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# Impaired Association of Retinal Degeneration-3 with Guanylate Cyclase-1 and Guanylate Cyclase-activating Protein-1 Leads to Leber Congenital Amaurosis-1*

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**Background:** Defects in the function of guanylate cyclase 1 (GC1) cause Leber congenital amaurosis (LCA) type 1.
**Results:** GC1, GCAP1, and RD3 form a complex in the endoplasmic reticulum that targets GC1 to outer segments.
**Conclusion:** A subset of LCA1 is caused by impaired formation of the RD3-GC1-GCAP1 complex.
**Significance:** Understanding the molecular interaction of RD3 with GC1 and GCAP1 has potential therapeutic benefits for LCA1.

**One-fifth of all cases of Leber congenital amaurosis are type 1 (LCA1). LCA1 is a severe form of retinal dystrophy caused by loss-of-function mutations in guanylate cyclase 1 (GC1), a key member of the phototransduction cascade involved in modulating the photocurrents. Although GC1 has been studied for some time, the mechanisms responsible for its regulation and membrane targeting are not fully understood. We reported earlier that retinal degeneration 3 (RD3) protein interacts with GC1 and promotes its targeting to the photoreceptor outer segments (POS). Here, we extend our studies to show a direct association between RD3 and guanylate cyclase activating protein 1 (GCAP1). Furthermore, we demonstrate that this functional interaction is important for GC1 targeting to POS. We also show that most LCA1-causing mutations in GC1 result in lost GC1 interaction with RD3 or GC1 being targeted to the plasma membrane. Our data suggest that GC1, GCAP1, and RD3 form a complex in the endoplasmic reticulum that targets GC1 to POS. Interruption of this assembly is likely the underlying mechanism for a subset of LCA1. This study offers insights for the development of therapeutic strategies to treat this severe form of blindness.**

Leber congenital amaurosis (LCA)[2] consists of a group of hereditary retinal dystrophies that are characterized by severe loss of visual function, with an occurrence of about 1:100,000 (1). LCA accounts for ~5% of all retinal dystrophies (2, 3). It is diagnosed by fundus examination, extinguished scotopic and photopic ERGs, and retinal degeneration in young patients (4). Nineteen causative genes have been identified for LCA (5–8). Twenty four mutations in guanylate cyclase 1 (*GC1*) have thus far been reported in LCA1 patients (4). Loss of GC1 function is the most common feature of LCA1, and it is thought to be the cause of photoreceptor death in LCA1 patients (1, 9–14). LCA12 is another severe form of retinal dystrophy caused by mutations in the gene encoding retinal degeneration 3 (RD3) protein (15). We showed earlier that RD3 is crucial for the stability and expression of GC1 and that the interactions between RD3 and GC1 are essential for GC1 trafficking to the photoreceptor outer segment (POS) (15).

In vertebrate photoreceptor cells, phototransduction begins with photon absorption by the photopigments. Rhodopsin or cone opsin triggers hydrolysis of cGMP by activating the phototransduction cascade. This closes the cGMP-gated cation channels (CNG) in the plasma membrane (PM) and hyperpolarizes the membrane (16–18). The role of GC1 in cGMP production is crucial for the recovery of phototransduction (19).

Impaired targeting of retinal membrane proteins is known to trigger degenerative diseases like retinitis pigmentosa and ciliopathies (20, 21). The proteins comprising the POS discs are made in the inner segment (IS) on the rough endoplasmic reticulum (ER) followed by post-translational modifications that occur in the ER and Golgi apparatus (22, 23). Newly synthesized OS proteins must transport to OS through the connecting cilium (24). A GC1 trafficking model proposes a vesicular mechanism (25) that aids other integral and peripheral membrane proteins in moving from the ER to the POS (4). We previously showed that GC1 traffics to the POS by interacting with RD3 (15). We also showed that RD3 negatively regulates GC1, probably during protein trafficking (26). However, the exact mechanism of GC1 trafficking and OS targeting is still unknown.

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[2] The abbreviations used are: LCA, Leber congenital amaurosis; GC1, guanylate cyclase 1; RD3, retinal degeneration 3; POS, photoreceptor outer segment; GCAP1, guanylate cyclase-activating protein 1; ROS, rod outer segment; IS, inner segment; OS, outer segment; IP, immunoprecipitation; PM, plasma membrane; ER, endoplasmic reticulum; MBP, maltose-binding protein; IHC, immunohistochemistry.

Guanylate cyclase-activating protein 1 (GCAP1) has been shown to regulate the activity of GC1 (27–29). However, studies using immunoprecipitation (IP) or direct binding assays have not established the physical interaction of GCAP1 with any other proteins. The most likely explanation for this finding is that the structure of GCAP1 varies highly under different conditions. GCAP1's binding with its partners occurs transiently under certain fluctuating ionic conditions (19, 30) known to occur during phototransduction. $Mg^{2+}$ and $Ca^{2+}$ levels significantly impact GC1's activation by GCAP1 (28).

Here, we report that GCAP1 directly binds to RD3 and that the presence of GC1, GCAP1, and RD3 in a complex is essential for GC1 to be successfully targeted to the POS. We show that 10 out of 24 LCA1 mutants in GC1 do not bind to RD3 *in vitro*. Our study suggests that a number of GC1-associated LCA1 disease phenotypes are caused by impaired RD3-GC1-GCAP1 complex formation and GC1 trafficking to the OS.

## EXPERIMENTAL PROCEDURES

*Animals*—All animal work was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Vision Research. All protocols were reviewed and approved by the IACUC of the University of Oklahoma Health Sciences Center.

*cDNAs and Antibodies*—The human *GC1* cDNA in pRcCMV was a gift from Dr. Alexander Dizhoor (Pennsylvania College of Optometry, Elkins Park, PA). Full-length human *GCAP1* was generated by PCR using retinal cDNAs as templates in enhanced GFP vector (Addgene). Human *GCAP2-Myc* was purchased from Origene (MR202065). The GCAP-6B12 monoclonal antibody was generated in our laboratory from mice immunized with an affinity-purified GST fusion protein containing the C-terminal 45 amino acids of the mouse GCAP1 that had no homology with GCAP2. Polyclonal antibodies for RD3 and GC1 were generated against the last 16-amino acid C-terminal peptide of the mouse GC1 and RD3; details are presented in Table 1 (15).

*In Vitro Expression of RD3, GCAP1, and GC1*—COS-7 cells were transfected with *rd3*, *GC1*, and GCAP1 constructs. Single, double, and triple transfections were performed with 0.1 $\mu$g of human RD3, 0.2 $\mu$g of GC1, and 10 ng of GCAP1 plasmids using the calcium phosphate procedure (15). At 22 and 48 h post-transfection, cells were fixed with 4% paraformaldehyde for 10 min, washed with 1× PBS, and used for immunohistochemistry (IHC) and biochemical analyses.

*Photoreceptor Outer Segments (POS) and Immunoprecipitation (IP)*—We prepared POS from retinas of post-natal 21 (P21) wild-type (WT), $GC1^{-/-}$, $GCAP^{-/-}$, and $rd3^{-/-}$ mice as described previously with slight modifications (35). Briefly, retinas were fresh-frozen in liquid nitrogen in the presence of 10% sucrose and kept at −70 °C until the time of use. Three independent samples, each containing 3–5 retinas, were used. Retinal samples were homogenized in buffer A (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA) with protease inhibitors (Roche Applied Science) containing 47% sucrose in a Dounce tissue homogenizer, and centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatant containing crude POS was diluted with buffer A and centrifuged at 11,000 × *g* for 30 min at 4 °C. Then the pellet was resuspended in 47% sucrose in buffer A and placed at the bottom of a discontinuous sucrose gradient layered with equal amounts of 37, 32, and 28% sucrose and centrifuged at 31,000 × *g* for 70 min at 4 °C. The band at the 28 and 32% interface contained purified ROS (POS), and the bands at the 37 and 47% interface contained non-ROS (POS) membranes, which also contained inner segment proteins. These were collected, diluted with buffer A, and pelleted by centrifugation at 27,000 × *g* for 30 min at 4 °C. Purified ROS and non-ROS pellets were resuspended to a final concentration of 1 $\mu$g/$\mu$l total protein in buffer A. IP studies using immunoaffinity matrix containing 6B12 antibody coupled to CNBr-activated Sepharose beads (GE Healthcare) were performed as described earlier (15).

*Direct Binding Analysis*—Human RD3 was cloned in pMAL-p5X vector (New England Biolabs, N8109S) containing a maltose-binding protein tag. *Rd3-MBP* was used for the following experiments. 1) For binding of bacterial RD3-MBP with endogenous bovine GCAP1, We incubated 1 $\mu$g of RD3-MB with 1 mg of bovine lysate for 2 h at 4 °C. We then added 60 $\mu$l of equilibrated 6B12-conjugated beads to the mixture and incubated for 30 min. Beads were washed and eluted with 1% SDS. 2) For binding of bacterial RD3-MBP with bacterial His-GCAP1, we incubated 1 $\mu$g of His-GCAP1 with 1 $\mu$g of RD3-MBP for 2 h in the presence of 1 mM $CaCl_2$ and $MgCl_2$, which in our experience facilitates the binding of GCAP1 with its binding partners. Then we incubated the complex with 9D12 antibody-conjugated beads for 30 min. Beads were washed thoroughly and eluted with 10 mM amylose. The results were visualized by SDS gel and Western blotting. Approximately the same size of D2 loop of RDS (peripherin-2) was cloned in the same vector and served as a negative control under the same buffer conditions.

*Site-directed Mutagenesis and Immunofluorescence Microscopy*—We designed primers containing the mutations and performed site-directed mutagenesis by PCR with GeneArt site-directed mutagenesis system (Invitrogen A13282). We generated all known LCA1-causing GC1 mutants (4). The entire *GC1* cDNA was verified by sequencing. To avoid unwanted mutations in the vector backbone, the insert (*GC1*) for all mutants was excised and cloned in an unmodified vector. Cryosections from 21-day-old (P21) homozygous WT, $GC1^{-/-}$, $GCAP^{-/-}$, and $rd3^{-/-}$ mice, as well as COS-7 cells transfected with these genes, were labeled as described previously (42, 43). Details of the antibody concentrations are shown in Table 1. Samples were visualized under a ×40 objective with confocal microscopy. ImageJ software was used for quantification of IHC in COS-7 cells.

## RESULTS

*Protein Expression Patterns in WT, $GC1^{-/-}$, $rd3^{-/-}$, and $GCAP1^{-/-}$ Retinas*—In WT mouse retinas, GCAP1 is localized in the POS, IS, and the pedicles of the outer plexiform layer (Fig. 1*A*) (30). In $GC1^{-/-}$ and $rd3^{-/-}$ retinas, GCAP1 is mainly located in the IS and is absent from the POS (Fig. 1, *B* and *C*). As expected, there was no GCAP1 in $GCAP1/2^{-/-}$, hereafter called $GCAP^{-/-}$ (Fig. 1*D*). We initially found RD3 in the POS by mass spectrometry

**TABLE 1**
**The list of antibodies used in this study**

| | Reagent | Catalog. no. | Dilution used (Western blot) | Dilution used for ( IHC) | Generated by |
|---|---|---|---|---|---|
| 1 | RD3-62 | YZ 4162 | 1:1,000 | 1:100 | Yen-Zym antibody, LLC |
| 2 | RD3-9D12 | | 1:500 | | Generated in our laboratory |
| 3 | GC1-75 | YZ 4575 | 1:2,000 | | Yen-Zym antibody, LLC |
| 4 | GC1-2H6 | | 1:1,000 | 1:100 | Generated in our laboratory |
| 5 | GCAP1-6B12 | | 1:1,000 | 1:100 | Generated in our laboratory |
| 6 | Retinitis pigmentosa 1 | | | 1:200 | Kind gift of Dr. Eric Pierce, Harvard Medical School |
| 7 | RAB 11 | R5903 | | 1:200 | Sigma |
| 8 | Calretinin | AB5054 | 1:200 | | Chemicon |
| 9 | Na/K-ATPase | a5 | 1:1,000 | | Development Studies of Hybridoma Bank (DSHB) |
| 10 | Rhodopsin | | 1:5,000 | 1:1000 | Generated in our laboratory |
| 11 | Vybrant CM-DII | V-22888 | | 1:2000 | Invitrogen |
| 12 | Actin | A3854 | 1:50,000 | | Sigma |
| 13 | GFP | A11122 | | 1:500 | Invitrogen |
| 15 | CNGB1 | | | 1:200 | Kind gift of Dr. Steve Pittler, University of Alabama |

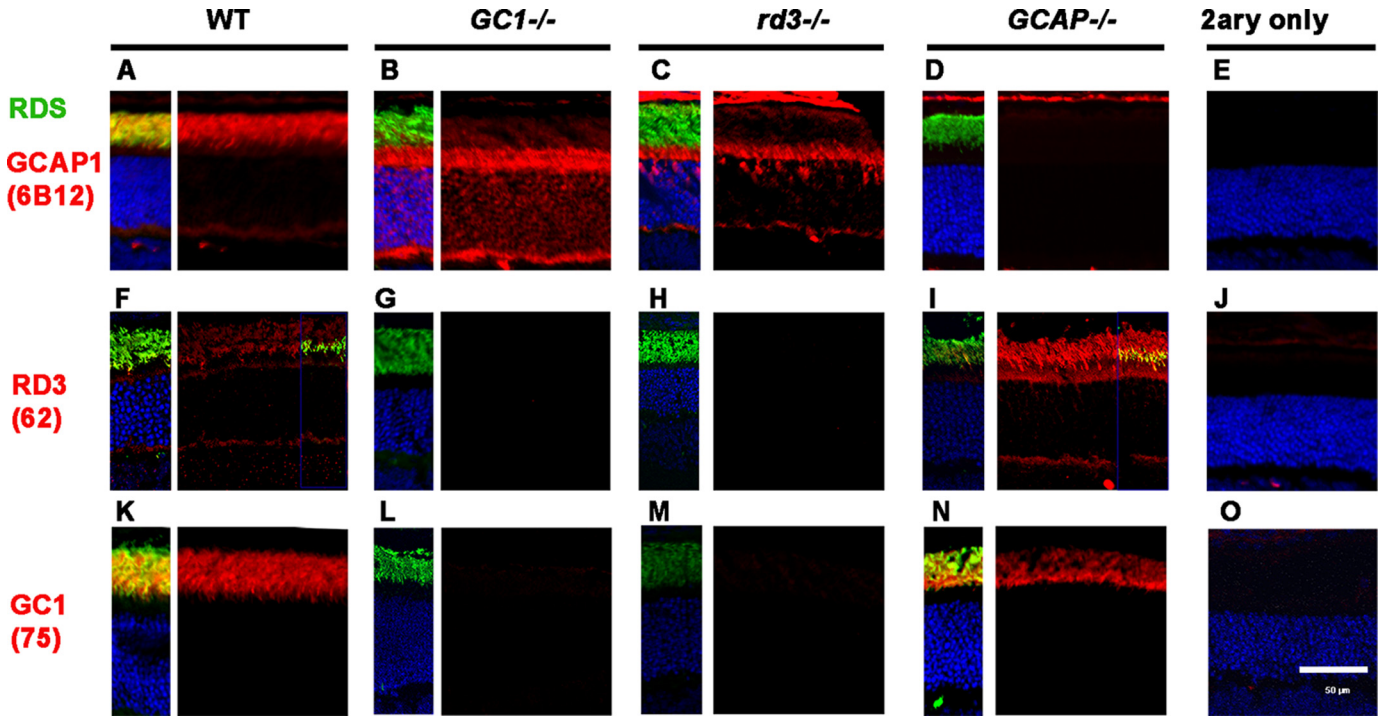


FIGURE 1. **Localization of GCAP1, RD3, and GC1 in WT, *GC1*$^{-/-}$, *GCAP*$^{-/-}$, and *rd3*$^{-/-}$ retinas.** 21-Day-old frozen fixed retinas were used to determine the expression pattern of RD3, GC1, and GCAP1 proteins in four WT, *GC1*$^{-/-}$, *GCAP*$^{-/-}$, and *rd3*$^{-/-}$ retinas. *A–D*, GCAP1 protein expression in WT (*A*), *GC1*$^{-/-}$ (*B*), *rd3*$^{-/-}$ (*C*), and *GCAP*$^{-/-}$ (*D*) retinas. Secondary antibody alone was used as a negative control (*E*). *F–I*, RD3 localization in WT (*F*), *GC1*$^{-/-}$ (*G*), *rd3*$^{-/-}$ (*H*), and *GCAP*$^{-/-}$ (*I*) retinas, and secondary antibody alone (*J*). *K–N*, GC1 localization in WT (*K*), *GC1*$^{-/-}$ (*L*), *rd3*$^{-/-}$ (*M*), and *GCAP*$^{-/-}$ (*N*) retinas, and secondary antibody alone (*O*). Retinitis pigmentosa 1 (RP1) antibody was used as a marker for axoneme of photoreceptor cells (44) (*I* and *F insets*). RD3 co-localizes with retinitis pigmentosa1 (*RP1*) in photoreceptor axoneme and is overexpressed in the axoneme of the *GCAP*$^{-/-}$ retina. Sections from the listed genotypes were co-labeled with RDS as a marker for photoreceptor POS.

and IHC using acid-purified polyclonal antibody raised against this protein (15, 31). Since then, we have generated several monoclonal and polyclonal antibodies against RD3. One of these antibodies works well in IHC without antigen retrieval, and it better reveals RD3 localization, as it labels POS, IS, axoneme, and outer plexiform layer (Fig. 1*F,* 62ab, see Fig. 2*C* for antibody specificity and characterization). Using this antibody, we found RD3 below the detection level in the *GC1*$^{-/-}$ retinas (Fig. 1*G*), while it accumulates in the POS of the *GCAP*$^{-/-}$ retinas and accumulates further in the photoreceptor axonemes (Fig. 1*I*, *inset*). This localization is consistent with our earlier report, but showed stronger labeling at the axonemes (15).

The localization of GC1 in the POS of WT retinas has been reported (32–34). We generated a new GC1 antibody (*75 ab,* see Fig. 2*B* for the specificity of the antibody), which confirms GC1 localization to the POS of rods and cones (Fig. 1*K*). As expected, GC1 is absent from *GC1*$^{-/-}$ (Fig. 1*L*) and *rd3*$^{-/-}$ retinas (Fig. 1*M*), confirming earlier findings (15). Interestingly, we found GC1 labeling in the POS of the *GCAP*$^{-/-}$ retinas, albeit at a lower intensity than in WT retinas (Fig. 1*N*). However, in this genotype, we consistently observed uneven labeling of GC1 throughout the POS with accumulation in areas closer to the IS. Some of these findings have been reported previously (30, 32–34), and we have included them here for comparison.

*Protein Levels in rd3*$^{-/-}$*, GC1*$^{-/-}$*, and GCAP*$^{-/-}$ *Retinas*—To assess GC1, GCAP1, and RD3 protein levels in WT and knock-out retinas we prepared POS- and IS-enriched fractions

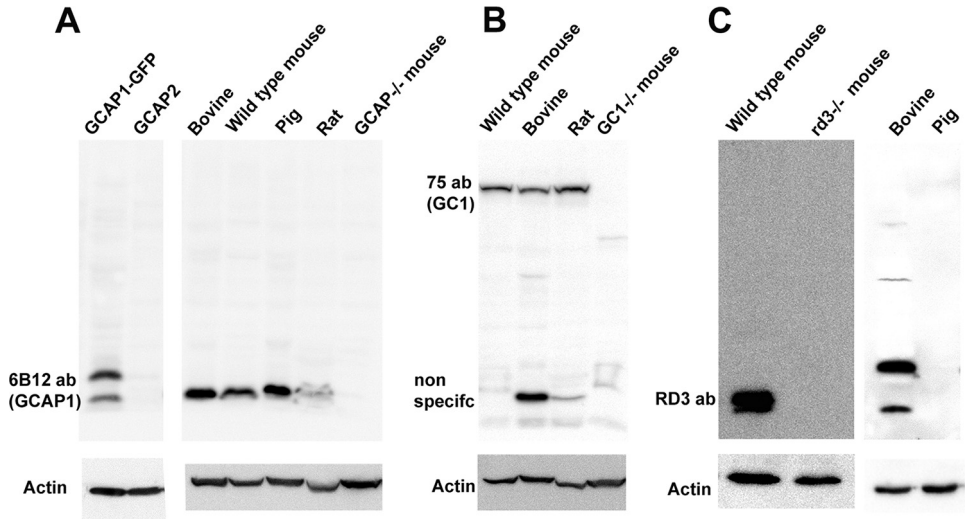


FIGURE 2. **Characterization of the 6B12 (GCAP1), 75 (GC1), and 62 (RD3) antibodies.** *A,* specificity of 6B12 against GCAP1 (in relation with GCAP2) was validated by expressing mouse GCAP1-GFP and mouse GCAP2-Myc in HEK293 cells. 6B12 did not recognize the GCAP2 protein. This was expected, as this antibody was raised against the C-terminal region (last 45 amino acids) of mouse GCAP1, which was quite different from GCAP2. The specificity of 6B12 was examined in bovine, mouse, pig, rat, and $GCAP^{-/-}$ mouse retinas. *B,* Western blot of 75 (GC1) antibody in WT mouse, bovine, rat, and $GC1^{-/-}$ mouse retinas, suggesting the specificity of the 75 antibody in mouse retina. The antibody detected an extra band in bovine retinas. *C,* RD3 antibody (62) was validated using retinas of WT and $rd3^{-/-}$ mice, cows, and pigs.

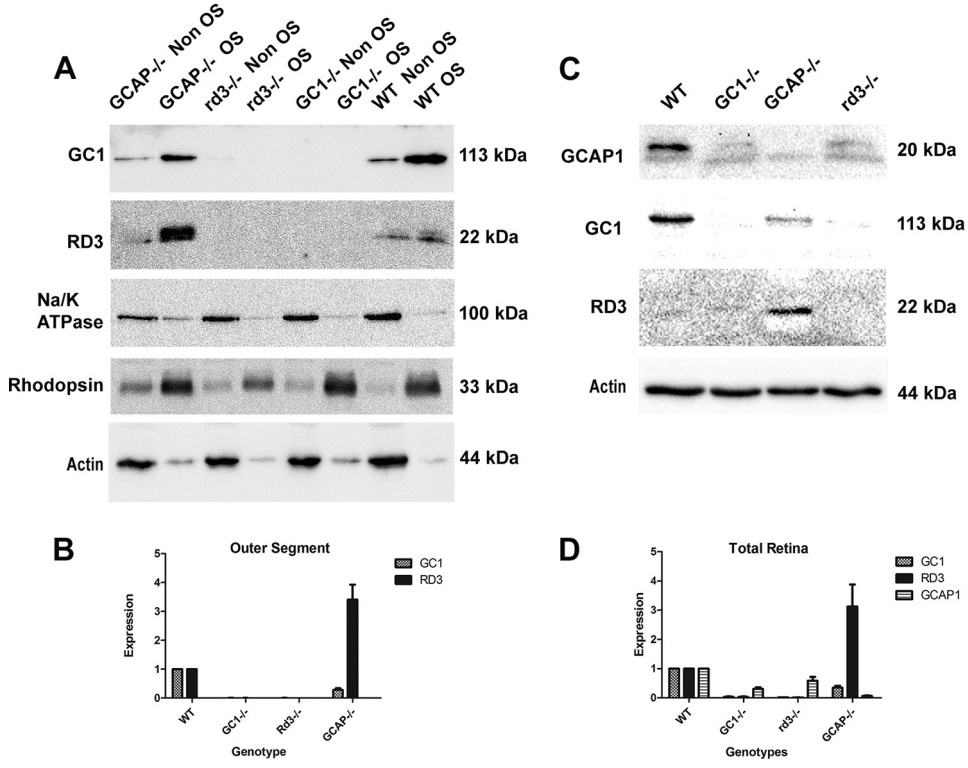


FIGURE 3. **Protein levels in WT, $GC1^{-/-}$, $rd3^{-/-}$, and $GCAP^{-/-}$ retinas by Western blotting.** *A,* proteins from POS- and IS-enriched extracts were quantified, and equal amounts were loaded on 10% SDS-PAGE under reducing conditions. To compare non-POS from different samples, actin was used as a loading control. Because of the lack of a proper loading control for comparing POS fractions between different samples, acrylamide gel was stained with Coomassie Blue after blotting. For each sample (*lane*), the entire stained proteins were used as a denominator. Membranes were probed with antibodies in the following order: RD3 (62), GCAP1 (6B12), GC1 (75), Na/K-ATPase (IS marker), rhodopsin (OS marker), and actin. *C,* we could not detect the GCAP1 protein in POS, so we used another method. Retinas were hypotonically lysed (see "Experimental Procedures"). After centrifugation for 10 min at 1000 × *g*, the pellets were discarded, and equal amounts of supernatant protein were loaded onto an SDS gel and immunoblotted for all three proteins. *B* and *D,* quantification of WT and the three mutants from *A* and *C*. The values for the mutants were compared with their WT counterparts, which were set to 100%.

using established methods (35). The GC1 level was 2.5 times lower in $GCAP^{-/-}$ POS than in WT POS (Fig. 3, *A* and *B*). The RD3 expression level in $GCAP^{-/-}$ POS was 3.1 times higher than in WT POS, confirming the IHC data (see Fig. 3 for quantification method). We could not detect GCAP1 in the POS or IS fractions by Western blotting, because most of the GCAP1 is in the soluble fraction and was lost during the process. To evaluate GCAP1 levels, retinal tissues were homogenized in hypo-

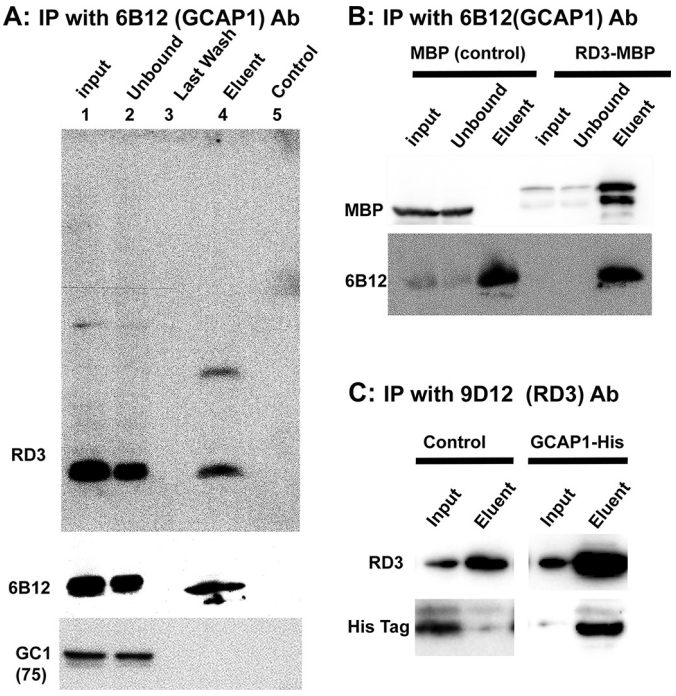


FIGURE 4. **Direct binding analysis of RD3 and GCAP1.** *A,* to detect whether RD3 and GCAP1 interact *in vivo*, a 6B12 (GCAP1)-conjugated affinity column was used to perform IP with Triton-solubilized bovine lysate. Incubation was carried out for 2 h at 4 °C. Different fractions were then separated by a 10% SDS gel and blotted. The blots were probed with RD3 antibody (Ab) and re-probed with 6B12 and GC1 antibodies. To verify the specificity of the interaction, unconjugated Sepharose 4B beads were used as negative control (*lane 5*). *B,* to determine whether RD3 and GCAP1 directly interact, RD3-MBP fusion protein was incubated with bovine lysate for 2 h at 4 °C. IP was performed with 6B12, followed by immunoblotting with anti-MBP and 6B12 antibodies. MBP tag (empty) was used as a negative control. *C,* to provide more evidence of whether the interaction of RD3 and GCAP1 is more direct rather than through other proteins, the mixture of bacterially expressed and purified full-length RD3-MBP and GCAP1-His proteins was incubated for 2 h at 4 °C in the presence of 1 mM $CaCl_2$ and $MgCl_2$, followed by IP on a 9D12 (RD3) immunoaffinity matrix. Input and bound fractions were analyzed by Western blotting with His and 9D12 antibodies. An irrelevant HIS-tagged protein, an RDS (perpherin) D2 loop of the same size, was used as a negative control under the same ionic conditions.

tonic buffer and centrifuged at 1,000 × *g* to remove the nuclei and unbroken cells. The GCAP1 level in the supernatant was 3.4 times lower in $GC1^{-/-}$ retinas than in WT retinas. RD3 and GC1 protein levels in the supernatant followed the pattern observed in isolated POS (Fig. 3, *C* and *D*). Thus, $GC1^{-/-}$ retinas have less RD3 and no GC1 and express significantly less GCAP1. In $rd3^{-/-}$ POS, GC1 and RD3 are below the detection level, and GCAP1 is reduced. In $GCAP^{-/-}$ POS, GC1 is expressed less than in WT retinas, and RD3 is highly overexpressed.

*RD3 Directly Interacts with GCAP1*—We previously showed that RD3 influences the localization and the expression level of GCAP1 (15). We next examined whether these proteins interact directly or through other proteins such as GC1. For this study, we generated a monoclonal antibody against GCAP1 (6B12) that does not recognize GCAP2 (Fig. 2*A*). Using this antibody, RD3 but not GC1 was IPed from bovine retinal extracts, demonstrating direct interactions between GCAP1 and RD3 (Fig. 4*A*). Considering the efficiency of our GCAP1 antibody (6B12), this result was unexpected. Studies using activity assays have demonstrated the interaction between GCAP1 and GC1 (28, 29, 36); however, the site of association has not been mapped at a biochemical level (see under "Discussion" for further explanation).

We further confirmed the interaction between RD3 and GCAP1 in a direct binding assay using recombinant RD3 tagged with maltose-binding protein (MBP) and bovine retinal lysate containing endogenous GCAP1. Purified RD3-MBP was incubated with bovine lysate for 2 h, after which the mixture was added to GCAP1 antibody (6B12)-conjugated beads. The beads were washed and eluted with 1% SDS, and RD3-MBP was detected in the eluent; the MBP control did not interact with endogenous GCAP1 (Fig. 4*B*). Next, we used the same strategy to verify the direct association between RD3 and GCAP1 by mixing purified bacterial RD3-MBP with purified His-GCAP1. We included 1 mM $CaCl_2$ and $MgCl_2$, which, based on our experience, reinforces the interaction of GCAP1 with its binding partners. Pulldown of the mixture with RD3 antibody (9D12) brought down both MBP-tagged RD3 and His-GCAP1 (Fig. 4*C*). RDS D2 loop-tagged with His served as a control to verify the specificity of the interaction (the same buffering conditions were provided). These results confirm that the interaction between RD3 and GCAP1 is direct and occurs post-synthesis.

*RD3, GC1, and GCAP1 Form a Complex When They Are Transiently Expressed in COS-7 Cells*—In COS-7 cells, RD3, GC1, and GCAP1 show different expression patterns when expressed individually (Fig. 5) than when they are co-expressed (Fig. 6). Single transfection of GCAP1 shows a uniform diffuse expression pattern. When GC1 is singly expressed, it exhibits ER localization, and RD3 shows a vesicular and punctated type of localization. However, when two of the three proteins are co-expressed, intracellular co-localization is observed (Fig. 6), with vesicular formation occurring when RD3 is co-expressed with either of the other two proteins. We next explored whether these three proteins form a complex, which could affect the properties or GC1 function.

We transiently transfected cells with the three cDNAs (*GC1, GCAP1,* and *RD3*) and examined their expression at several time points post-transfection. From the initial results, we selected 22- and 48-h post-transfection time points to assess their pattern of localization by IHC. At 22 h post-transfection, all three proteins were detected in vesicle-like structures distributed throughout the cells, and none were associated with the PM (Fig. 7*A*). However, at 48 h, all three proteins had reached the PM (Fig. 7*B*). Co-labeling the triple-transfected cells with trafficking marker (*RAB11*) allowed us to confirm the co-localization of GC1 and GCAP1 with RAB11. Fig. 7*C* shows the ER localization and vesicle formation of GC1 and GCAP1, clearly co-labeled with rab11 in vesicles spreading across the cells.

Fig. 7*D* illustrates the targeting of GCAP1-GFP, GC1, and RAB11 to the PM. Labeling the cells transfected with all three cDNAs with GC1, RD3, and PM marker (Vybrant CM-DII, Invitrogen) confirmed the co-localization of GC1 and RD3 to the PM at 48 h (Fig. 7*E*). The PM staining was performed with GC1, RD3, and GCAP1-GFP, although only two of three proteins can be imaged at a time, due to the limitation in the number of wavelengths used for IHC (Fig. 7*E*).

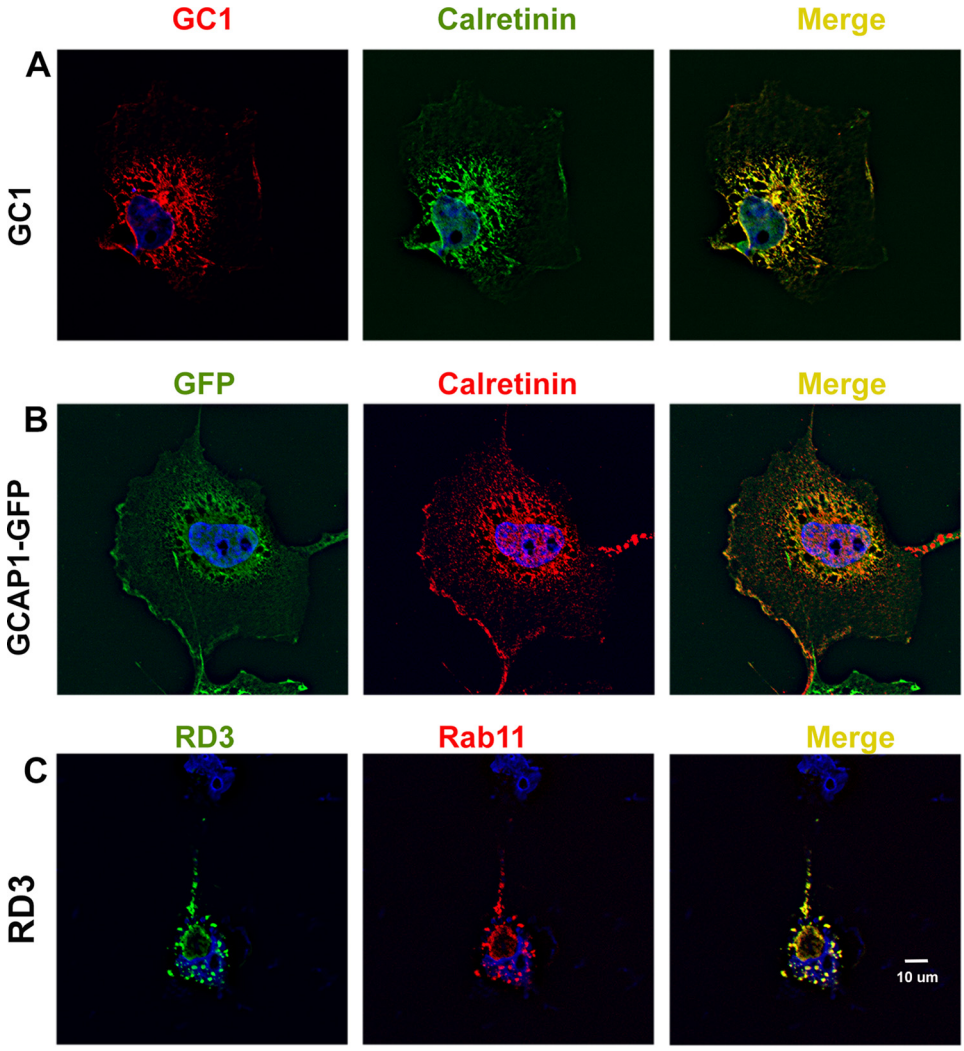


FIGURE 5. **RD3, GC1, and GCAP1 expression patterns in transfected COS-7 cells.** To monitor the expression patterns of RD3, GC1, and GCAP1 when these proteins are singly expressed in COS-7 cells, these cells were transfected with RD3, GC1, or GCAP1-GFP full-length cDNAs. After 48 h, cells were fixed and labeled with RD3, GC1, or GFP (GCAP1) antibodies. Calretinin and RAB11 were used as markers for ER and trafficking vesicles, respectively, as indicated. *A,* GC1 co-localized with the ER marker calretinin in a pattern characteristic of ER localization in GC1-transfected cells. *B,* GCAP1 co-localized with ER marker calretinin in a pattern characteristic of ER localization but in a more uniform pattern. *C,* RD3 co-labeled with trafficking marker Rab11 antibodies in cells expressing RD3.

*RD3-GC1-GCAP1 Complex Is Not Properly Formed and Targeted in GC1 (LCA1) Mutants*—To determine whether GC1-associated LCA1 mutations alter the binding/localization pattern, we co-expressed RD3, GCAP1, and four mutant forms of GC1 as follows: F565S, P858S, R995W, and M1009L known to cause severe form of LCA1 (8, 37). Each mutant formed vesicle-like structures that contained all three proteins. However, none of these vesicles targeted to the PM (Fig. 8). This observation suggests that the proper assembly of GC1 is critical for membrane targeting of GC1.

To further understand the impact of LCA1 mutations on RD3-GC1 binding capacity, we generated all known LCA1-causing GC1 mutants (4). We previously showed that RD3 directly binds to GC1 (15) and that the lack of RD3 in $rd3^{-/-}$ retinas led to lack of expression of GC1. Thus, we concluded that GC1 essentially needs RD3 to reach the membrane where its function is needed. The next question was how the binding of RD3 and GC1 is affected in GC1-associated disease mutations that led to loss of GC1 function. We sought to understand whether the LCA1 phenotypes are solely caused by the failure of RD3 to bind to GC1. In reciprocal pulldown experiments using RD3 and GC1 antibodies, we evaluated the effect of LCA1-causing mutations on the efficiency of their association. In both cases, an equal amount of extract (input) was used. It is important to mention that because the expression of GC1 mutants was always lower than their WT counterparts, we used more lysates of cells transfected with mutants. In Fig. 9*A*, RD3 antibody-conjugated CNBr-activated Sepharose beads were used to pull down WT or different GC1 mutants. Fig. 9*A* shows the input and bound (eluted) fractions for WT and each mutant. Compared with WT GC1, RD3 binding efficiency was significantly compromised in the following 10 LCA1 mutants: Y351C, L41F, F565S, R768W, P858S, A934P, L954P, R995W, M1009L, and H1019P.

Fig. 9*B* illustrates the IP between RD3 and WT and LCA1 mutant GC1, using GC1 antibody-conjugated CNBr-activated Sepharose beads. Using the same amount of lysate for

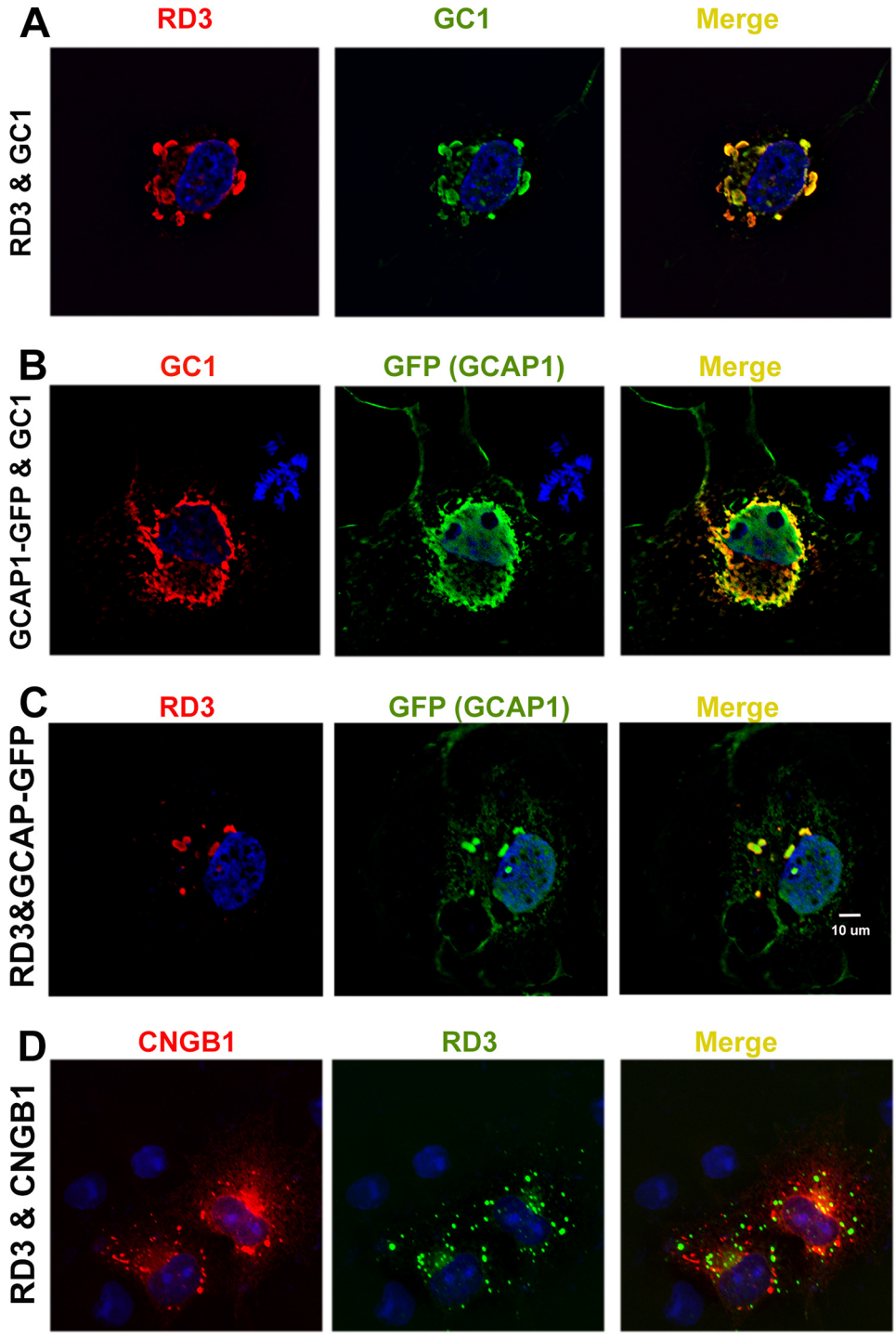


FIGURE 6. **Double expression of RD3/GC1, GC1/GCAP1, RD3/GCAP1, and RD3/CNGB1 in transfected COS-7.** The impact of RD3 on the localization of GC1 and GCAP1, when it is co-expressed with these two proteins in COS-7 cells, was evaluated in transfected cells expressing RD3/GC1 (*A*), GC1/GCAP1 (*B*), RD3/GCAP1 (*C*), or RD3/CNGB1 (*D*). Cells were labeled with antibodies to RD3, GC1, GCAP1, and CNGB1 as indicated. *A,* we previously showed (15) that in cells co-transfected with RD3 and GC1, RD3 could drive GC1 out of the ER into distinct vesicles that co-labeled with RD3 and GC1 antibodies (92% co-labeling). *B,* most GC1 was trapped in ER membranes when co-expressed with GCAP1-GFP in COS-7 cells. This combination seemed to lack the crucial elements needed to release the complex into the cytoplasm (96% co-labeling). Fusing GFP to GCAP1 did not change the localization of GCAP1 in single-cell culture (45). *C,* uniform expression pattern of GCAP1 turned into a distinct vesicular format that co-labeled with RD3 and GFP (GCAP1) antibodies when these two proteins were co-expressed in transfected COS-7 cells (90% co-labeling). *D,* distinct intracellular vesicles were labeled with RD3 and CNGB1 channel antibodies (as negative controls) in cells co-expressing RD3 and CNGB1 channels, which did not co-label. Combined, these experiments indicate that RD3 interacts with GCAP1 and GC1 and that this interaction is critical for subcellular localization.

WT and GC1 mutants, we confirmed the results in Fig. 9*A* and revealed a significantly lower ability of RD3 to bind to the 10 above-mentioned GC1 mutants. For Fig. 9, *A* and *B*, we performed statistical analysis using the Student's *t* test to examine the differences between the bound fractions of WT and LCA1 GC1 mutants (data not shown).

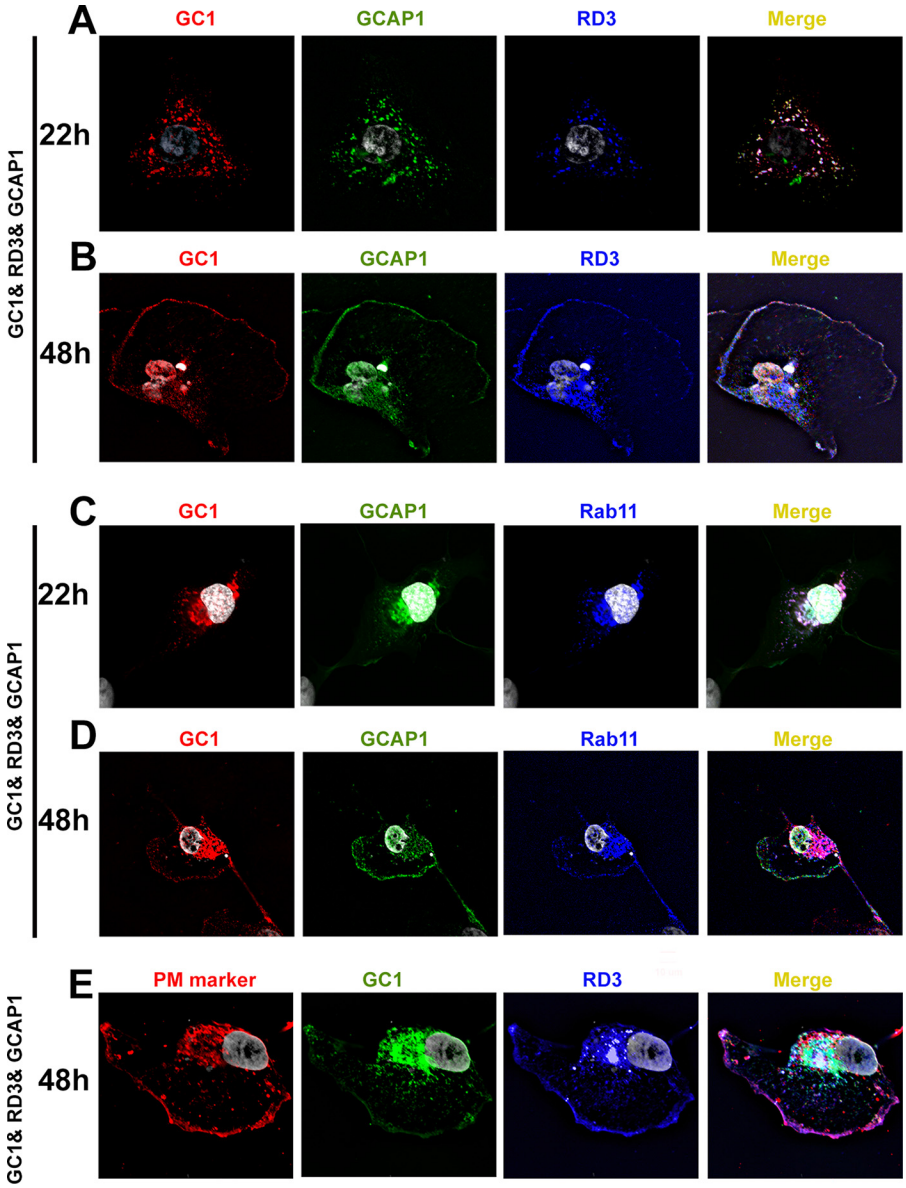


FIGURE 7. **Simultaneous expression of RD3, GC1, and GCAP1 proteins in transfected COS-7 cells.** RD3, GC1, and GCAP1 were simultaneously co-expressed in COS-7 cells and monitored 22 and 48 h after transfection. *A,* at 22 h post-transfection, cells were fixed and stained with GC1, GFP (GCAP1), and RD3 antibodies. The three proteins were co-labeled in distinct vesicles (98% co-labeling). Most cells did not show PM localization. Sparse faint PM labeling was observed. *B,* cells were fixed 48 h after transfection and stained with GC1, GFP (GCAP1), and RD3 antibodies. Most cells had all three cDNAs strongly showing the migration of RD3, GC1, and GCAP1-GFP to the PM (93% co-labeling). We observed scattered vesicles containing the proteins. *C,* expression pattern of trafficking marker RAB11 was evaluated in transfected cells with RD3, GC1, and GCAP1-GFP cDNAs after 22 h of transfection. Transfected cells were stained with GC1, GFP (GCAP1), and RAB11 antibodies. Intense immunoreactivity of the proteins was observed in distinct vesicles (98.5% co-labeling). We were not able to detect more than four wavelengths. Therefore, RD3 antibody was not used in this experiment. This limitation also applies to *D* and *E*. *D,* after 48 h of transfection, most cells receiving RD3, GC1, and GCAP1 cDNAs showed co-migration of the proteins and RAB11 to the PM. The co-localization of RD3-GC1-GCAP and RAB11 in both vesicles and the PM is evident. However, because we could not perform IHC with four antibodies, either RD3 or RAB11 is shown (89% co-labeling). At the latter time point, despite PM localization, we observed few vesicles with RD3, GCAP1, and GC1 immunoreactivity, as well as RAB11. *E,* at 48 h post-transfection, cells were stained with PM marker and GC1 and RD3 antibodies. Intense immunolabeling was observed in PM (96% co-labeling).

## DISCUSSION

Here, we report that RD3 and GCAP1 directly bind to each other and can complex with GC1. This association facilitates GC1 trafficking to the POS. The trafficking machinery transfers the RD3-GC1-GCAP1 complex to the PM, and this process requires members of the RAB family of proteins, including RAB5 and RAB11 (Fig. 7) (15). LCA1 mutants do not bind to RD3 as efficiently as the WT GC1. In transfected COS-7 cells, the RD3/GC1 (LCA1 mutants)/GCAP1 failed to reach the PM (Fig. 8). This observation suggests that a subset of LCA1 is caused by the improper assembly of these proteins, leading to impaired GC1 trafficking/catalytic activity where it is needed for phototransduction.

*RD3, GC1, and GCAP1 Are Trafficked with the Same Cargo Vesicles; They Need Each Other to Target to the OS*—With no RD3 in *rd3*$^{-/-}$ retinas, GCAP1 and GC1 do not target to the POS (15). In *GCAP*$^{-/-}$ retinas, GC1 accumulates in photoreceptor axoneme and can reach the POS to some extent. How-

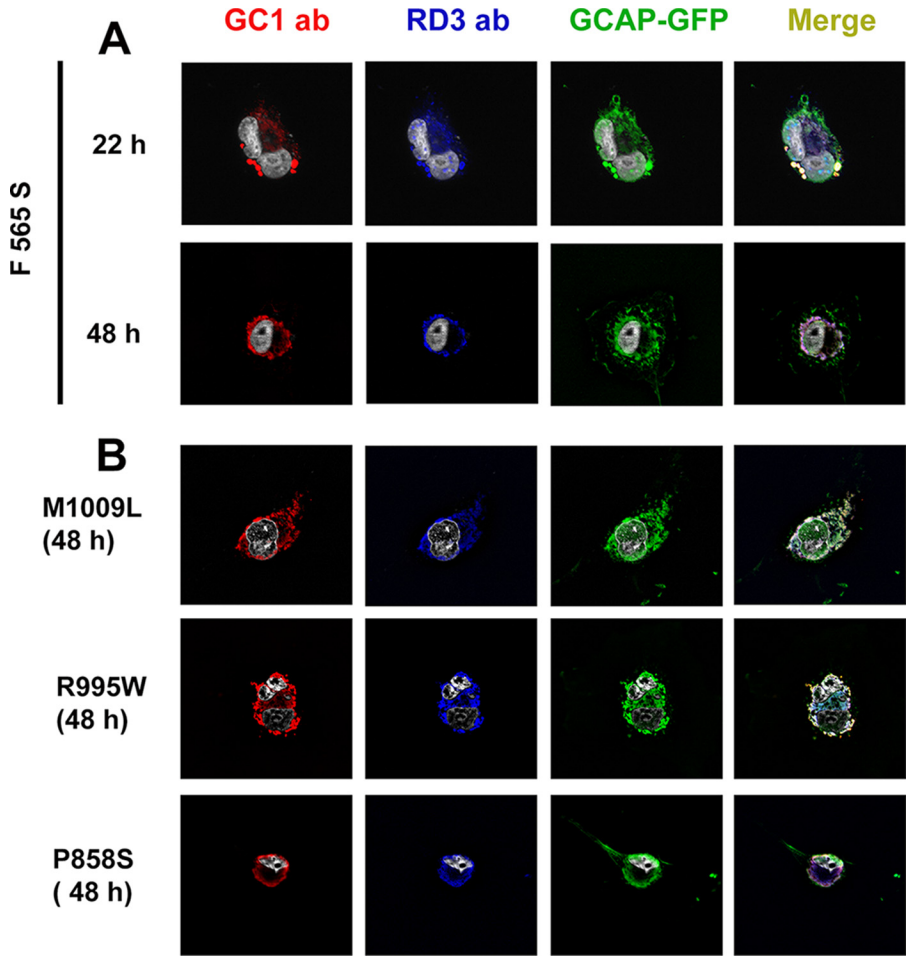


FIGURE 8. **Co-expressed RD3, GCAP1-GFP, and LCA1 mutants of GC1 accumulate in the ER membrane.** *A,* co-migration of RD3, GCAP1-GFP, and F565S-GC1 was evaluated in cells transfected with the cDNAs for these proteins. Cells at 22 and 48 h post-transfection were fixed and probed with GC1, GFP (GCAP1), and RD3 antibodies. Most cells that expressed all three proteins strongly co-localized in ER as well as in vesicle-like structures (Merge), but no PM localization was observed. *B,* co-expressed RD3, GCAP1-GFP, and LCA1 mutants of GC1 (P858S, R995W, and M1009L) accumulate in the ER membrane. Cells at 22 h post-transfection showed co-localization of the three proteins in a similar pattern to that seen with an F565S mutant (see *A*; data not shown). Cells at 48 h post-transfection were fixed and probed with GC1, GFP (GCAP1), and RD3 antibodies. These three proteins co-localized but did not spread throughout the cytoplasm or target to the plasma membrane.

ever, GC1 is unevenly distributed in the POS of $GCAP^{-/-}$ retinas (Fig. 1*M*). This observation suggests that GCAP1 is crucial for GC1 targeting and contradicts the model proposed earlier by Karan *et al.* (4), in which GC1 targeting does not depend on GCAP1. The axoneme of $GCAP^{-/-}$ retinas shows strong labeling of RD3 compared with that of WT retinas. This shows that these proteins are involved with each other in some manner and suggests that up-regulation of RD3 in the absence of GCAP1 is an attempt to compensate for the lack of GCAP1. In $GC1^{-/-}$ retinas, GCAP1 cannot target to the POS (Fig. 1*B*) and RD3 is below the detection level (Fig. 1*G*). This means that the lack of any one of the three proteins interrupts POS targeting of the other two. These proteins traffic along the axoneme of photoreceptor cells via the same mechanism, probably by the same cargo vesicles.

Our findings regarding the level of GC1 in $GCAP^{-/-}$ contradict the results shown by Mendez *et al.* (36), who found GC1 levels unchanged in $GCAP^{-/-}$ retinas. We consistently observed GC1 down-regulation in total retinal lysates from $GCAP^{-/-}$ and in POS preparations. This discrepancy is not due to the extraction procedure, as we see significant up-regulation in RD3 levels in the same samples. However, it is likely due to the type of antibody or the extraction procedure used by the aforementioned authors. Our IHC data and biochemical analyses show a strong connection between RD3, GC1, and GCAP1.

*RD3-GC1-GCAP1 Complex Assembly Is Required for GC1 Trafficking to the PM*—Previous reports from GC1 activity assays indicate that GCAP1 and GC1 interact (28, 38). However, this interaction has not been biochemically demonstrated, except by Koch (29), who used chemical cross-linkers to show their associations. The unstable interaction of GCAP1 with GC1 and its dependence on environmental ionic conditions likely explain this inconsistency. Using our well characterized and effective GCAP1 antibody allowed us to isolate and fix GCAP1 without affecting GCAP1's interaction with its binding partners. Because the existing GCAP1 antibodies were not specific and did not work with the pulldown assay, we generated a GCAP1 monoclonal antibody (6B12). Using 6B12 in bovine retinas, we showed that RD3 interacts with GCAP1 *in vivo* (Fig. 4*A*). This is the first time that the interaction of GCAP1 with any binding partners has been demonstrated biochemically.

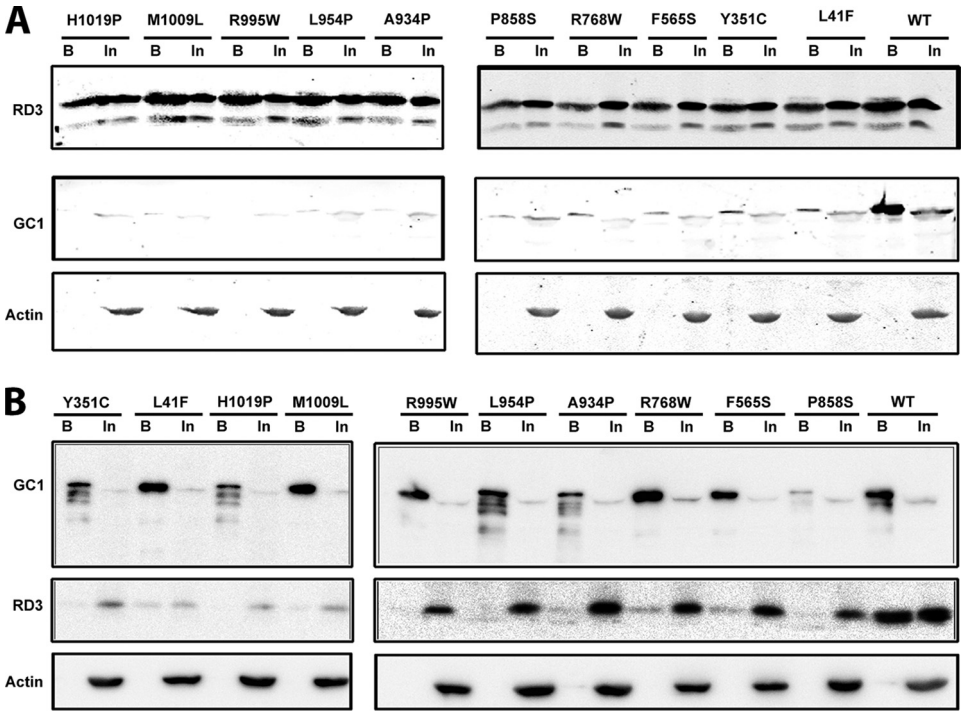


FIGURE 9. **RD3 does not bind to 10 of 24 known LCA1 mutants.** To understand the impact of disease-causing mutations on the RD3-GC1 binding efficiency, WT RD3 was co-expressed with WT and GC1 (LCA1) mutants in HEK293 cells. Forty eight hours post-transfection, cells were solubilized in 1% Triton and used as input for IP with both RD3 and GC1 antibodies. Equal amounts of input were used. The input (*In*) and bound (*B*) fractions are shown for both WT and each mutant. *A,* RD3 antibody-conjugated CNBr-activated Sepharose beads were used to pull down GC1. The same blot was subsequently probed with three antibodies in the following order: GC1 (2H6), RD3 (9D12), and actin antibodies. *B,* IP between WT RD3 and WT and GC1 LCA1 mutants using GC1 antibody-conjugated CNBr-activated Sepharose beads. The order of antibodies for *B* was as follows: RD3 (9D12), GC1 (2H6), and actin. Statistical analysis was performed using Student's *t* test to examine the difference between the bound fractions of WT and GC1LCA1 mutants. Results showed that, compared with WT GC1, the binding efficiency was significantly compromised between RD3 and the 10 following LCA1 mutants: Y351C, L41F, F565S, R768W, P858S, A934P, L954P, R995W, M1009L, and H1019P (data not shown).

Using a GCAP1–6B12 affinity column, we showed that endogenous GCAP1 binds to RD3-MBP (Fig. 4*B*). This further demonstrates that these proteins directly interact after their synthesis. This might also suggest that the two proteins interact with their mature forms as the interaction has a high binding capacity. Despite applying various ionic conditions, we have not found a biochemical interaction between GCAP1 and GC1 *in vivo*. There are three potential explanations for this finding. First, GC1 and GCAP1 binding could be influenced by other environmental factors, which we have not provided in our IPs. Second, binding might occur in a certain retinal fraction/localization, and using whole retina lysates may dilute out the form of GC1 that interacts with this complex. Finally, the GC1-GCAP1 binding is unstable in the presence of detergent. Taken together, data from IHC and direct binding assay suggest that RD3 and GCAP1 directly interact.

We co-expressed RD3, GC1, and GCAP1 in COS-7 cells and observed that the three proteins co-localized in vesicles that were targeted to the PM by 48 h post-transfection (Fig. 7). Although double-expressed RD3-GC1 and RD3-GCAP1 co-localized in large vesicles, they did not traffic to the PM (Fig. 6), showing that all three proteins were necessary for PM targeting. We further demonstrated that this trafficking machinery acquires members of the RAB family, a well established marker for endosome recycling. Based on these observations, COS-7 cells provide some insight into general aspects of protein translocation in cells. This cell system could be used as a model to study GC1 trafficking to the PM of IS.

*LCA1 Mutants Cannot Bind to RD3*—We assessed the impact of LCA1-causing mutations on the binding capacity of the complex by expressing 24 known LCA1 mutations *in vitro* and performing a binding assay of RD3 with all of the GC1 mutants. Ten mutants radically lost their ability to bind RD3, and four lacked the RD3-GC1 binding site, due to early termination. Thus, 14 mutants could not form a functional RD3-GC1-GCAP1 complex. This may explain why the vesicle-like structures found in cells transfected with RD3, GCAP1, and mutant GC1 did not disperse through the cytoplasm or traffic to the PM. The proper assembly of this complex seems to significantly affect GC1 trafficking. Previous reports (6, 39) considered a lack of GC1 activity stemming from mutations in GC1 to be the cause of LCA1. However, improper targeting leading to protein dysfunction is also likely. We showed that RD3 binding decreases GC1 activity (26). It is likely that RD3 may inhibit GC1 activity during POS trafficking to prevent GC1 from producing unnecessary cGMP in the IS. Thus, mutant forms of GC1 do not target to the POS, and RD3 remains in a "negative impact mode" to GC1. This condition prevents targeting of GC1 and leads to LCA1. Perrault *et al.* (40) evaluated RD3 mutation in patients with LCA or early-onset severe retinal degeneration and identified three *Rd3* mutations as follows: c.112C.T, c.136G.T, and a 2-bp deletion in c.137–138. The LCA12 phenotype strongly resembles LCA1.

Our previous studies showed that RD3 mutations do not affect RD3-GC1 binding capacity (26). The data suggest that

the difficulty in vision in studied probands may not be caused by mutations in RD3 protein alone. Rather, those mutations likely affect the binding capacities of RD3-GC1 or RD3-GCAP1. More research is needed to understand the underlying mechanism. Recently, Molday *et al.* (41) restored GC1 activity in *rd3*$^{-/-}$ mice with a viral gene transfer system, confirming our results, and described a role for RD3. This raises hope that simultaneous gene delivery of RD3, GC1, and GCAP1 may be a curative treatment option for early childhood LCA1.

*Conclusion*—We evaluated the strong possibility that RD3 is an important factor in LCA1. We presented evidence that RD3 and GCAP1 are likely required for *in vivo* POS targeting of GC1 as part of a GC1-GCAP1-RD3 complex. The clear regulatory role of RD3 in LCA1 makes this study scientifically and clinically important. The ionic conditions in the retina are significantly different between the IS and POS and are altered in certain circumstances. We therefore suggest that the RD3-GC1-GCAP1 complex may undergo changes in response to alterations in ionic conditions during trafficking. This can regulate different properties, including targeting of this complex and LCA1 manifestation. Further *in vivo* work on different aspects of this complex is crucial for understanding the mechanisms of GC1 trafficking and LCA1 disease.

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