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Functional Consequences of a Rod Outer Segment Membrane Guanylate Cyclase (ROS-GC1) Gene Mutation Linked with Leber's Congenital Amaurosis†

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ABSTRACT: ROS-GC1 is the original member of the subfamily of membrane guanylate cyclases with two Ca^{2+} switches, which have been defined as CRM1 and CRM2. These are separately located within the intracellular domain of the cyclase. CRM1 switches on the enzyme at nanomolar concentrations of Ca^{2+} and is linked with phototransduction; the other stimulates at micromolar Ca^{2+} concentrations and is predicted to be linked with retinal synaptic activity. Ca^{2+} acts indirectly via Ca^{2+}-binding proteins, GCAP1 and CD-GCAP. GCAP1 is a modulator of the CRM1 switch, and CD-GCAP turns on the CRM2 switch. A Leber's congenital amaurosis, termed LCA1, involves F514S point mutation in ROS-GC1. The present study shows that the mutation severely damages its intrinsic cyclase activity and inactivates its CRM1 switch but does not affect the CRM2 switch. In addition, on the basis of the established modulatory features of ROS-GC1, it is predicted that, in two other forms of LCA1 involving deletion of nt 460C or 693C, there is a frameshift in ROS-GC1 gene, which results in the nonexpression of the cyclase. For the first time, the findings define the linkage of distinct molecular forms of LCA to ROS-GC1 in precise biochemical terms; they also explain the reasons for the insufficient production of cyclic GMP in photoreceptors to sustain phototransduction, which ultimately leads to the degeneration of the photoreceptors.

Leber's congenital amaurosis (LCA) is a genetic disease of photoreceptor degeneration (*1*). It results in either total or drastic loss of vision (*2*−*6*). A recent study has mapped four mutations, collectively termed LCA1, in a gene for LCA on a human chromosome 17p13.1 (*6*, *7*), where the gene of a rod outer segment membrane guanylate cyclase (ROS-GC1) also is localized (*6*). Each form is linked to a single ROS-GC1 point mutation: F589S, A52S, deletion of nt 460C, and nt 693C (*6*). It is believed that in all LCA1 mutations there is an impaired formation of cyclic GMP in the retina, resulting in the permanent closure of cyclic GMP-gated channels (*6*). However, this hypothesis has not been experimentally tested. In particular, the functional consequences of mutations in ROS-GC1 are unknown.

Synthesis of cyclic GMP in rod outer segments is catalyzed by a membrane guanylate cyclase ROS-GC. Two isoforms of this enzyme, ROS-GC1 and ROS-GC2, have been cloned

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(ref *8*: structure corrected in: GenBank accession number M 92432; refs *9*−*12*, reviewed in ref *13*). Immunostaining and in situ hybridization studies now indicate the presence of both ROS-GC forms in photoreceptor outer segments (*8*, *9*, *14*−*16*), but at present it is unclear whether the observed heterogeneity in ROS-GCs reflects two different modulatory mechanisms in the same cell or whether it is the result of a rod/cone specific expression pattern.

Because LCA1s cosegregate with the chromosomal locus of ROS-GC1, and not of ROS-GC2, LCA1s are ROS-GC1-specific abnormalities. Both ROS-GCs are modulated by Ca^{2+} via Ca^{2+}-binding proteins, GCAP1 (*17*−*21*) and GCAP2 (*9*, *11*, *22*−*26*), in a negative feedback loop. When photoreceptor cells are illuminated, hydrolysis of the internal transmitter cyclic GMP causes closure of the cyclic GMP-gated channels in the plasma membrane (*13*, *27*−*29*). Subsequently, the intracellular Ca^{2+} falls from ∼500 nM in a dark-adapted photoreceptor cell to below 100 nM (*30*−*33*) in the light-adapted state of the photoreceptor cell (*34*−*39*). This in turn causes Ca^{2+} to dissociate from binding sites in GCAPs, which can now stimulate the ROS-GC. Increased synthesis of cyclic GMP along with the inactivation of the transduction cascade leads to the reopening of cyclic GMP-gated channels. While the Ca^{2+}-dependent modulation of ROS-GC in outer segments is one of the several feedback loops that shape the recovery phase of the photoresponse and constitute the light-adapted state of the photoreceptor cell, another Ca^{2+}-regulated feature specific to ROS-GC1, outside of the outer segments, is emerging. Here the cyclase, instead of being inhibited, is stimulated by high (micromolar) concentrations of $[Ca^{2+}_i]$ via a Ca^{2+}-binding protein, CD-GCAP (*40*−*42*). This mode of regulation is opposite to that in phototransduction, does not occur in rod outer segments, and is, therefore, hypothetically linked to retinal synapse activity (*40*).

CD-GCAP belongs to the family of S100 proteins, which is distinct from the family of GCAP (*43*). The GCAP- and the CD-GCAP-regulated ROS-GC1 domains are distinct (*42*, *44*). Thus, ROS-GC1 has two Ca^{2+} switches, which according to their locations in calcium-regulated modules (CRMs), have been designated as CRM1 and CRM2 (*26*). CRM1 is linked to phototransduction and CRM2 presumably to the retinal synaptic activity. In the present investigation the basal and functional statuses of these switches in a LCA1, involving F514S mutation in the ROS-GC1 gene, have been investigated. The findings indicate that the mutation drastically damages the basal catalytic cyclase activity of the encoded enzyme and specifically makes CRM1 switch inoperative but does not affect CRM2 switch. This study for the first time precisely explains the biochemical consequences of a molecular defect in ROS-GC1 that results in a Leber's amaurosis, and the defect is directly linked to the impairment of phototransduction machinery.

MATERIALS AND METHODS

Construction of ROS-GC1 Mutants. Two phenylalanine-related ROS-GC1 mutants, F514S and F591S, were constructed. The numbering corresponds to the mature ROS-GC1 protein (*10*). Both mutations required a single nucleotide change T → C; TTC codon for phenylalanine was mutated to TCC codon for serine. The mutations were accomplished by the technique of "coupled priming mutagenesis". ROS-GC1 cDNA was subcloned into pAlter vector (Promega mutagenesis kit); the mutations were attained by using the selection ampicillin repair primer and the following mutagenic primers: 5′-GTTTGGCTGAAGAAATCCCCAG-GAGATCGG-3′ for F514S and 5′-AAGCTGGACTGGA TGTCCAAGTCTTCCCTC-3′ for F591S. The mutated recombinants were sequenced to confirm their identities and individually subcloned into pcDNA3 expression vector (Invitrogen).

Insertion of N-terminal FLAG Epitope into ROS-GC1 and Its Mutants. The FLAG (DYKDDDDK) (IBI Kodak) epitope was inserted into ROS-GC1 immediately after the signal peptide cleavage site, where a *Bgl*II restriction site was created by site-directed mutagenesis in ROS-GC1 cDNA at nucleotide position 220 (*10*). Two oligonucleotides, 5′-GATC-GACTACAAGGACGACGATGACAA-3′ and 5′-GATCT-TGTCATCGTCGTCGTTGTAGTC-3′, were annealed and ligated into the *Bgl*II site, and proper ligation was confirmed by sequencing. The construct is named FLAG-ROS-GC1. FLAG-tagged F514S and F591S mutants of ROS-GC1 were built by substituting the *Sfi*I/*Bam*HI fragment of FLAG-ROS-GC1 cDNA in pcDNA3 expression vector with the *Sfi*I/*Bam*HI fragment of the ROS-GC1 mutants.

Expression and Purification of Recombinant GCAP1. The coding sequence of the bovine GCAP-1 was amplified from a full-length cDNA (*20*) via polymerase chain reaction (PCR) using the following primers: sense primer 5′-CAGGGAT CCACCATGGGGAACATTATGGAC-3′, introducing a *Bam*-HI site and a *Nco*I site encompassing the codon for the starter methionine, and antisense primer 5′-AGGGAATTCTATCA GCCGTCGGCCTCCGCG-3′, introducing an additional stop codon and *Eco*RI restriction site. The product obtained after 15 cycles of PCR was subcloned into pBlueScript SK vector via the introduced *Bam*HI and *Eco*RI sites. One of the resulting clones was sequenced to exclude the possibility that errors had been introduced by the PCR.

A *Nco*I (filled in)−*Eco*RI fragment containing the GCAP1 coding sequence was inserted between *Nde*I and *Eco*RI sites of pET21a(+) (Novagen) and expressed in *Escherichia coli* BL21(DE3) strain carrying the plasmid pBB131 (a kind gift of Dr. J. Gordon) encoding yeast *N*-myristoyl transferase. Cells were grown in LB medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin, supplemented with 50 μg/mL myristic acid at an OD_{600} of approximately 0.5, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) thirty minutes later. Cells were harvested by centrifugation 4 h after induction and frozen in 50 mM Tris-HCl at −20 °C until use. The thawed cell suspension was lysed with 100 μg/mL lysozyme (SERVA) and 10 units/mL DNaseI (TaKaRa), and the reaction was terminated with 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylflouride (PMSF). The insoluble fraction, obtained by centrifugation at 370000*g* for 20 min, was solubilized in buffer containing 6 M guanidinium hydrochloride, 0.1 mM PMSF, and 2.5 mM DTT for 1 h at room temperature, filtered, and dialyzed at 4 °C against three changes of 10 mM sodium phosphate buffer containing 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate (EDTA) and 1 mM DTT, followed by two changes of 10 mM sodium phosphate buffer containing 200 μM $CaCl_2$ and 1 mM DTT. After dialysis and centrifugation at 370000*g* for 20 min, the supernatant was concentrated on centriplus (Amicon; 10 kDa cutoff) and

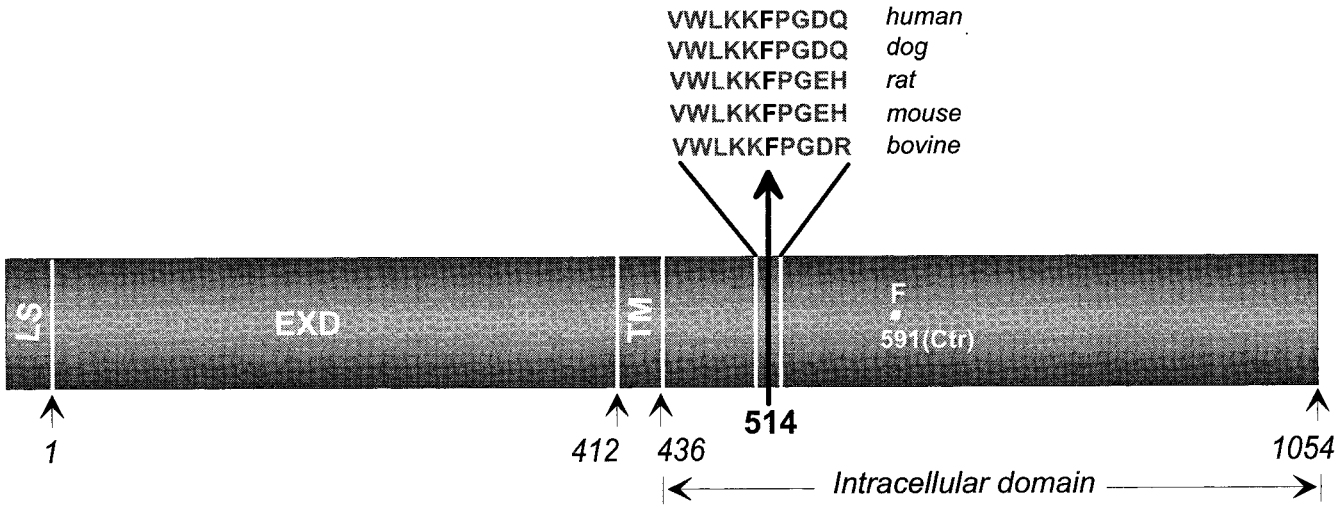

FIGURE 1: Schematic representation of the ROS-GC1 protein. The predicted domains are denoted by the following: LS, leader sequence; EXD, extracellular domain (intradiscal domain); TM, transmembrane domain. Numbers indicated correspond to the mature protein (*10*). At position 514 there is a phenylalanine in ROS-GC1 which is substituted by serine in some cases of LCA1 (Perrault et al., 1996). Alignment of the human, dog, rat, mouse, and bovine amino acid sequences (GenBank accession numbers: M92432/AJ222657, Y15483, L36029, L41933, L37089, respectively) in the region surrounding position 514 is shown above. The position of the control mutation, F591S, is indicated.

applied onto a Superdex75 XK16/60 gel filtration column (Pharmacia) in 10 mM sodium phosphate (pH 7.0) buffer containing 100 mM NaCl and 200 μM $CaCl_2$. Fractions containing GCAP1 were pooled and loaded onto an HR5/FPLC column (Pharmacia) of ceramic hydroxylapatite type I (BioRad). GCAP1 was eluted with a linear gradient of 0−100% 300 mM sodium phosphate (pH 7.0) in 10 mM sodium phosphate (pH 7.0) containing 200 μM $CaCl_2$, and purity was >90% on the basis of Coomassie staining after sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE). Purified GCAP1 was dialyzed against five changes of 50 mM $(NH_4)HCO_3$, followed by lyophilization for storage. Lyophilization did not significantly affect stimulating activity of GCAP1 on washed ROS membranes or membranes expressing wild-type recombinant (wt-r) ROS-GC1.

Expression Studies. 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 constructs by the calcium phosphate coprecipitation technique (*45*). Transfection with vector alone served as the control. Sixty hours after transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ buffer, scraped into 2 mL of cold buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed several times with the same buffer; the pellet represented the crude membranes.

Guanylate Cyclase Assay. The crude membranes were assayed for guanylate cyclase activities as described previously (*17, 46*). Briefly, membranes were preincubated on ice with or without GCAP1 or CD-GCAP [purchased commercially as S100B ($\beta\beta$) protein from Sigma] in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5) adjusted to the appropriate free Ca^{2+} concentrations with precalibrated Ca^{2+}/ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) solutions (Molecular Probes). Total assay volume was 25 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 225 μL of 50 mM sodium acetate buffer (pH 6.2) followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by radioimmunoassay (*47*). In membrane preparations from COS cells tranfected with the vector alone, the cyclase activity was negligible [0.3 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$].

Western Blot. After boiling in gel-loading buffer [62.5 mM Tris-HCl, (pH 7.5), 2% SDS, 5% glycerol, 1 mM β-mercaptoethanol (βME), and 0.005% bromophenol blue], 20 μg of membrane protein was subjected to SDS−PAGE in a buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. The proteins were transferred to immobilon membranes (Millipore) in the same buffer but containing 5% methanol. The blot was incubated in Tris-buffered saline (TBS pH 7.5) containing 100 mM Tris-HCl, 0.9% NaCl, and 0.05% Tween-20 (TBS-T) with 5% powdered nonfat Carnation milk (blocking buffer) overnight at 4 °C and rinsed with TBS-T. The anti-FLAG monoclonal antibodies (Sigma) were added at 1:300 dilution in the blocking buffer, and the incubation was continued for 1 h. After the blot was rinsed with TBS-T, the incubation was continued with the secondary antibody conjugated to horseradish peroxidase in blocking buffer (1:20000) for another hour. Finally the blot was treated with SuperSignal blaze chemiluminescent substrate (Pierce) for 5 min according to manufacturer's protocol. The immunoreactive band was detected by exposing the blot to Kodak X-ray film for 15 s.

RESULTS

F514S Mutation in ROS-GC1 Damages the Basal Cyclase Activity. The original report (*6*) identifies a missense mutation T → C in the human ROS-GC1 gene, which results in the conversion of phenylalanine-589 to serine (F589S). The GenBank sequence data on this protein, however, shows that there is no phenylalanine at position 589 (ref *6*: GenBank accession number AJ222657); instead, there is histidine. On

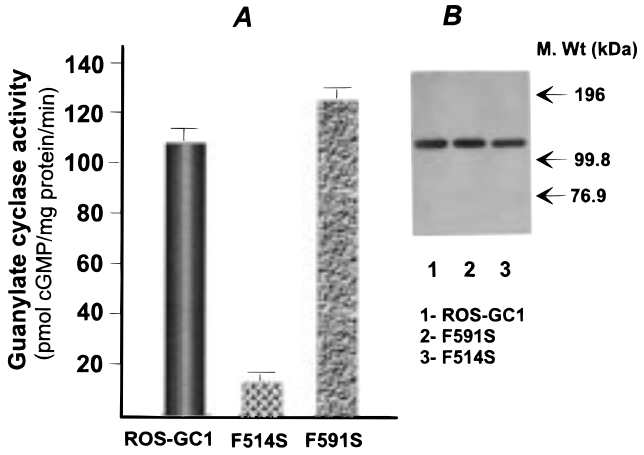

FIGURE 2: Expression of the wt-rROS-GC1, F514S, and F591S mutant proteins. (A) Guanylate cyclase activity. COS cells were individually transfected with the appropriate expression construct, and the membranes were prepared as described in Materials and Methods. These were assayed for guanylate cyclase activity (*46*). The experiment was repeated three times for reproducibility with three separate transfection experiments. (B) Western blot. Protein (20 μg) (COS cell membranes containing FLAG-ROS-GC1, FLAG-F514S, or FLAG-F591S) was electrophoresed on SDS−PAGE. The proteins were transferred to PVDF membranes, and the blot was incubated with anti-FLAG monoclonal antibody and with the secondary antibody as described in Materials and Methods. Immunoreactive bands were visualized by exposing the blot to Kodak X-ray film. The experiment was repeated two times with membranes prepared from separate transfection experiments. Arrows indicate the positions of the corresponding molecular weight markers.

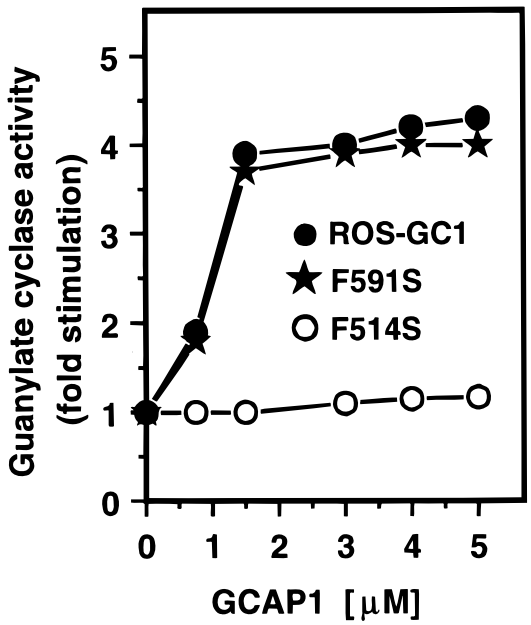

FIGURE 3: Effect of GCAP1 on the cyclase activity of the wt-rROS-GC1, F514S, and F591S mutant proteins. COS cells were transfected with the appropriate expression construct, and the membranes were prepared as described in Materials and Methods. These were assayed for guanylate cyclase activity in the presence of 10 nM Ca^{2+} and incremental concentrations of GCAP1. Each experiment was done in triplicate and repeated at least three times with separate membrane preparations. The depicted curves are from one representative experiment. Error bars are within the size of the symbol.

the basis of the unique location of the sequence GAAAT-***T***CCCA, (ref *6*, Figure 3a) the correct position of the mutated phenylalanine is 565. This corresponds to the bovine ROS-GC1 mutation, F514S (mature protein; ref *10*) (Figure 1).

ROS-GC1 is a highly conserved protein. Alignment of a 10 amino acid domain surrounding the mutated phenylalanine (F514) shows almost complete identity between its human, dog, rat, mouse, and bovine forms (Figure 1). The phenylalanine-514 in the bovine ROS-GC1, as indicated, is conserved in all species.

To determine the biochemical consequences of F → S substitution, we created F514S mutation in the wt-rROS-GC1. To eliminate the nonspecific consequences associated with an F to S substitution, we conducted an identical mutation involving F591S. The mutant proteins and wt-rROS-GC1 were expressed in COS cells, where under native conditions there is no expression of ROS-GC1. The leader sequence of all of the constructs was retained to ensure proper translocation and folding of the proteins. The cell membranes were tested for their basal guanylate cyclase activities.

The basal guanylate cyclase activities of the wt-rROS-GC1 and control F591S mutant were almost equal, about 120 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ (Figure 2A). In contrast, basal activity of the F514S mutant was about 10 pmol of cyclic GMP min^{-1} (mg protein)$^{-1}$, which was more than 10-fold lower than the wt-r and the control F591S cyclases (Figure 2A).

To assess if the decrease in F514S activity was due to the diminished expression of the protein, we determined the relative protein levels of the wt, control F591S, and F514S proteins in the cell membranes immunologically. A highly immunogenic FLAG epitope (DYKDDDDK), immediately after the signal peptide cleavage site (*48*), was inserted into all three proteins. The FLAG constructs were expressed in COS cells. To determine if the FLAG had any effect on the basal activities of the cyclases, we compared membranes of the FLAG-containing cyclases with the ones where the FLAGs were absent. There was no significant difference between the activities of cyclases with or without FLAG, 115−125 and 110−125 pmol of cyclic GMP min^{-1} (mg protein)$^{-1}$ for FLAG-tagged and untagged ROS-GC1, respectively, and 8−10 and 8−12 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for FLAG-tagged and untagged F514S mutant (four independent transfection experiments). This indicates that the FLAG does not influence the basal cyclase activity. The membranes were subjected to a Western analysis with antiFLAG monoclonal antibodies. The blots showed single immunoreactive bands of almost equal intensity (Figure 2B), indicating comparable expression of the proteins. Thus, the loss of the cyclase activity in LCA1 was due to the F514S mutation.

F514S Mutation Results in Loss of ROS-GC1 Modulation by GCAP1. To test whether the loss of basal cyclase activity in F514S mutation caused a parallel loss in the cyclase response to GCAP1, we incubated the F514S expressing cell membranes with a series of incremental concentrations of purified recombinant GCAP1 at a fixed (10 nM) Ca^{2+} concentration. The membranes expressing wt-rROS-GC1 and F591S served as controls. GCAP1 stimulated both wt-rROS-GC1 and F591S in a dose-dependent fashion, with almost identical stimulatory patterns (Figure 3). The maximal cyclase activation was about 4.5-fold over the basal value;

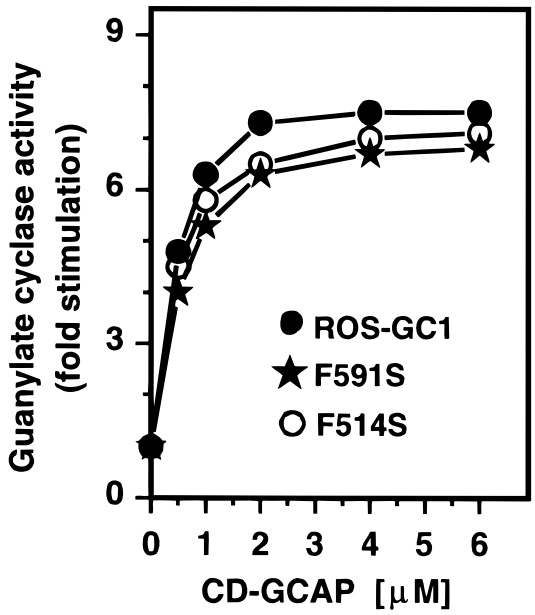

FIGURE 4: Effect of CD-GCAP on the cyclase activity of the wt-rROS-GC1, F514S, and F591S mutants. COS cells were transfected with the appropriate expression construct, and the membranes were prepared as described in Materials and Methods. These were assayed for guanylate cyclase activity in the presence of 1 mM Ca^{2+} and incremental concentrations of CD-GCAP. Each experiment was done in triplicate and repeated at least three times with separate membrane preparations. The depicted curves are from one representative experiment. Error bars are within the size of the symbol.

the half-maximal effective concentration (EC_{50}) of GCAP1 was reached at about 1 μM, and saturation at about 2.5 μM. These results indicate that the F591S mutation has no effect on the GCAP1-modulated ROS-GC1 activity. In contrast, GCAP1 concentration up to 5 μM (well into the saturation phase for wt-r ROS-GC1) elicited no response from the F514S mutant (Figure 3), indicating that the mutation damages the ROS-GC1 CRM1 switch, which under normal conditions is linked to phototransduction.

F514S Mutation Does Not Affect the CD-GCAP-Mediated Ca^{2+} Dependence of ROS-GC1. An intriguing feature of ROS-GC1, not shared by its other family member ROS-GC2, is that it has two Ca^{2+} switches, CRM1 and CRM2. CRM1 switch inhibits the enzyme at micromolar concentrations of Ca^{2+}, and the other stimulates. CRM1 is linked to phototransduction (vide supra), and it is likely that CRM2 switch is linked to retinal synaptic activity (*40*). CRM2 acts via a Ca^{2+}-binding protein, CD-GCAP, which is identical to S100B protein, a member of the S100 protein family (*43*). To determine the consequences of F514S mutation on the Ca^{2+} stimulatory switching mechanism, we incubated the membranes from the cells expressing F514S with a series of incremental concentrations of purified CD-GCAP at fixed (1 mM) Ca^{2+} concentration. In parallel, under identical conditions, the membranes expressing wt-rROS-GC1 and F591S were also processed. CD-GCAP stimulated all three forms of cyclases in a dose-dependent fashion with almost identical stimulatory patterns (Figure 4). In each case, the EC_{50} of CD-GCAP was reached at about 0.8 μM, and saturation at about 2 μM. Thus, F514S mutation does not alter the ability of the cyclase catalytic core to respond to CD-GCAP.

DISCUSSION

The present study was undertaken to determine the direct biochemical consequences of LCA1 on the ROS-GC1 mutation involving F514S. The findings indicate that the mutation causes a major loss in the intrinsic activity of the enzyme and also incapacitates the enzyme in its modulation by Ca^{2+}. These modifications in enzyme features provide a molecular explanation for the impaired phototransduction in LCA1. Furthermore, the study indicates that the mutation is selective for phototransduction, because it does not damage the CD-GCAP-mediated activation of the enzyme, suggesting that the retinal synapse-related functions are not ceased in LCA1. The bases of these conclusions are discussed below.

A fundamental operational principle of the phototransduction machinery is the maintenance of the optimum level of cytoplasmic cyclic GMP in the DARK STATE. This keeps fractional (~5%) cyclic GMP channels open (*13*, *28*, *49*). The estimated cyclic GMP level in the Dark-resting level is ~4 μM resulting from a balanced synthesis and hydrolysis of cyclic GMP. The LCA1 mutation reduces the intrinsic ROS-GC1 activity by more than 10-fold. Then only one tenth of the required cyclic GMP will be produced. Therefore, restoration of the dark state would be slower than that in the normal photoreceptors.

Under native conditions of phototransduction, closure of the channels in response to photoexcitation results in the fall of $[Ca^{2+}_i]$ to less than 100 nM (*30*−*33*). GCAP1 binds ROS-GC1 and stimulates its activity by more than 5-fold; the cyclic GMP is replenished and the channels opened. The LCA1 mutation, however, changes ROS-GC1. The enzyme no longer responds to GCAP1, resulting in impairment of the recovery and light adaptation states of the photoreceptors. Thus, the mutation has not only destroyed the major basal activity of the cyclase; it has also completely eliminated its ability for Ca^{2+} modulation by GCAP1.

Furthermore, the decreased level of cyclic GMP resulting in the permanent closure of cyclic GMP-gated channels is envisioned to have another consequence. The Ca^{2+} ions will not enter the cell through the channel, yet they will be extruded via the Na^+/Ca^{2+}, K^+ exchanger (*32*). The cell will have a persistent low level of intracellular Ca^{2+}, which will adversely affect the integrity of photoreceptors. Thus, the LCA1 mutation has not only destroyed the phototransduction machinery; it has also destroyed the site which houses it.

The immunocytochemical presence of ROS-GC1 outside ROS in the retinal synaptic layer (*14*, *15*) and its ability to be stimulated by micromolar concentrations of Ca^{2+} have suggested that ROS-GC1, besides phototransduction, is also linked to retinal synaptic activity outside of the photoreceptor outer segments (*40*). This raises an important physiological issue: How does LCA1 mutation in ROS-GC1 affect the CD-GCAP-mediated Ca^{2+} activity? The results demonstrate that the mutation does not affect it. Hence, the predicted Ca^{2+}-dependent retinal synaptic activities will not be impaired. The LCA1 model for the first time clearly dissects at the physiological level the two Ca^{2+}-dependent differential activities of ROS-GC1. The results provide direct confirmation of the earlier conclusions where the distinctness of the two activities was shown by genetic remodeling/expression studies of ROS-GC1 (*17*, *42*−*44*).

Besides F514S mutation, three other ROS-GC1 mutations occur in LCA1 (1) deletion of nt 460C; (2) deletion of nt 693C; and (3) A52S. The first two mutations are frameshift in nature. They will result in premature termination of the protein (*6*), and there will not be any expression of functional ROS-GC1 in these types of LCA1. Thus, like F514S LCA1, it is predicted that photoreceptors of these LCA1s will lack phototransduction because the cyclic GMP-gated channels will remain closed. Similar to these LCA1s, a null mutation in the ROS-GC1 gene has been observed in the retinal degeneration (*rd/rd*) chicken, and it has been predicted that this causes insufficient production of cyclic GMP and results in the absence of phototransduction (*50*).

It is predicted that there will not be any biochemical consequences of the third LCA1 ROS-GC1 mutation, involving A52S. This is because the mutation is in the extracellular domain of ROS-GC1. This domain has no role in the Ca^{2+}-regulated and intrinsic cyclase activity of ROS-GC1 (*17*, *22*). This prediction is in accord with the finding that the original study could not relate this mutation directly to LCA1 and commented that "it is difficult to decide whether we are dealing with a disease-causing mutation or a rare polymorphism" (*6*).

In conclusion, these reconstitution studies in a heterologous system for the first time have explained the biochemical consequences of the F514S mutation of LCA1 in the guanylate cyclase, and have directly linked the cyclase mutation with the phototransduction machinery. Furthermore, the results have uncovered an unexpected ROS-GC1 feature: Despite its breakdown in linkage with phototransduction, F514S mutation does not damage the predicted cyclase linkage to the retinal synapse, which occurs through the CRM2 switch.

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