Molecular Characterization of S100A1-S100B Protein in Retina and Its Activation Mechanism of Bovine Photoreceptor Guanylate Cyclase†

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Received January 2, 1996; Revised Manuscript Received February 21, 1996⊗

ABSTRACT: In contrast to the membrane guanylate cyclases which are stimulated by extracellular ligands, rod outer segment membrane guanylate cyclase (ROS-GC) activity is modulated intracellularly by calcium in two ways: one, where it is inhibited, and the other, where it is stimulated. The former way is linked to the phototransduction, and physiology of the second is unknown. In both ways calcium modulation of the cyclase occurs through the calcium binding proteins: through guanylate cyclase activating proteins (GCAPs) in the case of phototransduction, and through the recently discovered calcium-dependent GCAP (CD-GCAP) in the case of the other way. The kinase-like domain of ROS-GC is critical for the phototransduction-linked process. The present study shows the expression of α and β chains of S100A1-S100B protein in the bovine retina and demonstrates that this protein stimulates ROS-GC activity in a dose-dependent fashion, that the stimulation is calcium dependent with an EC_{50} of 17 μM, and that the kinase-like domain is not involved in the calcium-modulated cyclase activation. Instead the involved domain resides at the C-terminal segment, between amino acids 731 and 1054. Thus, this S100A1-S100B protein-mediated calcium-modulated signal transduction mechanism is novel. Furthermore, this study provides the molecular understanding of the two transduction processes mediated by the same ROS-GC, one linked to the low and the other to the high calcium levels.

The recent discovery of a calcium binding protein, termed calcium-dependent guanylate cyclase activating protein (CD-GCAP) (Pozdnyakov et al., 1995), has demonstrated that the rod outer segment membrane guanylate cyclase (ROS-GC) may not be linked only to the calcium-modulated process of phototransduction but also to the other unknown biological processes. In the case of phototransduction, the linkage of ROS-GC with calcium is through guanylate cyclase activating proteins (GCAPs), GCAP1 (Gorczyca et al., 1994; Palczewski et al., 1994) and GCAP2 (Dizhoor et al., 1994, 1995). These proteins, at low (below ∼100−200 nM) free calcium, stimulate ROS-GC activity and, at higher concentrations, inhibit it. A recent preliminary report indicates that the most critical ROS-GC-modulated site is its intracellular kinase like domain (Duda et al., 1996). In contrast to GCAPs, CD-GCAP at high (micromolar) calcium concentrations stimulates ROS-GC activity (Pozdnyakov et al., 1995). This indicates that ROS-GC is able to transduce more than one calcium-modulated intracellular signal, one involving stimulation and the other inhibition of the cyclase activity.

The present study supports this concept by showing the presence of S100A1-S100B protein in retina and by demonstrating that these proteins stimulate ROS-GC activity at high calcium levels, and that this mechanism of activation is distinct from that linked with phototransduction and may occur in certain neural cells other than ROS.

EXPERIMENTAL PROCEDURES

Materials. S100A1-S100B protein was purchased from Calbiochem. The purity of this protein authenticated by the vendor was in excess of 99.5%; SDS−PAGE revealed only one protein-stained band. The lyophilized protein (1 mg) was reconstituted in 2 mL of 50 mM Tris-HCl, pH 7.0/50 mM NaCl. The solution was concentrated in Centricon-10 to ∼0.5 mL, diluted to 2 mL with the above buffer, and concentrated again. After three additional concentration/dilution cycles, the final buffer volume was adjusted to 0.6 mL. Anti S100A1 and anti S100B monoclonal antibodies, calmodulin, and troponin C were purchased from Sigma, cell culture media and Superscript reverse transcriptase were from GIBCO-BRL, Taq polymerase and Fmol sequencing kit were from Promega Corp., and [α-^{32}P]dATP, [γ-^{32}P]ATP, and [^{125}I]NaI were from ICN.

Reverse Transcriptase PCR (RT-PCR) Amplification. (A) cDNA Synthesis. Twenty micrograms of total RNA (DNase-treated) isolated from bovine retina was employed individually for one reverse transcriptase reaction in a mixture containing 1 μM random 9-mers (Stratagene), 5 units/μL Superscript reverse transcriptase, 0.125 mM each of dNTPs, 0.5 unit/μL RNasin (Promega), 25 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9 at 25 °C), and 0.1% Triton X-100. The reaction mixture was incubated at 37 °C for 45 min, and the reaction was stopped by heating at 70 °C for 10 min.

(B) PCR Amplification of the Transcripts for the α and β Chain of the S100A1-S100B Protein. The 149 bp fragment of S100A1-S100B α chain was amplified from cDNA (*vide supra*) using the following primers: 5′-GGCAAGG-GAAGGGGA(CT)AA(AG)TA(CT)AA-3′ (nucleotides 31−

† This study was supported by USPHS Awards EY 10828 and NS 23744.

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0006-2960(96)00007-4 CCC: $12.00

54; Figure 1: primer S1α) and 5′-ACTTCCCCATCTCC-(AG)TT(CT)TC(AG)TC-3′ (complementary to nucleotides 137−179; Figure 1: primer S2α) (Zimmer et al., 1991). The 200 bp β chain fragment of S100A1-S100B was amplified using primers 5′-GTTGCCCTCATTGA(TC)GC(ACGT)TT-(CT)CA-3′ (nucleotides 145−167; Figure 1: primer S1β) and 5′-ATAAACTCCTGGAA(AG)TC(AG)CA(CT)TC-3′ (complementary to nucleotides 322−344; Figure 1: primer S2β) (Kuwano et al., 1986). The amplification was performed in a reaction mixture of 50 mM KCl, 10 mM Tris-HCl (pH 9 at 25 °C), 2 mM $MgCl_2$, 0.2 mM each of dNTP, 1 μM of each primer, and 2.5 units of Taq DNA polymerase, with a thermal profile as follows: 1 min at 94 °C, 1 min at 53 °C, and 1 min at 72 °C for 35 cycles. Amplified products (one-fifth aliquot of the reaction mixtures) were electrophoresed on 2% agarose gels containing 0.5 μg/mL of ethidium bromide. The proper bands were excised from the gels, and the cDNAs were individually purified and sequenced using the Fmol sequencing kit (Promega) according to the manufacturer's protocol. The sequencing and amplification primers were the same, except the sequencing primers were ^{32}P-end-labeled.

Construction of ROS-GC Deletion and Hybrid Mutants. For creation of ROS-GCext$^-$, two *Hpa*1 restriction sites were created by oligonucleotide-directed mutagenesis at nucleotide positions 241 and 1446 (Goraczniak et al., 1994) in ROS-GC cDNA subcloned into pAlter1 vector (Promega Corp. mutagenesis kit). The 1.2 kb *Hpa*1 fragment was excised and the remaining part was religated, resulting in deletion of the predicted extracellular domain, amino acid residues 8−409. For ROS-GCkin$^-$, two *Hpa*1 sites were generated at positions 1557 and 2409, the 852 bp fragment was excised with *Hpa*1, and the remainder was religated, resulting in deletion of amino acids 447−730. To construct the hybrid mutant consisting of atrial natriuretic factor receptor guanylate cyclase (ANF-RGC) (amino acids 1−745) and ROS-GC (amino acids 731−1054), an *Hpa*1 restriction site was introduced into ANF-RGC cDNA at nucleotide position 2638 and another *Hpa*1 site into ROS-GC at position 2409. The mutated ANF-RGC cDNA was subcloned into pBluescript and the mutated ROS-GC cDNA into pcDNA3. The 2.4 kb *Kpn*1/*Hpa*1 fragment of ROS-GC was replaced by the corresponding fragment of ANF-RGC cDNA. All the constructs were sequenced to confirm their identities and correct ligations. The mutant cDNAs were finally cloned into the *Kpn*1/*Xba*1 sites of the pcDNA3 vector for COS cell expression.

Expression of ROS-GC and Mutants. COS-7 cells [simian virus 40 (SV40)-transformed African Green Monkey kidney cells] maintained in Dulbecco's modified Eagle's medium with penicillin/streptomycin and supplemented with 10% fetal bovine serum (Sigma) were transfected with appropriate expression constructs using calcium phosphate coprecipitation technique (Sambrook et al., 1989). At 60 h posttransfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ buffer, scraped into 1 mL of the same buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed three times with the same buffer. The pellet represented the crude membranes.

Guanylate Cyclase Assay. The crude membranes were assayed for guanylate cyclase activity (Paul et al., 1987). Briefly, membranes were preincubated on ice bath with or without S100A1-S100B in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, 50 mM Tris-HCl, pH 7.5, 0.4 mM EGTA, and varying concentrations of Ca^{2+}. The concentrations of free calcium at every data point were directly measured using Calcium Green-5N fluorescent probe (Molecular Probes Inc.: Calcium Calibration Kit 3, 1 μM to 1 mM). Total assay volume was 40 μL. The reaction was initiated by the addition of the substrate solution containing 4 mM $MgCl_2$ and 1 mM GTP. Incubation (37 °C, 10 min) was terminated by the addition of 360 μL of 50 mM sodium acetate buffer, pH 6.25, followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP formed was quantified by a radioimmunoassay (Nambi et al., 1982).

RESULTS AND DISCUSSION

Molecular Expression of S100A1-S100B Protein in the Bovine Retina. S100A1-S100B protein is a heterodimer, composed of α and β subunits [reviewed in Hilt and Kligman (1991) and Schafer et al. (1995)]. To determine the expression of these subunits in the bovine retina, reverse transcription of RNA isolated from this tissue was performed. This was followed by amplification of the 149 bp fragment corresponding to the α subunit (nucleotides 31−179) and 200 bp fragment corresponding to the β subunit (nucleotides 145−344). Because the nucleotide sequences of these subunits are not known, the partially degenerated primers were designed according to the published rat sequence (Zimmer et al., 1991; Kuwano et al., 1986). Amplification of each cDNA fragment yielded a single band of the predicted size, as visualized on an agarose gel. The cDNAs from these bands were individually purified and then subjected to direct sequencing. The nucleotide sequence of each fragment was translated into the corresponding amino acid sequence and compared with that of the known bovine α and β subunit sequences (Figure 1). The sequences of the amplified fragments gave an identical match to the corresponding α and β subunit fragments, previously determined by protein sequencing (Isobe et al., 1978, 1981).

Earlier immunological studies (Kondo et al., 1984; Iwanaga et al., 1985; Michetti et al., 1985) indicated the presence of S100 in retina, and recently a calcium protein placental homolog (Capl) has been purified to apparent homogeneity from bovine retina (Polans et al., 1994). The present report, however, provides the first direct molecular evidence for the expression of S100A-S100B protein in the bovine retina and the first disclosure of the nucleotide sequence of the α and β subunit fragments in any bovine tissue.

Expression of Catalytic Cyclase Activities of ROS-GC and Its Mutants in Transfected COS Cells. For this study, ROS-GC and its mutants: ext$^-$ (deleted extracellular domain, amino acid residues 8−409), kin$^-$ (deleted kinase-like domain, amino acid residues 447−730), and hybrid (consisting of the extracellular, transmembrane and kinase-like domains of ANF-RGC and the C-terminal domain of ROS-GC) (Figure 2) were transiently expressed in COS cells. In all mutants, the leader sequence was retained to ensure proper translocation of the expressed protein. The expression results show that the basal guanylate cyclase activity of the wild-type cloned ROS-GC, deletion mutants, and of the hybrid ranged between 50−150 pmol of cyclic GMP/(min·mg of protein) and was 100−150-fold higher than control cells transfected with vector only. It is, therefore, concluded that all the cyclases are expressed to about the same levels, translocated through the membrane and correctly folded.

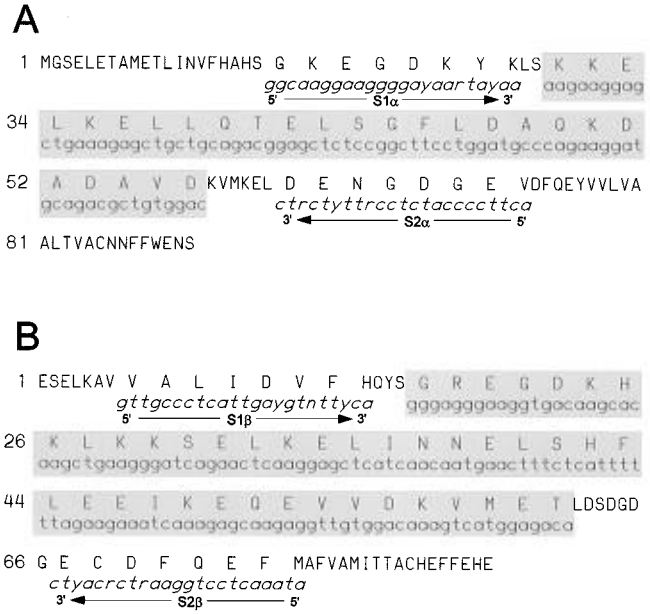

FIGURE 1: Partial nucleotide and deduced amino acid sequence of α (A) and β (B) subunit of the S100A1-S100B protein. The 149 bp fragment of the α subunit and 200 bp fragment of the β subunit were amplified from the bovine retina cDNA and sequenced as described in Experimental Procedures. Nucleotide and deduced amino acid sequences of the amplified fragments are shadowed and are aligned with the sequence of α and β subunits previously determined by protein sequencing (Isobe et al., 1978, 1981). Primers used for amplification and sequencing are denoted as S1α, S2α, S1β, and S2β, and their sequences are given in italics (y = C or T; r = A or G; n = any nucleotide).

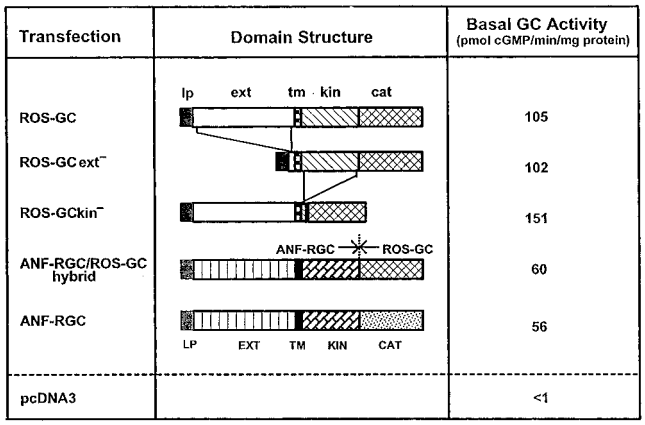

FIGURE 2: Schematic representation of ROS-GC, of deletion mutants, and of the hybrid, and their specific activities in COS particulate fractions. The predicted domains of ROS-GC are denoted by ext, extracelllular domain; tm, transmembrane domain; kin, area with tyrosine kinase seqence similarity; and cat, catalytic domain. For the hybrid, the vertical broken line depicts the divide between ANF-RGC (to the left) and ROS-GC (to the right). Domains of ANF-RGC are capitalized. The right-hand column of the figure depicts the cyclase activity of these expressed proteins as measured in crude membranes of COS cells.

Stimulation of ROS-GC Activity by S100A1-S100B Protein. In order to determine whether ROS-GC is a transducer of the S100A1-S100B signal, the cloned ROS-GC was expressed in COS cells, and the cell membranes were incubated with a series of incremental concentrations of purified S100A1-S100B protein at a fixed high (1 mM) calcium concentration. S100A1-S100B stimulated ROS-GC activity in a dose-dependent fashion with a half-maximal activation occurring at ~1.1 μM and saturation at ~4 μM (Figure 3A). The stimulatory effect of S100A1-S100B on the cyclase

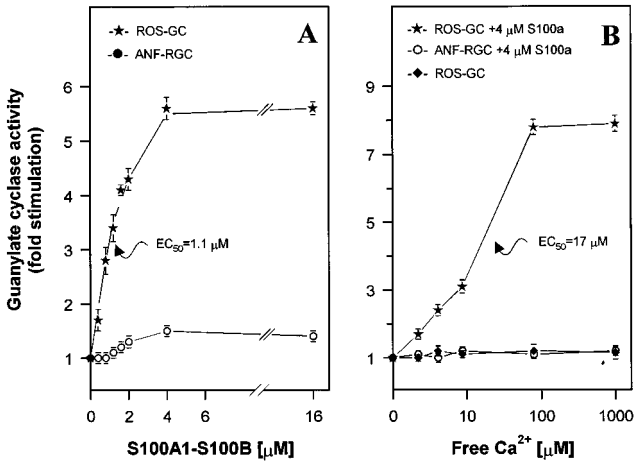

FIGURE 3: Effect of S100A1-S100B (A) and free Ca^{2+} (B) on ROS-GC activity. COS cell membranes were prepared and assayed for guanylate cyclase activity in the presence of (A) 1 mM free Ca^{2+} and S100A1-S100B, as indicated, or (B) 4 μM S100A1-S100B and free Ca^{2+}, as indicated. Each experiment was done in triplicate and repeated three times with separate membane preparations. The results shown (±SD) are from one representative experiment.

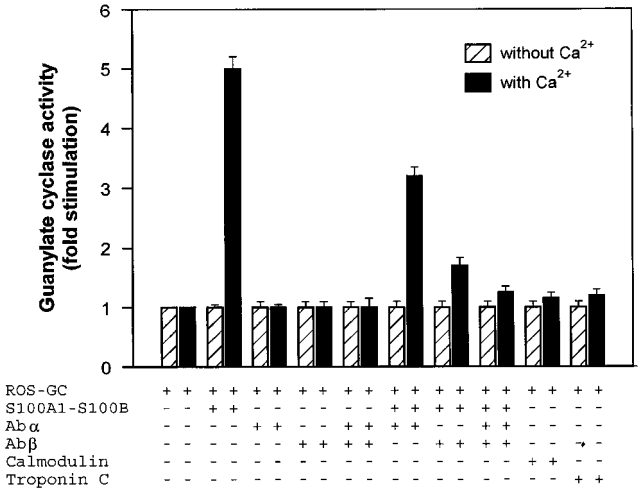

FIGURE 4: Effect of S100A1-S100B, anti S100A1 (Abα), and anti S100B (Abβ) monoclonal antibodies, calmodulin, troponin C, and Ca^{2+} on ROS-GC activity. S100A1-S100B was preincubated with or without antibody for 1 h on ice bath. COS cell membranes expressing ROS-GC were prepared as described in Experimental Procedures and assayed for guanylate cyclase activity in the presence of indicated additions. The experiment was done in triplicate, and results shown represent mean ± SD.

activity was calcium-dependent: it increased as free calcium increased with a half-maximal stimulation (EC_{50}) occurring at ~17 μM (Figure 3B). In the absence of S100A1-S100B, the cyclase did not respond to any concentration of calcium (Figure 3B). This indicated that ROS-GC activity is modulated by high calcium levels through S100A1-S100B. That the S100A1-S100B effect is specific for ROS-GC was indicated by the finding that under similar conditions this protein did not influence the ANF-RGC activity (Figure 3A) and that ANF does not influence the ROS-GC activity (Goraczniak et al., 1994), and neither calmodulin nor troponin C in the absence or presence of calcium influenced the cyclase activity (Figure 4). The specificity of S100A1-S100B was further attested by using its monoclonal antibody probes directed against S100A1 and S100B subunits. These mixed monoclonal antibodies completely blocked the ability of the protein to stimulate ROS-GC (Figure 4). Individually, S100A1 antibody blocked about 20% and S100B antibody

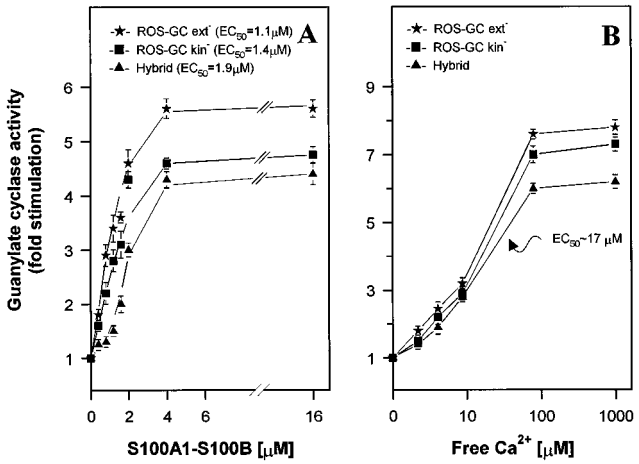

FIGURE 5: Effect of S100A1-S100B on cyclase activity of ROS-GC mutants. (A) Stimulation of the three mutants by S100A1-S100B at constant 1 mM free calcium. (B) Stimulation by incremental increase of free calcium. The assay conditions are defined in Figure 3.

blocked about 70% ROS-GC maximally stimulated cyclase activity (Figure 4). It is thus concluded that ROS-GC activity is modulated by high levels of calcium and that the modulation is specifically mediated by S100A1-S100B.

These results obtained with recombinant ROS-GC are in general agreement to those reported recently for the native bovine ROS-GC particulate fractions where S100 proteins activated the cyclase with a half-maximal value of about 35−40 μM (Margulis et al., 1996), a value approximately twice to that observed here for the ROS-GC activation.

Localization of the ROS-GC Modulated Domain. To localize the S100A1-S100B-modulated domain in ROS-GC, expression studies with three ROS-GC mutants were conducted: the ext$^-$ (deleted-extracellular domain), kin$^-$ (deleted kinase-like domain); and hybrid (extracellular, transmembrane and "kinase-like" domains of ANF-RGC and the C-terminal domain of ROS-GC) (Figure 2). These mutants were transiently expressed in COS cells, and their membrane fractions were appropriately treated and studied for their cyclase activities. At a fixed calcium concentration of 1 mM, S100A1-S100B stimulated the guanylate cyclase activity of all the mutants in a dose-dependent fashion with similar EC_{50} values: ~1.1 μM for ext$^-$, 1.4 μM for kin$^-$, and 1.9 μM for hybrid (Figure 5A). These EC_{50} values were almost identical to that observed for the wild type ROS-GC value of 1.1 μM. Similarly, the maximal achievable cyclase activity of the wild type and its mutants was observed at the same concentration of S100A1-S100B protein, i.e., ~4 μM. The maximal cyclase stimulation of the wild type and ext$^-$ were comparable, but they were about 20% lower in the kin$^-$ and hybrid mutants. Again, the patterns of S100A1-S100B-mediated cyclase stimulation of all mutants were comparable to the wild type ROS-GC (compare Figure 5 with Figure 3). In all cases, the EC_{50} value for calcium was ~17 μM. These results demonstrated that the extracellular domain of ROS-GC had no detectable role, and, similarly, the kinase-like domain had only a minimal role in the calcium-dependent S100A1-S100B-modulation of ROS-GC activity. Therefore, the cyclase-modulated domain must be present in the cyclase carboxyl region. This conclusion was supported with the studies of the hybrid whose only ROS-GC component was the C-terminal segment, amino acids 731−1054. Cyclase activity of this mutant was responsive to S100A1-S100B, the extent and pattern of stimulation was almost identical to those of the wild-type ROS-GC and its deletion mutants. It needs to be recalled that the hybrid responds to ANF (Duda et al., 1996), and that ANF-RGC does not respond to S100A1-S100B (Figure 3A). It is, therefore, concluded that the carboxylic domain consisting of amino acids 731−1054 is essential for mediating the S100A1-S100B activity. In a preliminary report, it is indicated that, in the phototransduction-linked ROS-GC activation, the kinase-like domain is most critical for the calcium-modulated cyclase activity of ROS-GC (Duda et al., 1996). This, then, indicates that the high calcium-modulated signal transduction mechanism is novel, and that ROS-GC may be linked to at least two calcium-modulated signaling pathways. Finally, for almost a decade S100 proteins had been considered as intracellular biological mediators of calcium. The present study provides a potential mechanism of their action.

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BI960007M