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Review Article

RD3: A Challenge and a Promise

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Abstract

Photoreceptor cells are highly specialized cells that convert light energy into electric signals. Ten percent of their outer segment membranes (approximately 77 cm2 of membrane) are renewed every day. Therefore, photoreceptor cells must possess an extraordinary trafficking system to provide material needed to build up outer segment discs through a 0.3 µm diameter connecting cilium. The mechanism to traffic membrane proteins in the retina and corresponding degenerative diseases is still elusive. The retinal degeneration 3 (*Rd3*) is the gene responsible for a murine autosomal recessive hereditary retinal degeneration, which is known as Leber Congenital Amaurosis 12 (LCA12). Degeneration of photoreceptor cells begins at about two weeks of age and completes between two and four months. We generated the first antibody against RD3, and through a protein-protein interaction analysis, discovered that the RD3 protein interacts directly with guanylate cyclase 1 (GC1), and partially expresses in the outer segment. We also detected the major binding site between these two proteins and realized that RD3 is directly involved in the trafficking of this crucial protein. In a separate study; we reported that RD3 negatively regulates GC1, which is crucial for efficient trafficking of GC1. During the trafficking path, RD3 prevents unnecessary production of cGMP. It is possible that RD3 may still be involved in regulating GC1 even after targeting. Several mutations that cause visual difficulties have been reported for the mouse and human ortholog of RD3. The symptoms these mutations cause are very similar to those reported for a more severe form of blindness referred to as LCA1. Therefore, RD3 might cause a broader range of retinal diseases. Gene replacement of RD3 has been shown to restore the GC1 across the retina. This makes RD3 a novel therapeutic target for retinal targeting of impaired degenerative diseases.

INTRODUCTION

Malfunctioning of protein targeting in the retina has been linked to a number of retinal degenerative diseases such as retinitis pigmentosa and ciliopathies [1-3]. Nevertheless, the key elements and the pathways, which execute the targeting of membrane proteins, remain elusive [4-7]. One fundamental reason for this obscurity, which has slowed down the progress of the growth of this field and prevents performing developmental studies, is the complexity and uniqueness of the mammalian retina. This is because mammalian photoreceptor cells tolerate drastic qualitative changes such as remodelling, inter segmental fusion, and polarity loss when they are dissociated and therefore generation of an *in vitro* model for disc renewal and trafficking has not yet been accomplished [8].

Leber Congenital Amaurosis (LCA) is the earliest and most severe form of all inherited retinal dystrophies, characterized by blindness or severe visual impairment from birth. LCA accounts for at least 5% of all retinal dystrophies and is one of the main causes of blindness in children, which is characterized by severe loss in visual function, nystagmus, sluggish pupils and abolished or absent electroretinogram (ERG). So far, 17 different genes have been associated with autosomal recessive forms of LCA. Fundamental cell and molecular mechanisms in photoreceptor cell structure, function, and survival are regulated by these proteins such as phototransduction, vitamin A metabolism, vesicle trafficking, protein assembly, ciliary structure and transport, photoreceptor development and morphogenesis, guanine nucleotide synthesis, and outer segment phagocytosis [9-11]. Currently, no curative or preventative treatments for this debilitating disease exist [10].

Without a doubt, retinal degeneration3 (RD3) is one of the most important findings in vision research in the past decade. A stop codon within Rd3, which causes premature termination of this protein, leads to manifestation of Leber Congenital Amaurosis 12 (LCA12 [12]. This crucial protein, which has been overlooked for a long time, has helped to understand the mechanism of targeting of membrane proteins, particularly guanylate cyclase 1 (GC1). The latter plays a central role in the survival of photoreceptor cells as well as the visual cycle as its function controls the influx of $Na^{2+}$and Ca^{2+} into photoreceptor outer segments by associating with cyclic nucleotide-gated channel in the plasma [13]. Loss-of-function mutations in the GC1 gene cause another form of LCA, namely LCA1, which accounts for 20% of all LCA cases [9,10]. The guanylate cyclase

activating protein (GCAP1) is a well-known regulator of GC1 activity in an anionic dependent manner [14-16]. We showed that this protein is very much affected by the absence of RD3 as in *Rd3*$^{-/-}$ retina, GCAP1 is mis-localized to the inner segment [13]. This review will summarize what is currently known about the structure and function of RD3, and we will present an overview of the published and unpublished findings. Moreover, the current understanding of the disease related role of RD3 will be described with an emphasis on broader roles for this protein, which might be involved in other diseases directly related to the malfunction of GC1 in photoreceptor cells.

HISTORY

The *Rd3* mutation was originally found among mice captured in Valle di Poschiavo in Switzerland in 1969. The mouse strain carrying this mutation was originally known as "tobacco mouse" [17]. The mice were brought to Jackson Laboratory and were bred into four different lines: *RBF/DnJ*, *RBJ/DnJ*, *4Bnr* and *30Rk/J*. The extent of degeneration among these four genotypes varies significantly [12]. In the *Dnj* strain, degeneration is very aggressive starting from post natal (P) 14 days, and by three weeks of age, most of the photoreceptors will have been eliminated. The *4Bnr* strain has mild degeneration and massive cell death that does not commence before four weeks. The other two genotypes possess even slower rates of degeneration. Moreover, retinal degeneration in pigmented mice is slower than in the albino mice carrying this mutation. *Michael Danciger et al*. [18] suggested that genetic modifiers contribute to such non-uniformity. For many years RD3, had been overlooked in such a way that it was used for production of hybridoma because the spleen is slightly larger and found to be more efficient for the aforementioned usage [17].

GENETICS

Chang et al. [17] mapped the *Rd3* gene to mouse chromosome 1 at 10 ± 2.5 cM distal to Akp1. This was located between markers D1Mit292 and D1Mit51. Due to closeness of *Rd3* to USA2A, the field originally considered *Rd3* as a mouse ortholog of human USH2A [19]. This gene carries mutations responsible for Usher II a retinal degeneration/hearing loss syndrome. Crossing *4Bnr* mice with C57BL/6J mice enabled *Danciger et al*. [18] to narrow down the location of the *Rd3* gene between the markers D1MIT292/ D1MIT209 and D1MIT510, a far enough distance to exclude *Rd3* from being a USH2A ortholog. By using meiotic linkage and linkage disequilibrium mapping, *Kukekova et al*. [20] reduced the map of chromosome 1 to only 230 kb, which contained the three genes *TRAF5*, *C1orf36*; and *SLC30A.* The authors suggested that these genes could be potential positional candidates for *rcd2* (rod cone dysplasia type 2*).* Further analysis showed that the reduced canine *rcd2* interval overlapped with murine retinal degeneration 3 *(Rd3)*. Unlike mouse and human *Rd3,* each has a single known transcript, *Kukekova et al.* [20] detected three canine retinal *Rd3* splice variants and proposed that a sequence alteration was the cause of canine *rcd2*.

STRUCTURE

Rd3 mRNA contains 4287 bp of which only 588 bp contains the coding sequence, and the rest is N and C terminal un-translated regions. The RD3 gene codes a 195 amino acid protein and produces a 22-23 kDa protein [13]. It contains an N-terminal mitochondrial targeting signal, a possible coiled-coil domain, and two potential phosphorylation sites [21]. Our unpublished data, as well as reports from other groups [21] indicate that *Rd3* mRNA is expressed only in the retina. In situ hybridization from our lab performed on adult mouse retina shows strong expression of the *Rd3* gene in the inner segment (unpublished data) as well as outer plexiform layer, but *Lavorgna et al*. [21] showed *Rd3* mRNA in the outer nuclear layer, the inner nuclear layer, and the ganglion cell layer. *Friedman et al*. [12] reported that mouse and human *Rd3* has three exons. Using a positional candidate approach, a homozygous transition C to T in c.319 in the third exon of mouse *Rd3* causes a premature stop codon at the 107th amino acid, which alters CGA to TGA, thus converting arginine to a stop codon. The same group detected this mutation in all Rd3 mutant lines mentioned above. In the human form of this mutation, however, the stop codon occurs slightly earlier as the 99th amino acid turns out to be a stop codon [12].

LOCALIZATION

The first ever localization reported for RD3 was by *Friedman et al*. [12], who reported that this protein was associated with leukemia gene product (PML) bodies in the nucleus when they expressed GFP-tagged RD3 in COS1 cells. By expressing *Rd3* cDNA in COS1, we detected a different localization than that reported by the mentioned group. We found that RD3 was localized in vesicular structures all across the cytoplasm [13]; however, the addition of GFP has been shown to cause mislocalization [22]. Using acid purification of one of our polyclonal antibodies, we detected the RD3 protein in mouse retina mostly in the outer segment. Our continuous sucrose gradient data supported the immunohistochemistry results, where RD3 was sedimented in the same fractions as the outer segment proteins were. The only conclusion from our localization study is that RD3 is partially localized in the outer segment. We believe the localization of RD3 is not conclusive. Correct localization of this protein is critical and will certainly help to identify its function. Due to the importance of this issue, we have generated several monoclonal and polyclonal antibodies and have set this as a top priority.

FUNCTION

In 2006, we prepared five fractions of Rod Outer Segments (total ROSs, ROS membranes, soluble proteins, disk membranes, and enriched plasma membranes) and performed a tandem mass spectrometry-based proteomics approach to identify unknown proteins in rod outer segment [23]. This experiment identified 4,335 peptides from which 516 unique proteins were classified into seven categories according to their known or predicted functions: phototransduction, metabolism, structural, housekeeping, transport, vesicular trafficking, and uncharacterized proteins. An unknown protein was amongst the last category, which at that time was called *C1ORF36*. One primary reason to investigate RD3 over other proteins was the strong engagement of this protein with the retinal membranes. This was evident from the two following observations: 1) the amino acid composition of RD3 with a low hydrophobicity suggested that RD3 would be soluble, but it was highly insoluble

and 2) we were not able to hypotonically release the RD3 from the surrounding membranes. The latter was achievable by lysing the expressed RD3 in 6M urea (data not shown). This data suggests that RD3 is strongly associated with, but not integrated in to the retinal membranes.

The preparation of antibodies against RD3 has always been challenging. The biggest obstacle stemmed from the inability of the N-terminus regions to boost immunogenicity in a mouse. When used, the middle and C-terminus produced antibodies against a nonspecific protein, which was detected by several of our antibodies with a much higher affinity than the conventional RD3 (22 kDa). The second challenge was that RD3, expressed in a bacterial system, was so insoluble that even small pieces of this protein with a Glutathione S-Transferase (GST) tag resulted in bacterial inclusion bodies. In our experience, other tags did not boost detectible immunogenicity against this protein, and thus restricted us to a small protein part for each fusion. We solved this issue by generating a larger number of overlapping fusion proteins. Finally, in the spring of 2009, after a long-term challenge, we generated the first specific monoclonal antibody against this protein [13]. We showed that it directly interacts with GC1 and GC2. GC1 is a membrane protein located in the light-sensitive outer segment of rod and cone photoreceptor cells. In vertebrate rods and cones, photon absorption by rhodopsin or cone visual pigments triggers the hydrolysis of cGMP by activating a transducin-phosphodiesterase 6 (PDE6) cascade, which results in hydrolysis of cGMP and closure of the cGMP-gated cation channels (CNG) in the plasma membrane and membrane hyperpolarization. To recover their function photoreceptor cells must quickly restore the level of cGMP by retinal-specific GC1. Therefore, the function of this enzyme in the production of cGMP is absolutely crucial for phototransduction and the visual cycle. Although the enzymatic properties and activity of GC1 have been studied, the molecular determinants that are responsible for regulation of this activity are incompletely understood. Similarly, even though abnormal targeting of GC1 in the retina underlies several degenerative diseases, the mechanisms of trafficking and outer segment targeting of this protein in photoreceptor cells remains unknown [14,24].

To validate the binding of RD3 and GC1 and to investigate further into the nature of interaction of these two proteins, we co-expressed these two proteins in HEK293 cells and used antibodies against both RD3 and GC1 to perform reverse immunoprecipitation. RD3 and GC1 strongly pulled down one another, which showed that the interaction is likely direct. We next looked into the impact of RD3 on GC1 by co-expressing these two proteins in COS 7 cells. GC1 single transfection resulted in endoplasmic reticulum localization and RD3, when expressed individually, showed a vesicular (punctuated) pattern of expression. Co-expression of these two proteins, however, changed the pattern of expression of these proteins in a way that both proteins turned into round vesicles spreading all across the cytoplasm, which co-labeled with ras-related proteins family member, rab11. Next, we generated deletion mutants of GC1 and co-expressed them with RD3 in HEK 293 cells. Using cyanogen bromide activated beads to conjugate with RD3 antibody and performing immunoprecipitation, we determined the binding site between the two proteins [13]. In a separate publication, [25] to evaluate the impact of RD3 on the activity of GC1, we measured the activity of GC1 in two separate assays. First, the GC1 activity in rod outer segment preparations (containing endogenous GC1 and GCAP1) were measured in the presence of increasing concentrations of 1D4-tagged RD3 purified from bacteria via a 1D4 column. In a subsequent set of experiments, the membrane fraction of hypotonically lysed cells transfected with GC1 was incubated with myristoylated GCAP-His purified from bacteria and RD3-1D4 purified from transfected HEK293 cells. We found that recombinant RD3 expressed in transformed human cell culture or in bacterial cells inhibits GC1/GCAP1 complex activity in HEK293 cell membranes and rod outer segment. This data demonstrates that RD3 is a negative regulator of GC1 activity.

Moreover, our unpublished data suggest that RD3 has another binding site in the cytoplasmic domain of GC1. The binding affinity through this binding site is much less than the main binding site. The latter is called "RD3-GC1 second binding site." This matter is currently being extensively studied in our laboratory. Together, RD3 regulates the function and trafficking of GC1 through one of the following two models (Figure 1). In model A, RD3 competes with GCAP1 in binding to GC1 therein preventing the activation of GC1. This happens during the trafficking of this complex and dark stage of phototransduction when GC1 remains inactive. In the second model (Figure 1B), RD3 binds to GCAP1, and by conferring conformational changes to this protein, regulates the activity of GC1. In fact, in both models GCAP strongly interacts with RD3, but the nature of this interaction is different in models A and B. It is possible that in the second model, RD3 binds to both GC1 and GCAP1 simultaneously, through the second binding site regulates the activity of this protein, and by the main binding site controls the trafficking of GC1 to the outer segment.

In a recent study by Molday et al. [11], they used an Adeno-Associated Viral Vector (AAV8) with a Y733F capsid mutation containing the mouse RD3 complementary DNA (cDNA), driven by human rhodopsin kinase promoter to subretinaly deliver the *Rd3* to photoreceptor cells of 14-day-old of two strains of *Rd3*

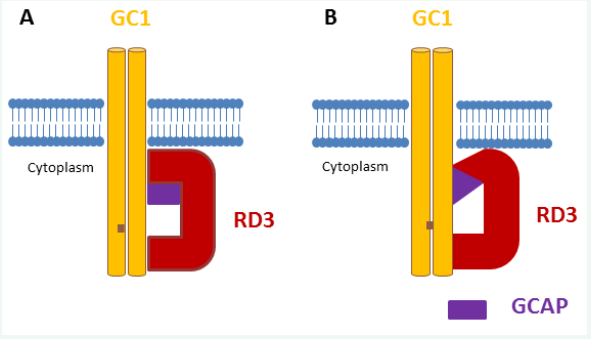

Figure 1 RD3 regulates GC1 in either of the following possible mechanisms: (A) the RD3-GC1 second binding site (see the text) competes with one or a combination of the GC1-GCAP1 binding site [26], and consequently negatively regulates the GC1 by preventing binding of GCAP1 to GC1. The major RD3-GC1 binding sites (amino acid 1055-1068) [13] regulate the targeting of GC1 to photoreceptor outer segment; or (B) RD3 interacts with GCAP1 and causes conformational changes in this protein; therefore, GCAP1 cannot activate GC1. In this model, the RD3-GC1 main binding site (amino acid 1055-1068) regulates the targeting of GC1 to outer.

mice; *4Bnr/J* and *In (5)30Rk/J*. Immunofluorescence microscopy was applied to determine whether *Rd3* transgene expression restores endogenous GC1 expression and localization to the photoreceptor outer segment layer. Strong GC1 expression was detected in the outer segment of treated mice two weeks post-injection with expression throughout most of the retina. They monitored the prolongation of the GC1 expression in treated retinas and observed the strong GC1 expression for at least seven months. In treated retinas, GCAP1 was restored in the inner segment and to some extent reached the outer segment of photoreceptor cells. To determine the impact of *Rd3* delivery on photoreceptor survival, *Molday et al*. [11] measured the thickness of outer nuclear layer of photoreceptor cells by counting the number of photoreceptor nuclei rows. In healthy retinas, the number of rows in the outer nuclear layer is 10-11. They observed that the treated retinas predominantly have six to eight rows of nuclei. In contrast, the untreated eye was reduced to a single layer. Scotopic and photopic ERG was further used in treated and untreated *Rd3*-/-mice by the mentioned group to evaluate the impact of AAV-mediated *Rd3* delivery on both rod and cone visual function. There was a strong scotopic and photopic response in the treated eye and a significantly reduced response in the untreated eye at 51 days post-injection. They suggested that the aberrant labelling of GC1 in the inner segment is an indication that RD3 is essential for targeting of GC1. This is the *in vivo* conformation of the work performed by *Azadi et al*. [13] who initially reported that RD3 traffic GC1 to outer segment. The work performed by *Molday et al*. [11] is a "proof of concept" for AAV-mediated gene therapy as a potential therapeutic treatment for LCA12, and raises the hope that the diseases caused by the lack of GC1 function can be cured by subretinal gene delivery of *Rd3* to the retinal photoreceptor cells.

DISEASE

Friedman et al. [12] performed a mutation analysis on 461 probands with retinopathies and reported an alteration of G to A in exon 2 of *Rd3* in one patient, which produced a stop codon at amino acid 100 and resulted in a premature truncation. In the course of screening they found several other alterations: p.W6R, p.E23D, p.K130M. p.G57V, p.W6R/ E23D, p.R68W, p.R167K and p.D195V. They generated p.W6R, p.E23D, p.K130M, and p.W6R; E23D mutations in the RD3-GFP expression construct. The localization of the mutants did not differ from wild type counterpart. We constructed the 6 former mutants and performed a binding analysis with the full-length GC1 when we co-expressed them in HEK293 cells. The binding of GC1 and above mentioned mutants were the same as wild type [25], which confirmed the results from Friedman et al. [12]. It seems that the mentioned mutation can hardly be the cause for the vision difficulties of the probands carrying these mutaions. Perrault et al. [27] performed a comprehensive survey of *Rd3* mutations and their clinical expressions in 852 patients affected with LCA or early-onset and severe retinal degeneration. They identified three *Rd3* mutations: c.112C.T, which caused p.R38*; c.136G.T, which caused p.E46*; and a 2 bp deletion in c.137–138, which caused a stop codon in amino acid 84. They suggested that the LCA12 phenotype in studied probands were highly similar to those of LCA1. *Preising et al*. [28] studied retinal degeneration in a consanguineous Kurdish family with LCA/early onset retinal dystrophy. They observed a severe form of retinal dystrophy that showed up at a very early age. Taken together, the mutants carrying defected *Rd3* resemble LCA1 and due to overlapping in the mechanism of action, distinguishing these two diseases is not feasible.

CONCLUSION

Results from our laboratory as well as the data from others have revealed an understanding that RD3 is a very crucial protein for trafficking, regulation, and the manifestation of diseases that involve GC1. The overlapping symptoms in patients carrying *Rd3* mutations (LCA12) with LCA1 would raise the possibility that the same mechanism of disease may be the cause of both diseases. In both cases, we believe that the impaired targeting of GC1 is the causal factor of the diseases. We are currently investigating the molecular mechanisms behind the targeting of GC1. The outcome will identify pathways and the key molecules, which execute such a sophisticated mechanism of action. The knowledge from the latter could be adapted for unravelling the trafficking mechanisms of other important photoreceptor membrane proteins.

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