Regions in vertebrate photoreceptor guanylyl cyclase ROS-GC1 involved in Ca^{2+}-dependent regulation by guanylyl cyclase-activating protein GCAP-1

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Abstract The membrane bound guanylyl cyclase (GC) photoreceptor membrane GC1 (ROS-GC1) of photoreceptor cells synthesizes cGMP, the intracellular transmitter of vertebrate phototransduction. The activity of ROS-GC1 is controlled by small Ca^{2+}-binding proteins, named GC-activating proteins (GCAPs). We identified and characterized two short regulatory regions (M445–L456 and L503–I522) in the juxtamembrane domain (JMD) of ROS-GC1 by peptide competition and mutagenesis studies. Both regions are critical for the activation of ROS-GC1 by GCAP-1.

Key words: Phototransduction; Guanylyl cyclase; Calcium-binding protein; Guanylyl cyclase-activating protein 1

1. Introduction

In vertebrate photoreceptor cells, light induces the amplified hydrolysis of cGMP, which leads to the closure of cyclic nucleotide-gated (CNG) channels (for reviews, [1–4]). The closure of CNG channels prevents the influx of Ca^{2+} into the outer segment of the photoreceptor cell. Since Ca^{2+} is continuously extruded via a Na/Ca,K-exchanger, the cytoplasmic [Ca^{2+}] decreases after illumination from 500 to 50 nM. Changes in cytoplasmic [Ca^{2+}] are sensed by Ca^{2+}-binding proteins like calmodulin, recoverin and the guanylyl cyclase (GC)-activating proteins (GCAPs). GCAPs activate a membrane bound GC below 200 nM free [Ca^{2+}] to enhance the resynthesis of cGMP [5–11]. In vertebrate photoreceptor cells, two isoforms of a membrane bound GC are found [12–19]. They are synonymously termed rod outer segments GC1 (ROS-GC1) and ROS-GC2 or GC-E and GC-F, respectively. ROS-GCs serve a key function in phototransduction, since they synthesize the intracellular messenger cGMP under the control of a negative Ca^{2+} feedback. Since changes in cytoplasmic [Ca^{2+}] control the light sensitivity of photoreceptor cells, the regulation of ROS-GCs by Ca^{2+} and GCAPs is a key mechanism of light adaptation (for reviews, [4,20–22]).

The molecular control mechanisms by which GCAPs regulate the activity of ROS-GCs on the intracellular side are not understood in detail [9,23–25]. Previous experiments have indicated that two isoforms of GCAP, GCAP-1 and GCAP-2, are associated with ROS-GCs independent of [Ca^{2+}] [7,9,26–28]. Therefore, it was proposed that activation of ROS-GCs is triggered by a Ca^{2+}-dependent conformational switch in a stable ROS-GC/GCAP complex. As a first step to understand the activation mechanism at a molecular level, we sought to identify regions in ROS-GC1 that participate in target recognition and/or regulation by GCAP-1. Within the intracellular domain of ROS-GC1, the juxtamembrane domain (JMD) and the kinase homology domain (KHD) are necessary constituents for activation by GCAP-1 [9]. Deletion of the JMD and of the KHD of ROS-GC1 (S447–I730) results in loss of GCAP-1 sensitivity.

It was the aim of our study to identify short sequence motifs in ROS-GC1 that are critical for activation by GCAP-1. Two hypothetical regulatory domains in the JMD were identified by using a synthetic oligopeptide library and by characterizing corresponding mutants.

2. Materials and methods

2.1. Synthesis of oligopeptide library

Peptides encompassing the JMD (R437–G552; Fig. 1A) of bovine photoreceptor GC ROS-GC1 were synthesized with a multipin peptide synthesis kit (Chiron Mimotopes) according to Schrem et al. [28]. Each peptide was 12 amino acids long and overlapped the preceding one by 10 amino acids. Larger amounts of peptides #3a, #5, #34a and #36 were synthesized and purified according to Zoche et al. [29].

2.2. Preparation of ROS

ROS were prepared from fresh bovine retinae as described previously [10,12]. The predominant GC in our ROS preparation is ROS-GC1 (L. Molday, B. Molday and K.-W. Koch, unpublished observation).

2.3. GC assay

The activity of ROS-GC1 was determined by either a high performance liquid chromatography (HPLC) assay using a nucleotide separation and quantitation system [10,12] or by a radioimmuno assay [30]. Peptide competition experiments were carried out as previously described [28] in the presence of 0.1 mM ATP to increase ROS-GC1 activity independent of GCAP-1 [12].

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Abbreviations: GC, guanylyl cyclase; ROS, rod outer segments; ROS-GC1, photoreceptor membrane guanylyl cyclase 1; GCAP, guanylyl cyclase-activating protein; KHD, kinase homology domain; JMD, juxtamembrane domain; GC-A, atrial natriuretic factor receptor guanylyl cyclase; HPLC, high performance liquid chromatography

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2.4. Heterologous expression and purification of GCAP-1

Recombinant GCAP-1 was heterologously expressed in *Escherichia coli* and purified from inclusion bodies exactly as described previously [28].

2.5. Mutagenesis and expression

Four ROS-GC1 deletion mutants, Δ1 (deleted amino acids M445–L456), Δ2 (deleted amino acids R489–I522), Δ3 (deleted amino acids D507–R518) and K^- (deleted amino acids S447–I730), were constructed. The numbering corresponds to the mature ROS-GC1 protein [15]. Construction of the K^- mutant is described in [9]. The Δ1 mutant was generated by 'looping-out' nucleotides 1552–1587 in ROS-GC1 cDNA. To construct the Δ2 and Δ3 mutants, two *Hpa*I restriction sites were introduced into ROS-GC1 cDNA. For the former mutant, the sites were at nucleotide positions 1683 and 1785 and for the latter, they were at 1737 and 1774. The desired fragments were excised and the remaining parts re-ligated. All constructs were sequenced to confirm their identities. The mutated cDNAs were cloned into *Kpn*I/*Xba*I sites of the pcDNA vector for expression in mammalian cells. ROS-GC1 and mutants were expressed in COS cells as previously described [30].

3. Results

3.1. Screening of a ROS-GC1 oligopeptide library

Photoreceptor specific membrane bound ROS-GC1 is activated by GCAPs through its intracellular domain, a process that is different from the activation of hormone receptor GCs by extracellular ligands. A sequence comparison of ROS-GC1 with ROS-GC2 and the hormone receptor atrial natriuretic factor receptor GC (GC-A) revealed that the JMD (R437–G552; JMD in Fig. 1A) is highly homologous in ROS-GC1 and ROS-GC2, but is completely different in GC-A and ROS-GC1 (Fig. 1B,C). We reasoned that the JMD contains regions that are specific for the regulation by GCAP-1. In order to identify critical amino acid stretches within the JMD, we synthesized a peptide library of overlapping 12-mer oligopeptides (Fig. 1A). Peptides were screened for their ability to inhibit GCAP-1-mediated activation of ROS-GC1 in purified ROS.

Inhibitory effects of peptides (0.3 mM) on ROS-GC1 activity were tested both at 24 μM [Ca^{2+}] (2 mM CaEGTA) and at 1 nM free [Ca^{2+}] (2 mM EGTA). This allowed us to differentiate between peptide effects on the basal state of ROS-GC1 (at high [Ca^{2+}]) and on the activated state of ROS-GC1 (at low [Ca^{2+}]). Two small regions represented by peptides #1–5 (except #4) and #35/36 showed a significant inhibitory effect on the ROS-GC1 activity at low [Ca^{2+}] (Fig. 2A). ROS-GC1 activity at high [Ca^{2+}] was also diminished by #35/36 and, to a lesser extent, by #2 and #5 (Fig. 2B). It was puzzling that peptide #4 had no effect, although peptides #3 and #5 were the most effective. A HPLC analysis of #4 showed an inhomogenous product after synthesis.

3.2. Peptide competition studies

In order to confirm the results of the screening, we tested the inhibitory peptides in titration series at high and low [Ca^{2+}]. We also included extended versions of peptides #5 and #35 (Fig. 1, peptides #3a and #34a). Peptides #5 and #34a were the most effective and inhibited ROS-GC1 in a concentration-dependent way (Fig. 3A,B). Half-maximal inhibition was observed at concentrations (IC_{50}) of 100 μM (#5, without Ca^{2+}), 250 μM (#34a, without Ca^{2+}), 260 μM (#5, with Ca^{2+}) and 150 μM (#34a, with Ca^{2+}). Peptide #5 inhibited ROS-GC1 more effectively at low [Ca^{2+}] than at high [Ca^{2+}], whereas #34a was slightly more efficient at high

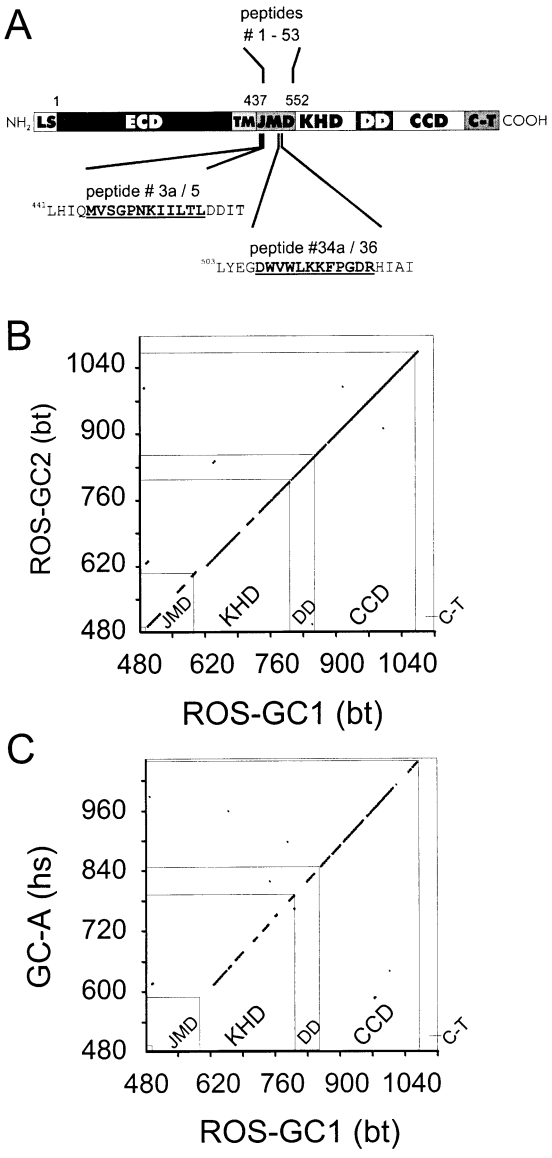

Fig. 1. A: Domain structure of bovine ROS-GC1. The location of overlapping peptides and their corresponding numbers are indicated (e.g. peptides #1–53 encompass R437–G552). The abbreviations are: LS, leader sequence in the unprocessed protein; ECD, extracellular (intradiscal) domain; TM, transmembrane domain; KHD, kinase homology domain; DD, hypothetical dimerization domain; CCD, cyclase catalytic domain; C-T, C-terminus. Positions of peptides #3a and #34a are indicated. Positions of #5 and #36 are typed in bold letters and are underlined. Comparison of amino acid sequences of bovine ROS-GC1 [15] with the corresponding parts of bovine (bt) ROS-GC2 [18] (B) and human (hs) GC-A (GenBank accession number NM000906) (C) illustrated in a dot blot matrix analysis. Analysis was performed with the program PDOTPLOT, version 3.13 (Dr W. Bönigk, IBI-1, FZ-Jülich) using the following parameter: matrix MDM [36], window 12 amino acids, cutoff score 300. Only the cytoplasmic parts of the proteins are compared. A high degree of sequence homology is seen as a diagonal black line.

[Ca^{2+}]. A peptide representing the region K762–L773 of the dimerization domain did not influence the ROS-GC1 activity at 0.3 mM and was used as a control in the titration set experiment in Fig. 3A,B. It was much less effective than peptides #5 and #34a.

The inhibitory effects of peptides #5, #34a and #36 were

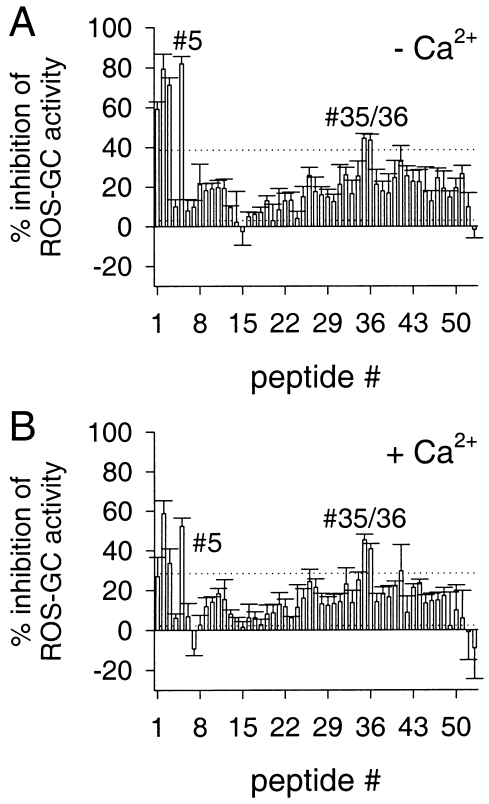

Fig. 2. Percentage inhibition of GCAP-1-dependent activation of ROS-GC1 at 1 nM [Ca^{2+}] (i.e. in the presence of 2 mM EGTA) (A) and 24 μM [Ca^{2+}] (B). Peptides at 0.5 mg/ml (0.3 mM) were added to a suspension of bovine ROS and incubated at the indicated free [Ca^{2+}]. Data represent the mean ± S.D. of 2–4 measurements. Dotted lines indicate the S.D. of the overall mean and serve as threshold criterion.

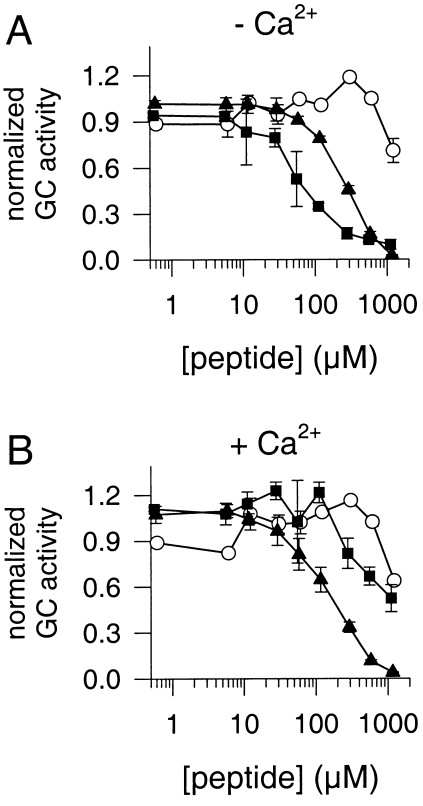

Fig. 3. Inhibition of ROS-GC1 activity in whole ROS as a function of the peptide concentration (■ #5, ▲ #34a, ○ control peptide). ROS-GC1 activity was determined at 1 nM [Ca^{2+}] (A) and at 24 μM [Ca^{2+}] (B). All data are the mean of three determinations ± S.D. Absolute values for GC activity were normalized to values obtained without peptides.

reversible. For example, ROS containing ROS-GC1 and endogenous GCAP-1 were incubated at low [Ca^{2+}] with either 320 μM #5 or 1 mM #36, which led to an inhibition of 80–90% compared to the control. Addition of 20 μM recombinant GCAP-1 to the same ROS suspension completely relieved the inhibition (not shown).

The results of the screening approach and the competition studies suggest that peptides #1–5 interfered mainly with the activated state of ROS-GC1 and that peptide #34a interfered with the basal and activated state of ROS-GC1. We conclude that #5 and #34a represent important regulatory sites within the JMD of ROS-GC1.

3.3. Characterization of ROS-GC1 mutants

We further focused on these two regions to investigate their role in regulating ROS-GC1 activity. The mutants Δ1 (M445–L456, corresponding to peptide #5), Δ2 (R489–I522, corresponding to peptide #34a plus flanking regions) and Δ3 (D507–R518, corresponding to peptide #36) were constructed. The expression level of all mutants in COS and HEK293 cells was similar to the wild-type as assessed by Western blotting (data not shown). All three deletion mutants showed a greatly decreased basal activity compared to the wild-type in COS cells. In contrast, deletion of a larger intracellular domain encompassing the whole KHD amino acids S447–I730 (designated K^-) does not lead to a reduction in basal ROS-GC1 activity (Fig. 4; [9]). GCAP-1 failed to activate all four mutants, whereas GC activity of the wild-type was stimulated 4–5-fold by addition of GCAP-1 above saturation (2 μM) (Fig. 5A). When expressed in HEK cells, the Δ1 mutant was slightly stimulated at higher concentrations of GCAP-1 (> 5 μM). However, basal activity and activation by GCAP-1 were also largely reduced when compared to the wild-type. To confirm the structural integrity of the mutants, we tested whether

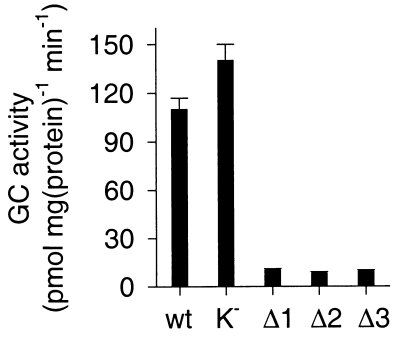

Fig. 4. Activity of heterologously expressed ROS-GC1 mutants in comparison to the wild-type. Mutants and wild-type were expressed in COS cells and membranes were assayed for basal GC activity at 10 μM [Ca^{2+}]. K^- denotes a mutant that lacks the JMD and the KHD. Δ1, Δ2 and Δ3 denote mutants that lack the regions represented by peptides #5, #34a and #36 (see text).

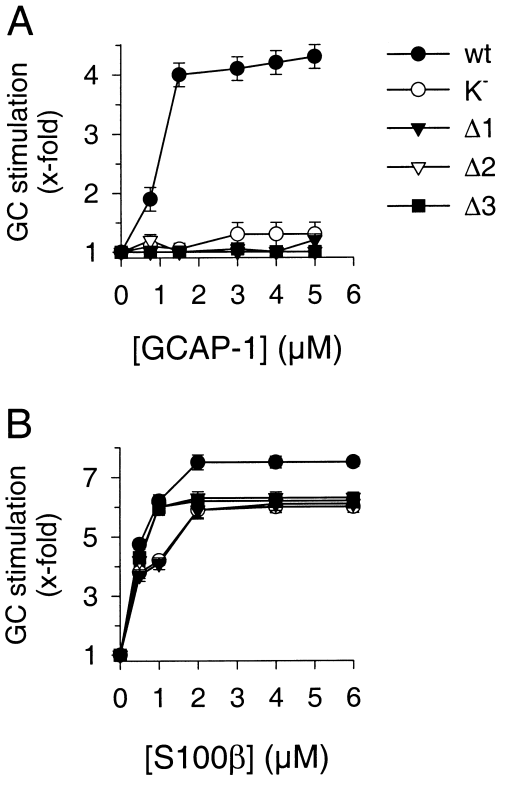

Fig. 5. Activation of ROS-GC1 wild-type and mutants by increasing concentrations of GCAP-1 (A) and S100β (B). ROS-GC1 was heterologously expressed in COS cells and assayed for GC activity at subnanomolar [Ca^{2+}] (A) and 10 μM [Ca^{2+}] (B). Activity is expressed as fold activation. Data points are the mean of triplicates. Each experiment was repeated three times.

ROS-GC1 mutants did respond to another EF-hand Ca^{2+}-binding protein S100β (also termed CD-GCAP). S100β is known to activate ROS-GC1 at micromolar [Ca^{2+}] [31,32]. It interacts with a different region than GCAP-1 [33]. S100β was able to activate all mutants to nearly the same relative extent as the wild-type (Fig. 5B). We conclude from these experiments that the ROS-GC1 mutants did not suffer from a general impairment of their function. Instead, they had selectively lost the ability to respond to GCAP-1.

4. Discussion

Our analysis of GCAP-1/ROS-GC1 interaction has revealed that two regions represented by peptides #5 and #34a are critical for activation of ROS-GC1 by GCAP-1. The two peptides acted differently on the Ca^{2+}-dependent activation of ROS-GC1 (Fig. 2 and 3). Since peptide #5 inhibited more effectively the activated state of ROS-GC1, we conclude that this region represents a GCAP-1-dependent transducer motif of ROS-GC1. The motif is necessary to cause Ca^{2+}-dependent activation of the cyclase catalytic domain.

One position in region #34a is of particular interest. Patients who suffer from the retinal disease named Leber's congenital amaurosis have a mutated form of ROS-GC1 [34], i.e. the phenylalanine in position 514 is mutated to a serine. A recent paper by Duda et al. [30] showed that ROS-GC1 with a F514S mutation has a decreased basal activity and has completely lost the sensitivity to GCAP-1. The mutant, however, responds normally to S100β. These results are in strong support of our finding that the sequence covered by peptide #34a represents a main regulatory site for GCAP-1.

Both regions are located in the JMD more than 300 amino acids upstream from the catalytic domain. Recently, Sokal et al. [35] identified a peptide in the catalytic domain of ROS-GC1 as a GCAP-1-binding site. Preliminary results from our laboratories indicated that region #34a represents a second interaction site for GCAP-1 (in preparation). We conclude that GCAP-1 interacts with more than one site in ROS-GC1. Peptide #34a and the peptide of Sokal et al. [35] are at remote distances in the primary structure within the cyclase molecule. We hypothesize that in the tertiary structure of ROS-GC1, the cyclase catalytic domain and the JMD form a multipoint attachment site for GCAP-1.

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