Structural and functional characterization of the rod outer segment membrane guanylate cyclase

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In the vertebrate photoreceptor cell, rod outer segment (ROS) is the site of visual signal-transduction process, and a pivotal molecule that regulates this process is cyclic GMP. Cyclic GMP controls the cationic conductance into the ROS, and light causes a decrease in the conductance by activating hydrolysis of the cyclic nucleotide. The identity of the guanylate cyclase (ROS-GC) that synthesizes this pool of cyclic GMP is unknown. We now report the cloning, expression and functional characterization of a DNA from bovine retina that encodes ROS-GC.

INTRODUCTION

In vertebrate retina, photoreceptor outer segment is the site of visual transduction. The inward cationic current in the dark-adapted outer segment is regulated by cyclic GMP (reviewed by Pugh and Lamb, 1993). A light flash on the rod outer segment (ROS) activates an enzyme cascade, resulting in the hydrolysis of cyclic GMP, which in turn decreases the dark current. Restoration of the dark current requires inactivation of the hydrolytic cascade and regeneration of the cyclic GMP. The enzyme guanylate cyclase (ROS-GC), which catalyses the formation of cyclic GMP, is therefore a critical participant in the recovery phase of the light response. At this stage, the complete structural identity of the ROS-GC is not known, although a 112 kDa cyclase has been purified from bovine ROS membranes (Hayashi and Yamazaki, 1991; Koch, 1991). Two properties that distinguish this cyclase from the guanylate cyclases of the natriuretic-factor receptor subfamily, two of which have also been cloned from retina (Kutty et al., 1992; Duda et al., 1992, 1993c; Ahmad and Barnstable, 1993), are its inhibition by ATP and insensitivity to the natriuretic factors (Hakki and Sitaramayya, 1990; Sitaramayya et al., 1991; Margulis et al., 1993).

Molecular cloning of a human retina cDNA encoding a membrane guanylate cyclase (retGC) has been reported recently (Shyjan et al., 1992). Hybridization studies *in situ* show its localization to photoreceptor inner segments and outer nuclear layer of the monkey retina. Whether this enzyme is present in ROS is not known. We have shown recently that the deduced amino acid sequence of this retGC shows similarities to the analysed amino acid sequence of bovine ROS-GC, but a 19-amino-acid segment in ROS-GC had no corresponding sequence in retGC (Margulis et al., 1993).

In the present paper, we report the molecular cloning, sequencing and expression of a cDNA from retina that encodes a membrane guanylate cyclase which is structurally and functionally identical with the bovine ROS-GC. Availability of this molecular probe should now make it easier to determine the precise role of ROS-GC in the recovery phase of the photo-transduction process.

EXPERIMENTAL

Molecular cloning

Because our many attempts to clone ROS-GC from the various commercially available bovine retina cDNA libraries failed, we constructed our own cDNA library.

Poly(A)$^+$ RNA was isolated from bovine retina by the Dynabeads oligo(dT) method (Dynal Protocol). First-strand cDNA synthesis was primed by a hybrid oligonucleotide which contains an 8 bp recognition sequence for endonuclease *Not*I (5′-GATGATCACGCCGGCGAATAT$_{16}$-3′), and the synthesis was performed by reverse transcriptase Superscript RTII, according to the manufacturer's protocol (Gibco–BRL). This was followed by the second-strand replacement synthesis using RNAase H, *Escherichia coli* DNA polymerase and *E. coli* DNA ligase. The double-stranded cDNA was blunt-ended with T_4 DNA polymerase. cDNA larger than 3 kb was isolated by centrifugation through a 10–30% (w/v) sucrose gradient, incubated with endonuclease *Not*I, and ligated into the pBluescript vector whose restriction ends were generated by endonucleases *Not*I and *Sma*I. The recombinant cDNA was transfected into the electro-competent *E. coli* cells (HB10B) by using an electroporation system. This cDNA library was used to isolate the full-length ROS-GC cDNA clone.

Two probes, 1 (nucleotides 2297–3125) and 2 (nucleotides 678–1286) of retGC (Shyjan et al., 1992), were used in two successive screenings of the cDNA library. These probes were generated from human retina cDNA library (Clontech) by PCR, using 21-mer primers. Approx. 1.5×10^5 bacterial colonies were screened by hybridizations to the randomly ^{32}P-labelled probes (Feinberg and Vogelstein, 1984). The hybridizations were performed at 56 °C by using a 1×10^6 c.p.m./ml in 5 × SSC (1 × SSC contains 0.15 M NaCl and 15 mM sodium citrate)/5 × Denhardt's solution/0.5% SDS containing 0.1 mg/ml denatured salmon sperm DNA. The stringency washing was done in 1 × SSC at 56 °C for 1 h. The first screening resulted in eight positive clones, the largest of which was 4.0 kb. Because this was the only clone which hybridized with the receptor-region probe (probe 2), it was used for subsequent sequencing. DNA sequencing was

Abbreviations used: ROS, rod outer segment; ROS-GC, ROS guanylate cyclase; retGC, retinal membrane guanylate cyclase; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; ATA, aurintricarboxylic acid.

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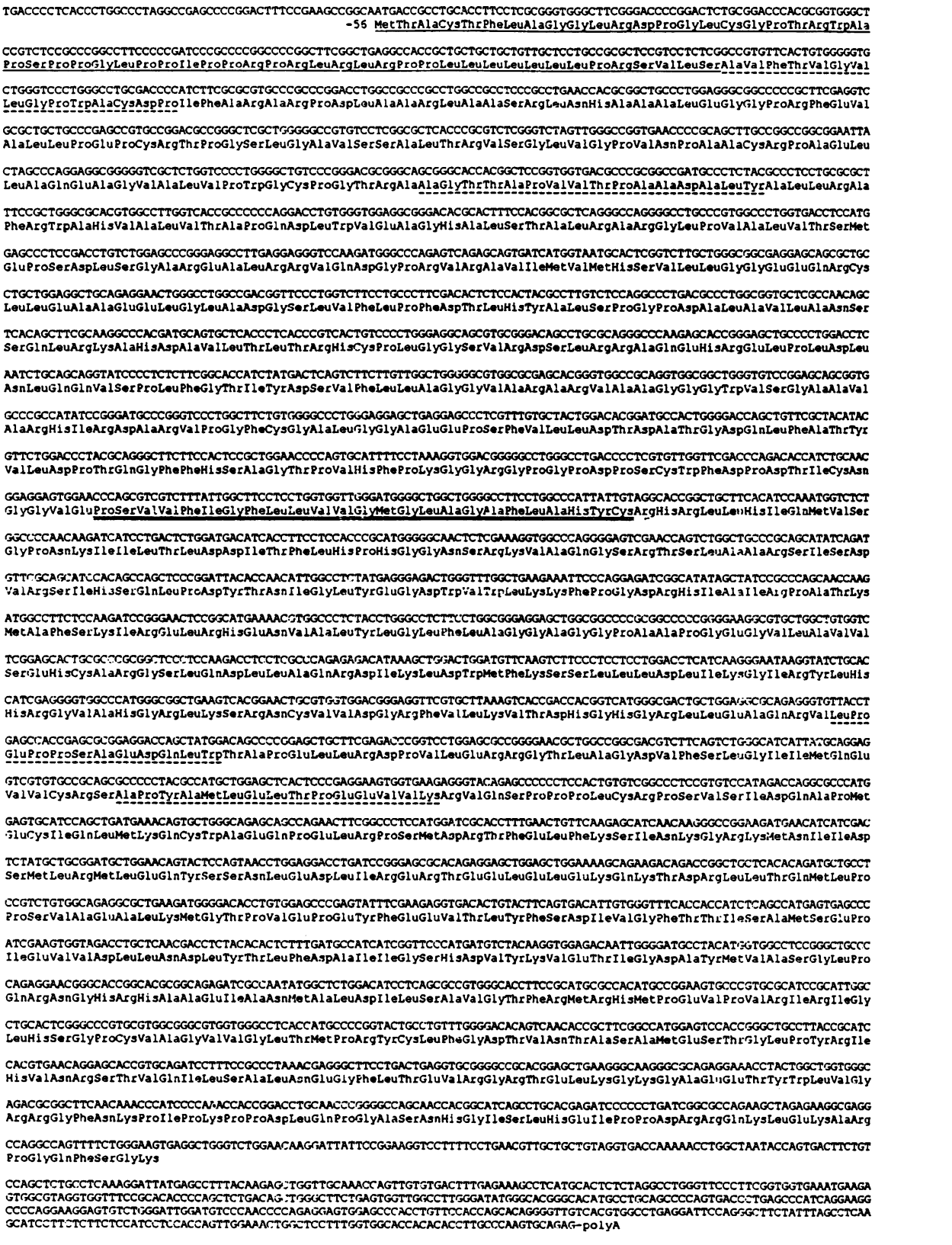

Figure 1 Nucleotide and deduced amino acid sequence of bovine ROS-GC

Nucleotides and amino acids are numbered at the left. The 56-amino-acid signal peptide is underlined, the transmembrane domain is doubly underlined, and bovine ROS-GC peptides that were isolated and sequenced (Margulis et al., 1993) are underlined by broken lines. The predicted first amino acid residue of the mature protein is numbered as 1, and the TGA stop codon is located at nucleotides 3382–3384.

performed by the dideoxy-chain-termination method (Sanger et al., 1977), using Sequenase 2.0 (United States Biochemicals), according to the manufacturer's protocol. Both strands of cDNA were sequenced by using internal primers synthesized by OLIGO-1000 synthesizer (Beckman).

Northern-blot analysis

Poly(A)$^+$ RNA was heated for 20 min at 65 °C in a denaturation buffer, which was 5.85% formaldehyde, 24.5% formamide, 3.5 mM EDTA, 17 mM Mops, 4.3 mM sodium acetate and 10 mM ATA (aurintricarboxylic acid). After addition of 0.1 vol. of sample buffer (0.25% Bromophenol Blue, 0.25% xylene cyanol FF, 50% glycerol, 1 mM ATA), the mRNA was applied to 1.0%-agarose gel containing 20 mM Mops, 5 mM sodium acetate, 1 mM EDTA, 0.22 M formaldehyde and 0.1 mM ATA and electrophoresed at 120 V for 2 h. The gel was dried and pre-hybridized at 64 °C for 2 h in 5 × SSC/5 × Denhardt's solution, containing 0.1 mg/ml denatured salmon sperm DNA. The gel was hybridized at 64 °C for 16 h in a solution identical with the prehybridization buffer, containing 1×10^5 c.p.m. of a ^{32}P-labelled probe/ml. The probe was a 369 bp antisense strand generated by PCR using a pair of primers corresponding to the nucleotide sequences 776–796 and 1125–1145 of ROS-GC (Figure 1). The probe was labelled with [^{32}P]dATP by asymmetric PCR reaction. After the hybridization, the gel was washed in 1 × SSC/0.1% SDS at room temperature (twice; 30 min each), followed by 0.5 × SSC/0.5% SDS at 55 °C (twice, 20 min each). A XAR5 (Kodak) film was exposed to the gel at −70 °C.

Expression studies

The *Sal*I/*Sac*I fragment containing the ROS-GC cDNA was cloned into the *Xho*I/*Sac*I site of pSVL (Pharmacia) to create the pSVL-ROS-GC expression vector.

COS-7 cells (simian-virus-40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin and 10% fetal-bovine serum, were transfected with the expression vector by the calcium phosphate technique (Sambrook et al., 1989). At 60 h after transfection, the cells were washed twice with 50 mM Tris/HCl, pH 7.5, containing 10 mM $MgCl_2$, scraped into 2 ml of cold buffer, homogenized, centrifuged for 15 min at 5000 ***g***, and washed with the same buffer. The pellet represented the crude membranes. These membranes were treated with 0.1 μM atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) or C-type natriuretic peptide (CNP) and/or with ATP (800 μM) for 10 min. Cells treated identically, except that they were transfected with the pSVL vector alone, served as control. The guanylate cyclase activity was measured as described by Paul et al. (1987).

RESULTS AND DISCUSSION

Using two successive PCR-constructed probes corresponding to the nucleotide sequence regions 2297–3125 and 678–1286 of retGC (Shyjan et al., 1992), we characterized a 4.0 kb cDNA clone from a bovine retina cDNA library that encodes a membrane guanylate cyclase protein. The nucleotide and the deduced amino acid sequence of this cDNA revealed an initiation codon situated at nucleotides 52–54. This determines the initiation of a 1054-amino-acid open reading frame, of which the first 56 amino acid residues represent the N-terminal hydrophobic signal peptide. The theoretical molecular mass of this protein is 120361 Da; if the putative signal-peptide sequence is excluded, the calculated molecular mass of the mature protein is 114360 Da.

The amino acid sequence of the N-terminal end and three internal fragments of the bovine ROS-GC (Margulis et al., 1993) [Ala-Val-Phe-Thr-Val-Gly-Val-Leu-Gly-Pro-Trp-Ala-*-Asp-Pro (N-terminal); Leu-Pro-Glu-Pro-Pro-Ser-Ala-Glu-Asp-Gin-Leu-Trp (internal fragment I); Ala-Pro-Tyr-Ala-Met-Leu-Glu-Leu-Thr-Pro-Glu-Glu-Val-Val-Lys (internal fragment II); and Ala-Gly-Thr-Thr-Ala-Pro-Val-Val-Thr-Pro-Ala-Ala-Asp-Ala-Leu-Tyr-Ala-Leu-Leu (internal fragment III)] have identical matches in the deduced amino acid sequence of the cloned ROS-GC (Figure 1): the N-terminal sequence matches with amino acids 1–15, the internal fragment I with residues 646–657, the internal fragment II with residues 693–707, and the internal fragment III with residues 107–125 (Figure 1). It is therefore concluded that the cloned cDNA encodes the bovine ROS-GC.

To determine the tissue specificity of ROS-GC, Northern-blot analysis of poly(A)$^+$ RNAs from selected bovine tissues such as retina, adrenal gland, testes and brain was performed. An antisense probe corresponding to nucleotides 776–1145 was used for these studies. A single 7.5 kb mRNA was detected in the retina, and none was detected in the other bovine tissues tested (Figure 2).

These results indicate the retina-specific localization of the cloned ROS-GC. An interesting part of the above study is the unusually large size of the ROS-GC mRNA, indicating that less than 50% of the mRNA portion comprises the coding region.

Hydropathy analysis of the deduced amino acid sequence of ROS-GC indicates the existence of a distinct hydrophobic domain consisting of 25 amino acids followed by three basic amino acids, indicative of a stop transfer sequence (Sabatini et al., 1982). This domain therefore represents the membrane-spanning domain of the protein, separating the N-terminal 411-amino-acid extracellular region from the C-terminal 614-amino-acid intracellular region. A domain in the intracellular region, covering residues 437–758, shows sequence identity with the tyrosine kinase family, and is therefore termed the 'kinase-like' domain; and residues 759–1010 cover a region which has a high degree of sequence identity with the conserved catalytic regions of other guanylate and adenylate cyclases. This therefore represents the catalytic domain of the protein.

This predicted topographic model of ROS-GC is supported by the protein-chemistry studies: the N-terminal residue (Ala) identified from the sequence studies of bovine ROS-GC is located at position 1 of the predicted amino acid sequence of the cloned ROS-GC; and the model is identical with that of the other members of the membrane guanylate cyclase gene family [reviewed by Sharma et al. (1994); Wong and Garbers (1992)].

The sequence of the extracellular region of the cloned protein reveals one potential site for N-linked glycosylation (based on the conserved sequence of Asn-Xaa-Ser/Thr) and 17 cysteine residues; eight cysteines are located in the extracellular domain, one in the transmembrane domain and eight in the cytoplasmic portion of the protein.

The bovine ROS-GC shows two distinct functional characteristics: first, its activity is inhibited by ATP-Mg or ATP-Mn (Sitaramayya et al., 1991); secondly, its activity is unaffected by ANF (Hakki and Sitaramayya, 1990). The latter feature distinguishes ROS-GC from the natriuretic-factor receptor subfamily of guanylate cyclases whose members, ANF-RGC and CNP-RGC, are stimulated by their respective ligands, ANF and CNP (Paul et al., 1987; Chinkers et al., 1989; Lowe et al., 1989; Koller et al., 1991; Duda et al., 1993c). To assess whether the cloned ROS-GC cDNA encodes a protein with these functional traits, the expression vector containing the cloned cDNA (pSVL-ROS-

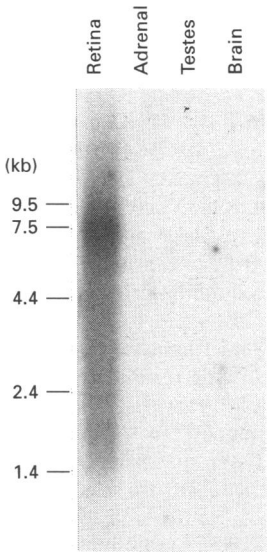

Figure 2 Northern-blot analysis of mRNAs isolated from various bovine tissues for ROS-GC

A portion (4 μg) of mRNA isolated from the indicated tissues was loaded into one lane, electrophoresed and hybridized to an antisense probe corresponding to nucleotides 776–1145 of ROS-GC as is described in the Experimental section. Positions of the molecular-size markers (RNA Leaders; Gibco–BRL) are indicated on the left.

GC) was incorporated into COS-7 cells. The particulate fractions of these cells were appropriately treated and analysed for cyclase activity.

The plasma membranes of the ROS-GC cDNA-transfected cells showed a 150-fold higher basal cyclase activity than did membranes of control cells transfected with pSVL alone (Figure 3 legend), indication that the encoded protein is a guanylate cyclase.

To determine the effects of ATP and natriuretic factors on ROS-GC activity, the plasma membranes of ROS-GC cDNA-transfected cells were incubated with ATP in the absence or presence of 0.1 μM ANF (rat, residues 8–33), BNP (rat BNP-32) or CNP (CNP-53). In the presence of ATP, the cyclase activity remained inhibited even when the individual hormones were present, and without ATP there was no rise in the cyclase activity above the basal level (Figure 3).

We therefore conclude, that the cloned guanylate cyclase is functionally equivalent to the wild-type ROS-GC.

At present, three forms of the membrane guanylate cyclase family, ANF-RGC (Duda et al., 1992; Kutty et al., 1992; Ahmad and Barnstable, 1993), CNP-RGC (Duda et al., 1993c) and retGC (Shyjan et al., 1992), have been cloned from human retina. A fourth human guanylate cyclase, termed STa-RGC, has been cloned from the intestine (de Sauvage et al., 1991). Amino acid sequence comparison indicates a significant overall structural identity of ROS-GC with the other three cyclases; there is 27% identity with STa-RGC, 30% with ANF-RGC, 31% with CNP-RGC and 79% with retGC (Figure 4). The level of identity rises to 37%, 40%, 41% and 88% in their intracellular regions; there is a respective identity of 25%, 32%, 32% and 85% between their 'kinase-like' domains; and an identity of 50%, 50%, 51% and 92% between their catalytic domains (Figure 4). The least identity with the other cyclases is found in the extracellular region: there is 14% identity with STa-RGC, 17% with ANF-RGC, 18% with CNP-RGC and 64% with retGC.

These results indicate that ROS-GC is structurally related to the other members of the membrane guanylate cyclase family. However, it bears a very close relationship with retGC. Indeed,

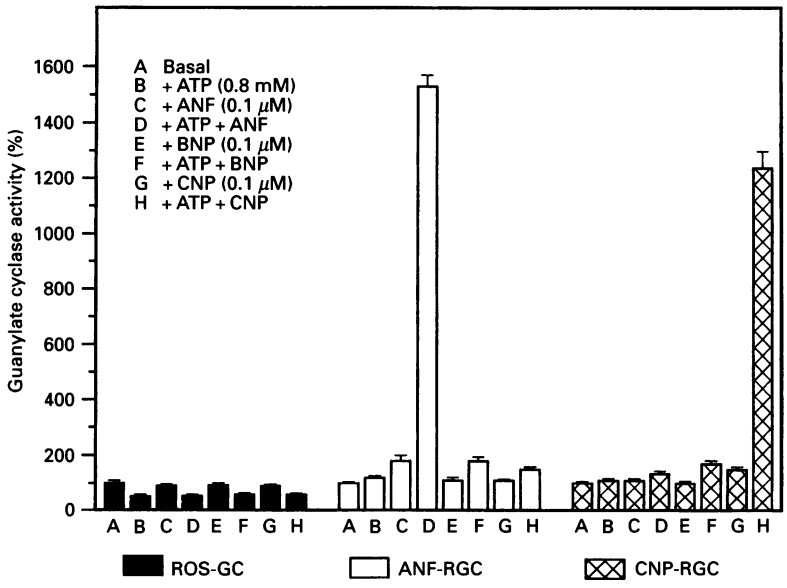

Figure 3 Effects of ATP and natriuretic peptide hormones on guanylate cyclase activity

Membranes of COS-7 cells transfected with ROS-GC cDNA, as described in the Experimental section, were assayed for guanylate cyclase activity in the presence of the indicated concentrations of ATP and/or natriuretic peptides, with 4 mM Mg^{2+} as a cofactor and 1 mM GTP as a substrate. Membranes of COS-7 cells expressing ANF-RGC or CNP-RGC (Duda et al., 1991, 1993c) were used as a control. In all cases the membranes were preincubated for 10 min in an ice-bath with the indicated additions, followed by assay at 37 °C. The basal guanylate cyclase activity in membranes expressing ROS-GC, ANF-RGC, CNP-RGC and pSVL vector alone was respectively 52, 21, 17 and 0.3 pmol of cyclic GMP/min per mg of protein. The experiments were done in triplicate and repeated twice. The data depicted are from one experiment; means ± S.D. are shown.

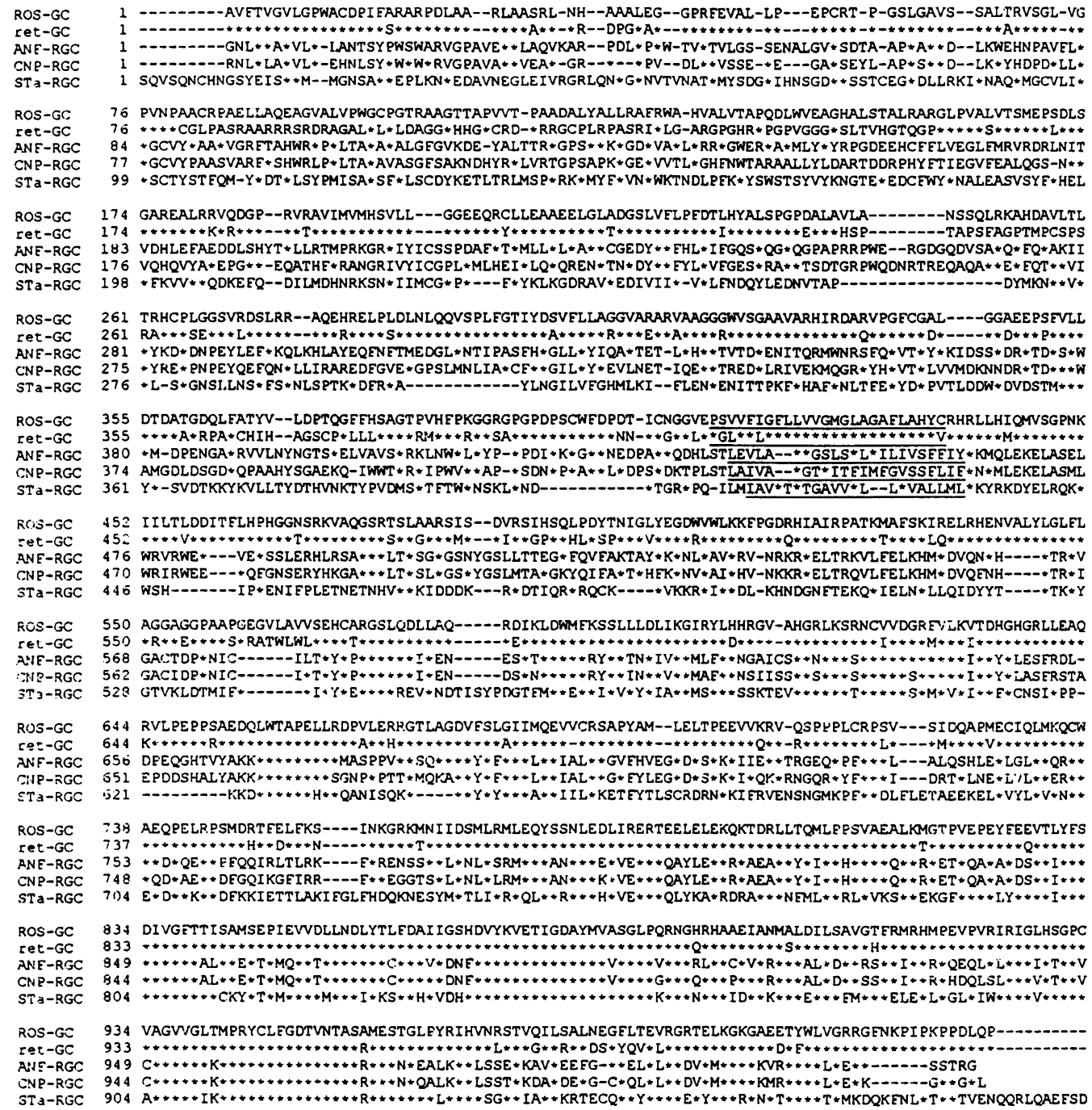

Figure 4 Amino acid sequence comparison of ROS-GC with retGC and other membrane cyclases

The deduced amino acid sequence of the mature bovine ROS-GC protein is aligned with the sequences of human retGC (Shyjan et al., 1992), ANF-RGC (Lowe et al., 1989), CNP-RGC (Duda et al., 1993c) and STa-RGC (de Sauvage et al., 1991). Identical amino acid residues between any two of the sequences are shown by asterisks; gaps introduced for the best alignment are shown by dashes. The transmembrane domain is underlined. [Program Pustell (Matrix PAM-250) was used for the sequence alignment.]

the identity between the two cyclases in the 'kinase-like' and the catalytic domains of the intracellular region is so high that it may be suggested that the minor structural differences between them are merely reflective of the species-specificity, i.e. retGC may represent the human ROS-GC.

Two features of retGC support this possibility (Shyjan et al., 1992). First, cyclic GMP production in retGC cDNA-transfected human embryonic kidney cells is not activated by the natriuretic factors ANF, BNP and CNP (Shyjan et al., 1992), suggesting that retGC is not a receptor for natriuretic factors. This attribute

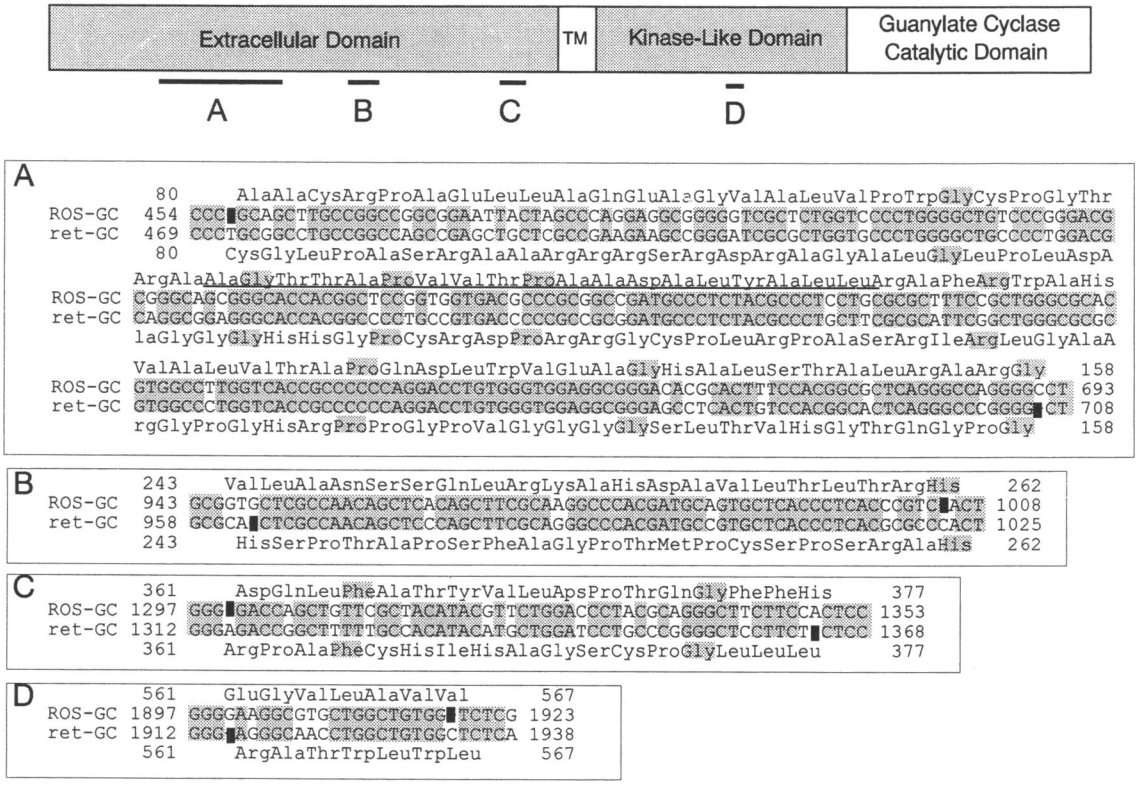

Figure 5 Graphical explanation of the origin of the four amino acid sequence motifs that differentiate ROS-GC and retGC

The location of the four different sequence motifs (A, B, C, D) in the extracellular and kinase-like domains is shown at the top of the Figure. Single nucleotide rearrangement (nucleotide deletion and insertion) resulting in a different reading frame for each of the segments is presented as A, B, C and D. Nucleotide deletions are marked as black boxes; identical nucleotides and amino acids are shaded. The sequence of the internal peptide III of bovine ROS-GC (Margulis et al., 1993) is underlined. Abbreviation: TM, transmembrane.

of retGC appears to be similar to that of ROS-GC, whose cyclase activity is also not affected by the natriuretic factors (see above, Figure 3). Secondly, hybridization studies *in situ* suggest selective localization of retGC in the inner segments of rod and cone photoreceptor cells and the outer nuclear layer (Shyjan et al., 1992). Though the status of its presence in the ROS layer is unknown, it is clear that retGC is also a photoreceptor cyclase.

An important feature that biochemically distinguishes the natriuretic-factor receptor subfamily from ROS-GC is the response to ATP; ATP, instead of stimulating, inhibits the ROS-GC activity (Sitaramayya et al., 1991). Cyclase activity of the presently cloned ROS-GC also is inhibited by ATP (see above, Figure 3), but this information is not available for retGC.

In terms of the structure–activity relationship, the non-responsiveness of the ROS-GC activity to the natriuretic factors deserves a comment. Previous studies have demonstrated that ATP is obligatory in the natriuretic-factor signalling, and the consensus sequence motif of the guanylate cyclases that defines the ATP-mediated event is Gly-Xaa-Xaa-Xaa-Gly (Duda et al., 1993a,c). This sequence motif is missing from ROS-GC (Figure 1), and also from retGC (Shyjan et al., 1992).

There are, however, several significant structural differences between ROS-GC and retGC. The amino acid sequence of ROS-GC at residues 80–158, 243–262, 361–377 and 561–567 has no identity with the corresponding sequences of retGC (Figure 4).

It is noteworthy that the identity of a 19-amino-acid segment within the above-mentioned residues 80–158 is established experimentally in ROS-GC by the protein sequence studies (Margulis et al., 1993).

Other notable differences are: (1) the predicted molecular mass of the mature ROS-GC protein is 114360, and 113870 for retGC; (2) the leader sequence of ROS-GC is five amino acids longer than that of retGC; (3) ROS-GC (mature) protein is longer than retGC by three amino acids; (4) ROS-GC contains one potential site for N-linked glycosylation, whereas none is found in retGC; (5) ROS-GC contains 17 cysteine residues, but 22 are found in retGC (notably, one cysteine is located in the transmembrane region of the ROS-GC, whereas none is present in the transmembrane region of retGC); (6) the predicted pI value (calculated for the extracellular region) of ROS-GC is 5.99, whereas it is 8.67 for retGC (Shyjan et al., 1992), indicating that this ROS-GC region is acidic and that of retGC is basic.

It is possible that ROS-GC and retGC are both functional and structural analogues. In fact, at the nucleotide level the structural identity between the two DNAs is even higher than at the protein level. The differences in the four sequence motifs that differentiate ROS-GC from retGC can be accounted for in each case by a single nucleotide deletion and a single nucleotide insertion (explained in Figure 5). This rearrangement causes the protein frame shift and the origin of distinctive protein fragments, A, B, C, D, in ROS-GC: the fragment A comprising residues 80–158 is the result of a nucleotide (T) deletion at position 472 and a nucleotide (C) insertion between nucleotides 706 and 707 in retGC; the fragment B (residues 243–262) is the result of a nucleotide (G) insertion between nucleotides 962 and 963 and a nucleotide (C) deletion at position 1022 in retGC; the fragment C (residues 361–377) is the result of a nucleotide (A) deletion at position 1315 and a nucleotide (A) insertion between nucleotides

1364 and 1365 in retGC; and the formation of the protein segment D (residues 561–576) is the result of a nucleotide (G) insertion between nucleotides 1914 and 1915 and a nucleotide (C) deletion at position 1933 in retGC.

In conclusion, the present study makes two important contributions. First, the complete identity of the ROS-GC has been revealed. This information should now make it possible to assess better the role of this enzyme in the recovery phase of the light response. Secondly, the ROS-GC and retGC may represent the photoreceptor cyclase subfamily.

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