Prolonged survival of the phosphorylated form of rhodopsin during dark adaptation of Royal College Surgeons rat

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Abstract To study rhodopsin (Rho) phosphorylation and dephosphorylation in Royal College of Surgeons (RCS) rat retina, specific antibodies toward major Rho phosphorylation sites in vivo, 334Ser or 338Ser, were prepared by immunization of authentic phosphorylated peptides in rabbit. Enzyme-linked immunosorbent assay identified that the raised antibodies exclusively recognized either the phosphorylated 334Ser or 338Ser site. In immunofluorescence labeling, both antibodies recognized photoreceptor outer segments in light-adapted retinas from Sprague–Dawley (SD), Brown–Norway (BN) and RCS rat. During dark adaptation, immunoreactivities toward phosphorylated 338Ser and 334Ser sites were diminished within several hours (0.2–2 h) in SD and BN rat retinas. However, those toward phosphorylated 338Ser and 334Ser sites were diminished within 4 to 7 days in RCS rat retinas. In vitro studies demonstrated decreased levels of both Rho phosphorylation and dephosphorylation reactions in RCS retinas. However, the dephosphorylation reaction was much more greatly affected than the phosphorylation reaction. Extremely prolonged survival of phosphorylated forms of Rho may contribute to persistent misregulation of phototransduction processes in retinal degeneration in RCS rat.
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Key words: Rhodopsin; Phosphorylation; Retinal degeneration; Immunocytochemistry

1. Introduction

Royal College of Surgeons (RCS) rat is the best studied animal model for human retinitis pigmentosa (RP) [1]. In terms of their molecular pathology of retinal degeneration, it was revealed that mutation of the gene encoding the receptor tyrosine kinase *Mertk* [2] causes an inability of phagocytosis of the shed tips of rod outer segment (ROS) debris by retinal pigment epithelium (RPE) [3,4]. Preservation of RCS photoreceptors was induced by viral gene transfer of *Mertk* gene [5], and mutations in the identical human homologue, *Mertk* gene, have been recognized in some patients with RP [6]. In terms of RCS photoreceptor cells, we recently found significantly reduced expressions of α-A crystallin and rhodopsin kinase (RK), which are thought to be involved in post-Golgi processing of opsin and rhodopsin (Rho) phosphorylation respectively, in RCS rats than those in the control

rats at the age of 3–4 weeks [7]. Therefore, we suggested that misregulation of phototransduction pathways in rod photoreceptors may be synergistically involved in the pathogenesis of RCS retinal degeneration with the primary deficit being in RPE phagocytosis.

In vertebrate retinal photoreceptors, it is known that phosphorylation and dephosphorylation of Rho is most importantly involved in the regulation of phototransduction cascade [8]. In terms of phosphorylation sites in Rho, in vitro and in vivo studies revealed that light dependent phosphorylation occurs at multiple C-terminal Ser residues (334Ser, 338Ser and 343Ser) [9–12]. We previously demonstrated that monophosphorylated Rho at 334Ser and 338Ser were predominantly found in mice retinas upon flash and continuous illumination, and these sites had different kinetics of dephosphorylation and spatial distribution within ROS [10]. Therefore, taken together, it is of great interest to study in vivo Rho phosphorylation and dephosphorylation in RCS retinal degeneration.

Here, to gain additional insight into photoreceptor functions during RCS retinal degeneration, we prepared specific antibodies toward major Rho phosphorylation sites, 334Ser and 338Ser, and studied in vivo Rho phosphorylation and dephosphorylation in RCS and control, Sprague–Dawley (SD) or Brown–Norway (BN), rat retinas utilizing these antibodies in conjunction with in vitro biochemical assays of Rho phosphorylation and dephosphorylation.

2. Materials and methods

Unless otherwise stated, all procedures were performed at 4°C or on ice using ice-cold solutions. RCS, SD or BN rats (Crea, Tokyo, Japan) reared in cyclic light conditions (700 lux, 12 h on/12 h off) were used. Authentic phosphorylated peptides, P-Rho334-peptide (DDEApSATASK), P-Rho338-peptide (CEASATApSKT), and non-phosphorylated peptides, Rho334-peptide (DDEASATASK) and Rho338-peptide (CEASATASKT) were purified by HPLC column (purities > 98%). Rat ROS and urea-washed ROS membranes were prepared using 200 freshly dissected retinas from dark-adapted BN rats as described previously [12]. Rho concentrations were determined in detergent solutions from the difference in absorbance at 500 nm before and after bleaching in the presence of NH_2OH by assuming ε_{500} to be 41 000 and the molecular weight to be 38 000.

2.1. Preparation of specific antibodies toward phosphorylated Rho at 334Ser or 338Ser

Specific antisera toward phosphorylated Rho at 334Ser or 338Ser were obtained by immunization of phosphorylated authentic peptides P-Rho334 peptide or P-Rho338 peptide chemically conjugated with bovine thyroglobulin and complete adjuvant to rabbit. Antisera were each further purified into IgG [7], and 0.1 mg of them were each incubated with urea-washed ROS (20 mg of Rho) at room temper-

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ature for 2 h. Then the mixture was ultracentrifuged at $100\,000\times g$ for 1 h. The resultant supernatant was used as specific antibody toward phosphorylated Rho at 334Ser or 338Ser.

2.2. Preparation of phosphorylated photolyzed Rho (P-Rho*)

After overnight dark adaptation, fresh ROS (5 mg of Rho) obtained from 6- to 8-week-old BN rats [12] was dissolved in 0.5 ml of buffer A (100 mM Na-phosphate buffer, pH 7.2, 5 mM MgCl$_2$, 100 mM KCl) containing 0.5 mM [γ-^{32}P]ATP (300 cpm/nmol) or cold ATP and incubated at 30 °C for 10 min under a 150-W lamp from a distance of 20 cm. The reaction was terminated by the addition of buffer B (200 mM Na-phosphate buffer, pH 7.2 containing 5 mM adenosine, 100 mM KF and 200 mM EDTA). P-Rho* pellet was obtained by centrifugation. For Rho phosphorylation kinetics, radioactive fresh P-Rho* pellets from four dark-adapted retinas (3-week-old SD, BN or RCS) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Rho band was cut out and its radioactivity was counted.

2.3. Immunofluorescence microscopy

Under deep ether anesthesia, rats kept under different illumination conditions were perfused with 300 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS), and retinas were dissected out and embedded in paraffin. After the deparaffinization, retinal sections vertically through the optic disc at 4 μm thickness were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h and then incubated successively with anti-P-Rho antibodies (1:500) and FITC-conjugated anti-rabbit IgG (Cappel, Durham, NC, USA) for 1 h each at room temperature.

2.4. Dephosphorylation of P-Rho*

Quantifying levels of dephosphorylation of P-Rho* was performed as described previously. [14] Briefly, radioactive rat P-Rho* pellet (5 mg of Rho) was extensively washed with hypotonic and isotonic buffers and incubated with fresh ROS (1 mg of Rho) from RCS rat (3 or 4 weeks old) or SD rat (3 weeks old) at 30°C under dark for 1 to 6 h. After the incubation radioactive counting was performed in scintillation cocktail after solubilization by perchloric acid.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Authentic peptides (50 μg/ml) in PBS were coated onto 96-well microtiter plate (Dynatech CO.) overnight at 4°C. After blocking with 1% BSA for 1 h at room temperature, the wells were incubated successively with anti-peptides antibodies at graded dilutions (0–25 000 times) and HRP-labeled anti-rabbit IgG (1:3000 dilutions, Cappel, Durham, NC, USA) for 1 h at 37°C. The wells were extensively washed with 0.035% Tween 20 in PBS and antigen-antibody binding was visualized with o-phenylenediamine as a substrate. The OD was measured at 492 nm.

3. Results

As shown in Fig. 1A,B, anti-P-Rho334 or anti-P-Rho338 IgG exclusively recognized P-Rho334 or P-Rho338 peptide, respectively, with no immunoreactivities toward other peptides. In Western blot analysis (Fig. 1C), these anti-P-Rho IgGs specifically reacted with P-Rho, but had no immunoreactivity toward the truncated form of Rho obtained by endoproteinase Asp-N, which was missing the C-terminal 19 amino acids, ^{330}DDEASATASKTETSQVAPA, containing all possible phosphorylation sites [13].

Using these specific antibodies, immunohistochemical study was performed. Immunoreactivities by these antibodies were specifically recognized retinal outer segments of light-adapted BN (lanes 2–4) and RCS rats (lanes 5–7), and those were diminished within 1 h and 7 days, respectively (Fig. 2). These changes in immunofluorescence labeling indicated light-dependent phosphorylation of Rho at 334Ser and 338Ser sites and their dephosphorylation after following dark adaptation. During the dephosphorylation of both sites, tips of ROS were
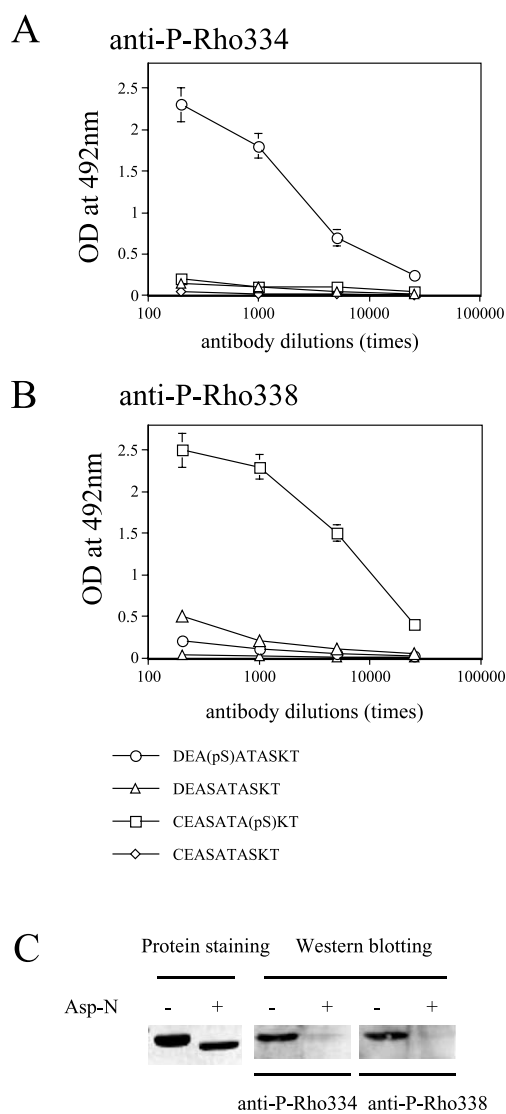

Fig. 1. Characterization of anti-P-Rho334 and anti-P-Rho338 IgG. Affinity purified anti-P-Rho334 or anti-P-Rho338 IgG were subjected to ELISA (A,B) using authentic peptides and Western blot analysis using endoproteinase Asp-N treated (enzyme:substrate = 1:1000 for overnight at room temperature) [13] or non-treated P-Rho* (C). In Western blotting, primary and secondary antibodies were probed at 1:2000 dilutions and specific antibody/antigen binding was visualized by ECL (Amersham)[7]. ELISA was performed in triplicate. Data are expressed as the means ± S.D.

slower than bases as similarly observed in our previous study using mice [10]. Using this method, kinetics of dephosphorylation of phosphorylated 334Ser and 338Ser sites of normal (BN and SD) and RCS rats were studied by comparing their changes in vertical lengths of immunofluorescence-labeled ROS during the dark adaptation (Fig. 3). In BN rat, dephosphorylation of 334Ser and 338 Ser sites went to completion within 1 or 0.2 h, respectively, and in SD rat, immunostaining by P-Rho338 was diminished until 1–3 h dark adaptation, while that toward P-Rho334 remained until 2–4 h dark adaptation. In RCS rat, however, dephosphorylation of both sites was much slower than in the normal control rat (SD and BN) retina. That is, immunostainings by P-Rho338 and P-Rho334 were diminished until dark adaptation for 4 and 7 days, respectively.
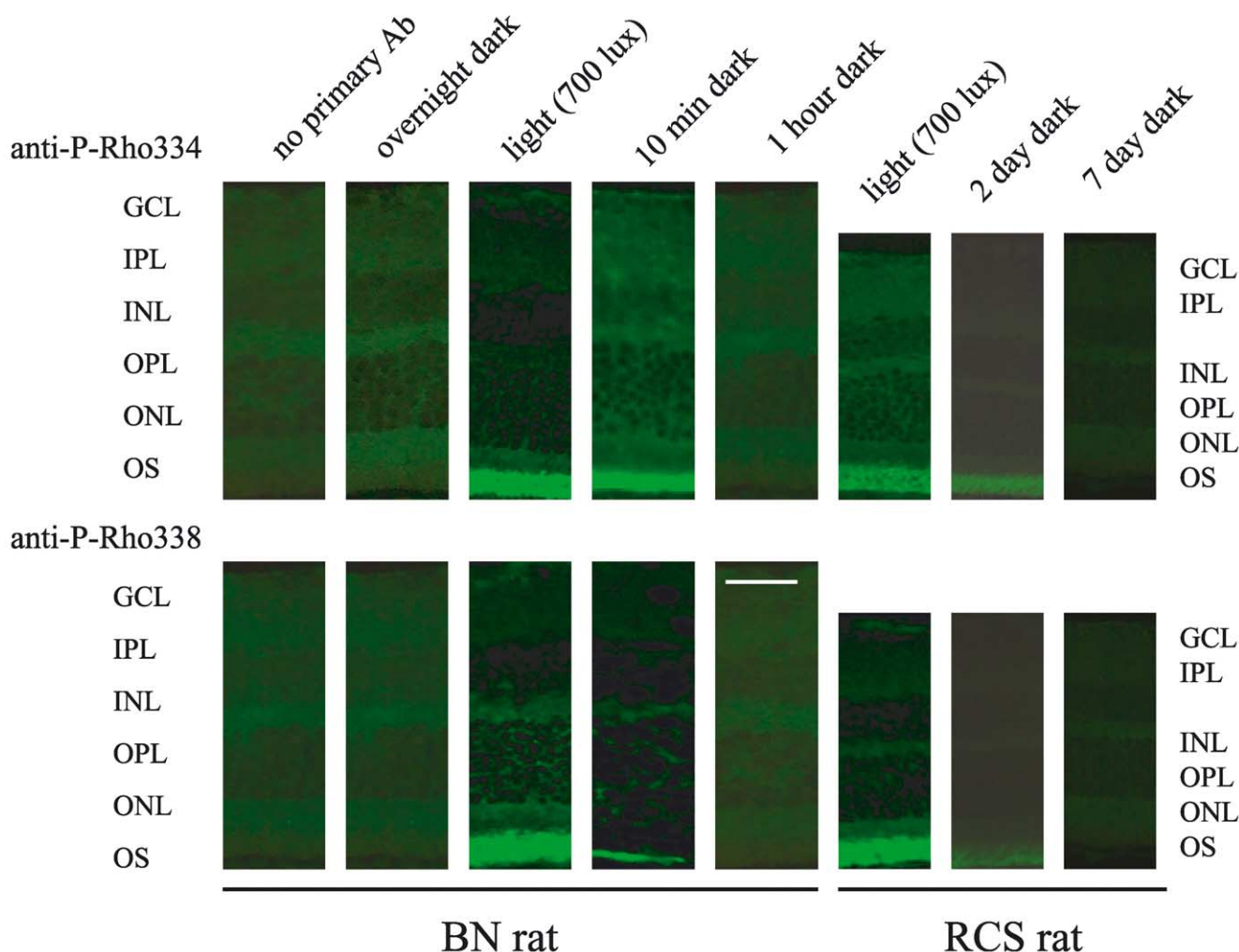
Fig. 2. Immunofluorescence labeling of anti-P-Rho334 and anti-P-Rho338 IgG in light- or dark-adapted rat retinas. Retinas obtained from 3-week-old BN and RCS rats kept under several illumination conditions were subjected to imunofluorescence labeling by anti-P-Rho antibodies. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment. Scale bar = 50 μm.

The in vitro biochemical assay of Rho phosphorylation revealed that levels of Rho phosphorylation in 3-week-old RCS rat were slightly lower than those in 3-week-old SD rat, and those of RCS rat decreased to approximately 20% of the initial levels in 3–5 weeks (Fig. 4, upper panel). In terms of dephosphorylation of P-Rho, approximately 40% of P-Rho was dephosphorylated by SD ROS (3 weeks old) during 6 h incubation, while almost no dephosphorylation was observed by incubation with ROS from 3- or 4-week-old RCS rat (Fig. 4, lower panel).

4. Discussion

In the present study, we studied Rho phosphorylation and dephosphorylation in normal (SD, BN) and retinal degeneration rats (RCS) using specific antibodies toward major phosphorylation sites of Rho in vivo, 334Ser and 338Ser, and found following observation; 1) Dephosphorylation of 338Ser and 334Ser went to completion within 0.2 and 1 h of dark adaptation in BN rat retina. This result is closely comparable with previous results obtained by biochemical assays

using mice [10,11]. 2) Dephosphorylation of both sites in albino species (SD rat) took slightly longer time periods (2–4 h). 3) However, dephosphorylation of these sites in RCS rat retina took place during several days of dark adaptation. In addition, biochemical assays demonstrated that in RCS retina, phosphatase activities were much lower than kinase activities in terms of Rho phosphorylation as compared with SD retina. Therefore, our data indicated that misregulation of the visual transduction cascade in photoreceptor by marked survival of phosphorylated forms of Rho may facilitate RCS retinal degeneration caused primarily by *Mertk* mutation. Similar to this mechanism, we have found that, in retinal degeneration model of cancer-associated retinopathy, anti-recoverin antibody was internalized into photoreceptor cells [15,16], and blocked recoverin function, Ca^{2+}-dependent regulation of RK, resulting in enhancement of Rho phosphorylation. In addition, Rho mutants within the C-terminus, in which 345Val and 347Pro are the most common sites of mutations causing autosomal dominant retinitis pigmentosa [17,18], were phosphorylated at significantly higher levels compared with wild type [19].

It was suggested that down-regulated or absence of Rho phosphorylation can cause retinal degeneration, based upon several studies using site-directed mutagenesis of Rho or transgenic mice carrying Rho with deleted phosphorylation sites. However, it was demonstrated that the C-terminus of Rho possesses several biochemical properties including gene product synthesis, transport and immunogenic properties, in addition to phosphorylation sites [20–22]. Therefore, retinal degeneration in these Rho mutants may be caused by mechanisms other than down-regulation of Rho phosphorylation. It was reported that RK mutations (deletion of the C-terminus) cause Oguchi disease, an autosomal recessive form of the congenital stationary night blindness [23–25], and a functional assay identified that such a RK mutant is an inactive form [26]. Oguchi disease is not a retinal degenerative disease but is clinically characterized by normal light adaptation and very
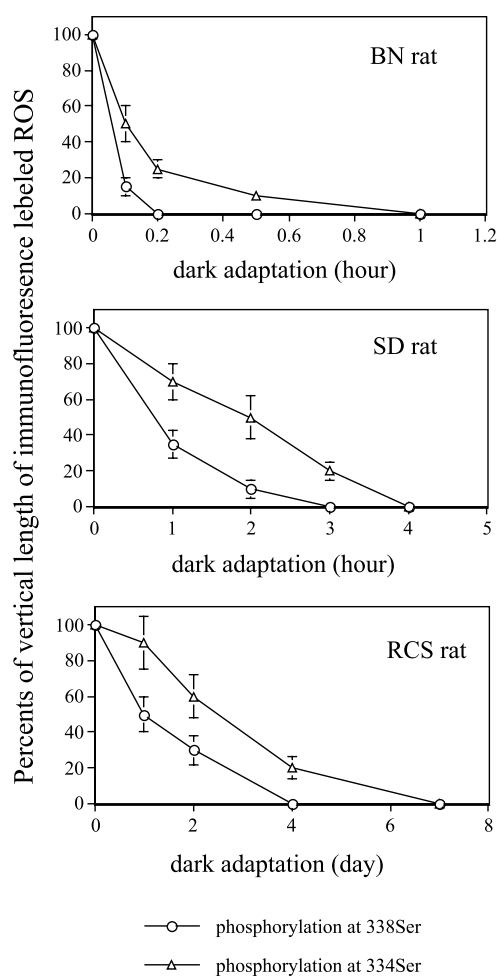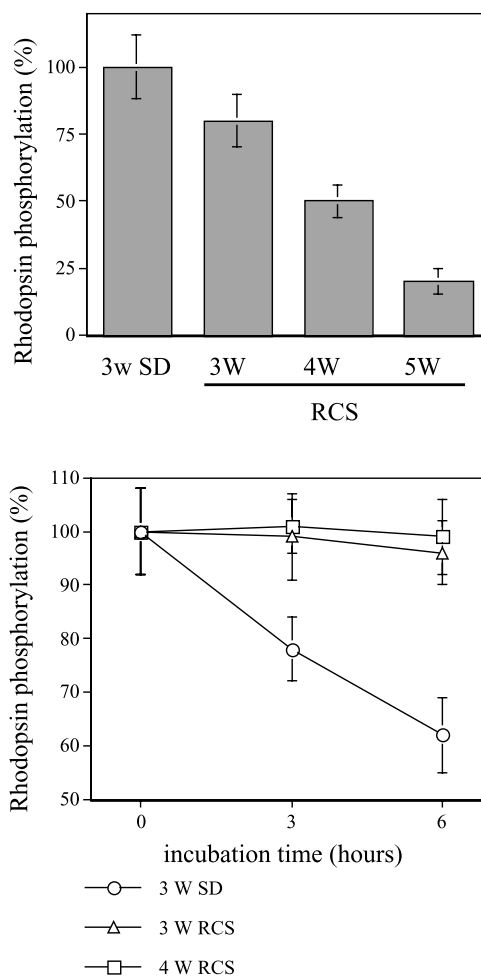

Fig. 4. Rhodopsin phosphorylation and dephosphorylation in vitro. Upper panel: Rho phosphorylation was performed using [γ-32P]ATP and ROS of RCS rats (3–5 weeks old) and SD rats (3 weeks old) as described in Section 2. After the reaction, the radioactive Rho bands in SDS–PAGE was counted and plotted. Lower panel: De-phosphorylation of Rho of 3-week-old SD and 3- to 4-week-old RCS rat ROS was studied over 6 h by incubation with radioactive P-Rho* pellet as described in Section 2. Experiments were performed in triplicate using fresh preparations. Data are expressed as the means ± S.D.

Fig. 3. Kinetics of dephosphorylation in phosphorylated 334Ser and 338Ser sites in BN, SD and RCS rats. Three-week-old BN, SD and RCS rats ($n = 15$ rats each) kept under illumination condition (700 lx) for 1 h were incubated under dark condition. At different time points (BN rats: 0, 0.1, 0.2, 0.5 and 1 h; SD rats: 0, 1, 2, 3 and 4 h; RCS rats: 0, 1, 2, 4 and 7 days), three rats (six eyeballs) in each category were subjected to imunofluorescence labeling by anti-P-Rho antibodies. Photographs of the sections were taken. Vertical length of photoreceptor outer segment layers and that of fluorescence labeling were measured at temporal points 1.0 mm apart from optic disc from six different points from six different eyeballs and their ratios were plotted. Data are expressed as the means ± S.D.

slow dark adaptation [25]. In addition, RK knock-out mice displayed significantly slow recovery from light stimuli, although the retinal morphology was normal [27]. Therefore, taken together with the above observations, we speculated that down-regulated Rho phosphorylation causes dysfunction of phototransduction in photoreceptors but not retinal degeneration, while misregulation of the phototransduction pathway by abnormally high levels of Rho phosphorylation may constitute a common mechanism causing photoreceptor cell death.

In conclusion, significantly prolonged survival of phosphorylated form of Rho found in RCS rat retina leads to misregulation of the visual transduction cascade and this may be an important mechanism responsible for retinal degeneration in RCS rat.

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