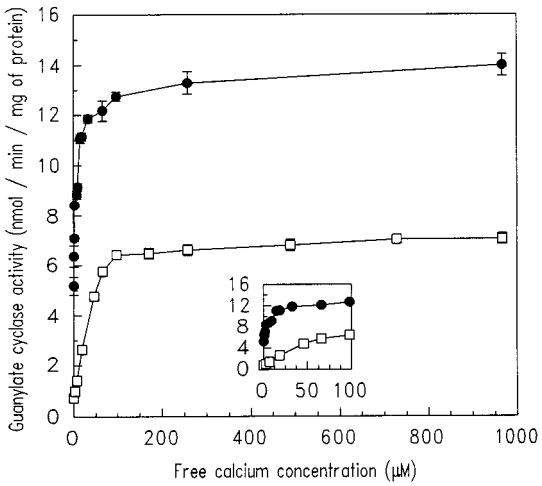

FIGURE 4: Calcium dependence of CD-GCAP and S100b in stimulating ROS-GC. Guanylate cyclase activity in washed ROS membranes was measured at 25 °C in the presence of saturating concentrations of CD-GCAP (12 μM) or S100b (20 μM) at the specified concentration of free calcium. The measurements were done in triplicate, each point represents the mean, and the error bars indicate the SD. Basal guanylate cyclase activity was 0.74 nmol/min/mg of protein. Insert shows data at 0−100 μM calcium with an expanded scale. Two experiments were done with similar results: (●) CD-GCAP, (□) S100b.

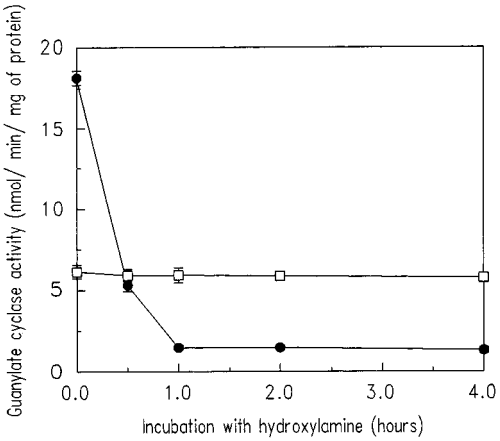

FIGURE 5: Influence of hydroxylamine on the cyclase-stimulating ability of CD-GCAP and S100b. Twenty-eight micrograms each of CD-GCAP and S100b in 50 μL were mixed with an equal volume of 2 M hydroxylamine, pH 7. 9, and incubated at 30 °C for a desired length of time. The samples were diluted 25-fold with water and filtered through Centricon-3. The concentrated samples were assayed for stimulation of cyclase activity in washed ROS membranes in the presence of 1 mM $CaCl_2$. The measurements were done in triplicate, each point represents the mean, and the error bars indicate the SD. Control experiments in which the proteins were incubated in 50 mM Tris, pH 7.9 instead of hydroxylamine under identical experimental conditions showed no significant decrease in activity. Basal guanylate cyclase activity in the washed membrane preparation used in this experiment was 0.78 nmol/min/mg of protein; (□) S100b, (●) CD-GCAP. Similar results were obtained in two independent experiments.

tion could not have been responsible for the higher activity and higher calcium sensitivity of CD-GCAP. This is also in agreement with the mass spectrometric data which showed no major difference between the molecular masses of S100β and CD-GCAP.

Figure 6 shows nondenaturing PAGE of hydroxylamine-treated and control preparations of CD-GCAP and S100b. It shows that S100b was unaffected by hydroxylamine, corroborating the activity measurements. It also shows that

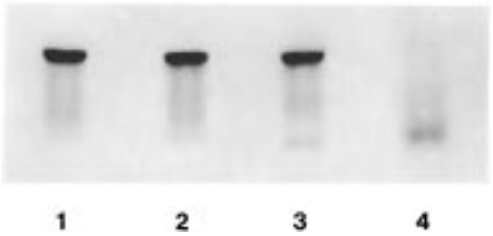

FIGURE 6: Influence of hydroxylamine on CD-GCAP and S100b. Preparations of the two proteins treated with hydroxylamine for 4 h as described in the legend to Figure 5 were electrophoresed under nondenaturing conditions and stained with Coomassie blue. Lanes: 1, control S100b; 2, treated S100b; 3, control CD-GCAP; 4, treated CD-GCAP.

CD-GCAP was degraded by hydroxylamine, accounting for the loss of activity. Amino acid sequence analysis of three peptides from the treated sample extracted from the gel showed that they had identical matches in S100β (italicized sequences in Figure 1). Taken together, these results suggested that the differential sensitivity of the two proteins to hydroxylamine is more likely to be due to conformational differences rather than to primary structure differences or post-translational modifications.

Convertibility of CD-GCAP to S100b and S100b to CD-GCAP. S100b preparations obtained commercially or freshly isolated from either brain or retina were identical in their ability to stimulate cyclase and insensitivity to hydroxylamine, and it suggested the possibility that the method employed in their purification might result in a conformation that distinguished them from CD-GCAP. The purification of S100b involved incubation with 1 mM zinc and zinc affinity chromatography on phenylsepharose (*13*). While the commercial sources would not disclose the method employed in purification, their citations include Baudier et al., (*13*). Purification through zinc affinity chromatography could have left the protein with tightly bound zinc resulting in a conformation less effective than CD-GCAP in activating cyclase and at the same time protecting it from hydroxylamine. Simple filtration and washing of S100b on Centricon membranes did not render S100b identical to CD-GCAP in activity and hydroxylamine sensitivity. It was therefore likely that tightly bound zinc was not easily removed by filtration. This is consistent with the report (*29*) that zinc binds S100b with high affinity (k_d in the range of 10^{-8}−10^{-7} M, compared to 10^{-5} M for Ca). Purification of CD-GCAP on the other hand involved heating the preparation for 3 min at 75 °C in the presence of 5 mM Ca and 5 mM Mg.

In order to test the influence of the purification protocols on the calcium sensitivity, amplitude of cyclase activation, and hydroxylamine sensitivity, purified CD-GCAP was incubated for 24 h with 1 mM zinc in 50 mM Tris-HCl, pH 6.8, 50 mM NaCl, and 10 mM 2-mercaptoethanol and subjected to zinc affinity chromatography on phenylsepharose as described by Baudier et al. (*13*). The purified protein is referred to as CD-GCAP→S100b. Purified S100b was heated for 3 min at 75 °C in 5 mM Mg and 5 mM Ca in 7 mg/mL BSA, 10 mM DTT, and 50 mM Tris-HCl, pH 8.0, dialyzed against 10 mM Tris-HCl, pH 8.0, concentrated, and purified by gel filtration on HPLC. The purified protein is referred to as S100b→CD-GCAP. All four proteins were changed on Centricon-10 into 50 mM Tris-HCl, 6.8, containing 50 mM NaCl and 10 mM 2-mercaptoethanol and tested for functional activities.

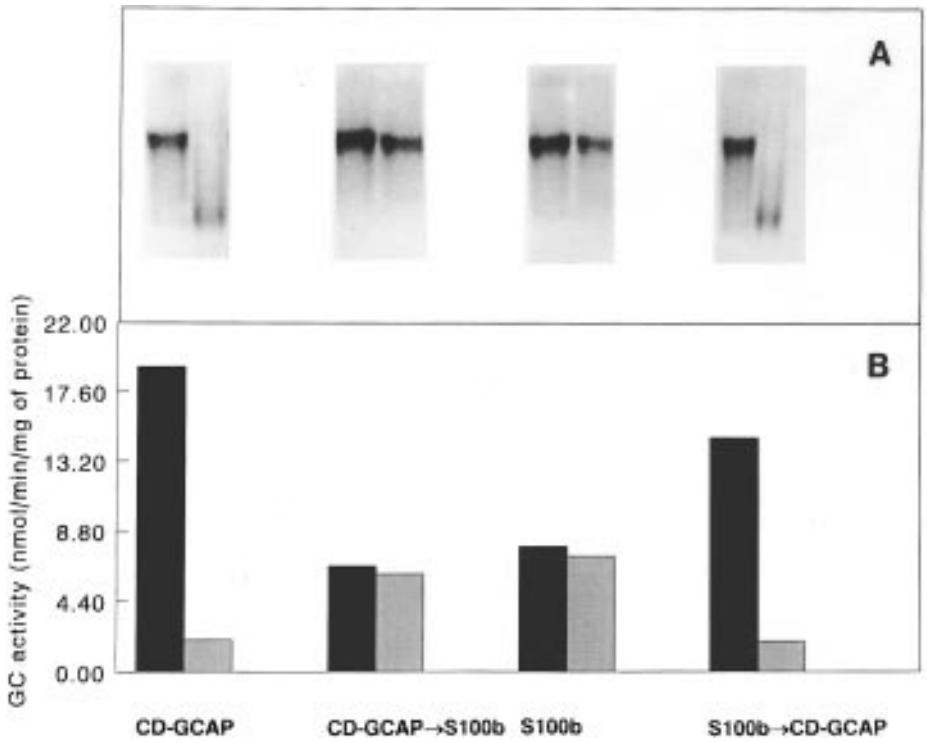

FIGURE 7: Effect of hydroxylamine on the stability (A) and activity (B) of CD-GCAP→S100b and S100b→CD-GCAP. CD-GCAP and CD-GCAP→S100b prepared from it and S100b and S100b→CD-GCAP prepared from it were treated with hydroxylamine as in Figure 6. The samples were electrophoresed under nondenaturing conditions and stained with Coomassie blue (A) and also assayed for cyclase-stimulating activity (B). In each pair in A and B, the first lane (or bar) is the control and the second lane (or bar) contains the hydroxylamine-treated sample. Basal activity of cyclase in the absence of activator preparations was 0.85 nmol/min/mg of protein.

Figure 7A shows the effect of hydroxylamine treatment on CD-GCAP, CD-GCAP→S100b, S100b, and S100b→CD-GCAP. The treatment broke down CD-GCAP and S100b→CD-GCAP but had no effect on S100b and CD-GCAP→S100b. This strongly suggested that the CD-GCAP and S100b conformations were interchangeable and that the zinc-bound conformation was resistant to hydrolysis by hydroxylamine.

Figure 7B shows the ability of the four preparations, all at 9 μM, to stimulate ROS-GC activity. Hydroxylamine almost totally abolished the activity of CD-GCAP and S100b→CD-GCAP but had no effect on S100b and CD-GCAP→S100b. These results corroborated the electrophoretic results in Figure 7A. In addition, the conversion of S100b to CD-GCAP resulted in nearly doubling its ability to activate cyclase. It might be noted here that the higher activity of CD-GCAP was one of the reasons for originally considering that it was different from S100b.

We then tested the effect of the conversion of S100b to CD-GCAP on calcium sensitivity in stimulating cyclase activity. From Figure 4 it is seen that at about 2 μM Ca concentration CD-GCAP stimulated cyclase activity half-maximally while S100b was nearly inactive. Table 1 shows a comparison of the calcium dependence of S100b and S100b→CD-GCAP prepared from it. While S100 had little or no effect on cyclase activity at 0.5 μM Ca, S100b→CD-GCAP increased it by 4-fold. At 2.7 μM Ca, S100b→CD-GCAP increased cyclase activity by nearly 8-fold while S100b increased it by about 60%. These results show that conversion of S100b to CD-GCAP increased its calcium sensitivity.

The above results demonstrated the interchangeability of hydroxylamine-sensitive (CD-GCAP) and -insensitive (S100b) conformations and also conclusively proved the identity of CD-GCAP with S100b. We suggest that zinc binding is what protects S100b from hydroxylamine. Heat treatment of the protein in Ca during the purification of CD-GCAP likely removes zinc and renders it hydroxylamine-sensitive. This hypothesis is also supported by another observation: Baudier and Cole (*30*) found that zinc protects cys84 from the hydrophilic solvent while calcium does not. It agrees with our finding that cys84 was one of the sites at which hydroxylamine broke CD-GCAP (Figure 1). It was not affected in S100b.

Table 1: Calcium Dependence of S100b and S100b→CD-GCAP[a]

free calcium conctn (μM)	GC activity (nmol/min/mg of protein) in the presence of 15 μg of	
	S100b	S100b→CD-GCAP
0.46	0.5	2.2
2.70	0.8	4.1

[a] Basal activity of GC in the absence of activators was 0.5 nmol/min/mg of protein and was unaffected by calcium concentrations in the range tested. Assays were done at 25 °C.

In addition, the sequences of two of the breakdown products after hydroxylamine treatment show that they came from the calcium-binding pockets of the protein, explaining the destruction of the calcium-dependent cyclase stimulatory activity of the protein by hydroxylamine.

The above results also suggest that zinc can down-regulate the CD-GCAP activation of ROS-GC. Whether such a mechanism of reducing cyclase activation has a physiological significance is unknown. It is also to be determined whether other activities in retina are influenced by S100b such as phosphorylation of proteins (*31, 32*) and whether zinc attenuates such influence.

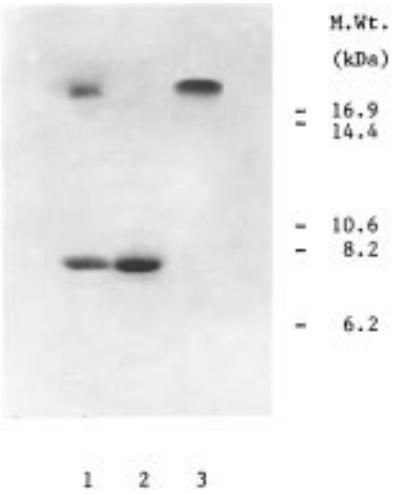

FIGURE 8: Preparation of S100β monomer and dimer. Monomer and dimer preparations were isolated as described in the Materials and Methods, electrophoresed in the absence of reducing agents, and stained with Coomassie blue. Lanes: 1, a mixed monomer/dimer preparation; 2, monomer; 3, dimer. The position of molecular weight standards is shown at the right.

Table 2: Ability of S100β Monomer and Dimer To Stimulate Cyclase Activity

activator conctn (μM)	GC activity (nmol/min/mg of protein)	
	monomer	dimer
none (control)	0.83	0.83
2	0.91	0.85
4	2.30	1.40
17	4.89	2.13
34	5.11	

S100b Dimer Is Less Active than Monomer in Stimulating Guanylate Cyclase. There was however a possibility that CD-GCAP is a better activator of cyclase than S100b, not because of a different conformation but because it contained a greater fraction of dimer. Dimer, but not monomer, of S100b was earlier shown to have neurite extension activity (*23*) and glial cell mitogenic activity (*33*). In our preparations of S100b and CD-GCAP the amount of dimer was usually negligible, amounting to less than 10% of the total protein even in preparations stored in the absence of reducing agents and electrophoresed under similar conditions (Figure 2). But the treatment of the S100b preparation with 5 mM Ca at 75 °C in converting it to CD-GCAP could have increased the dimer concentration contributing to greater activity of the preparation. In order to test this possibility, monomers and dimers of S100β were prepared (Figure 8) and tested separately in assay mixtures devoid of reducing agents. The results shown in Table 2 demonstrate that the dimer was about 50% less active than monomer in stimulating cyclase activity. Therefore a higher fraction of dimer in the preparations of CD-GCAP could not have accounted for its higher activity as compared to that of S100b.

The above results point to a difference between the extracellular and intracellular functions of S100b. The dimeric, oxidized form is preferred as the extracellular regulator of neurite growth and glial mitogenicity (*23, 33*), while the reduced monomeric form is preferred as the intracellular regulator of guanylate cyclase. It should however be noted that the protein which appeared as a monomer after electrophoresis (Figure 8), performed at high ionic strength (*19*), may actually exist as a noncovalently self-associated dimer or tetramer at lower ionic strength even under fully reducing conditions (*10, 34*−*36*). The nature of self-association is presently unknown (*35*). If we consider these associations, the molar concentrations of the monomer used in our experiments would be lower than what we calculated and would reinforce our conclusion that the monomer/noncovalently associated form appears to be more effective in stimulating cyclase than the disulfide-bound dimer.

CONCLUSIONS

S100b is one of the calcium-binding proteins originally isolated from brain. CD-GCAP is a calcium-binding protein isolated from bovine retina and shown to stimulate photoreceptor membrane guanylate cyclase. Structural, physical, immunological, and molecular biological evidence suggested that CD-GCAP is identical with S100b. The prominent differences between the proteins—in their level of activation of cyclase and hydroxylamine sensitivity—were found to be interchangeable and dependent upon their conformations.

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