Low Expression of αA-Crystallins and Rhodopsin Kinase of Photoreceptors in Retinal Dystrophy Rat

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PURPOSE. The Royal College of Surgeons (RCS) rat has been extensively characterized as a model for inherited retinal dystrophy such as retinitis pigmentosa. In the present study, compositions of retinal proteins were compared between RCS (rdy−/−) and control (rdy+/+) rats during progression of the disease to understand the molecular pathologic course of the retinal degeneration.

METHODS. Protein mapping was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional (2D)-PAGE using whole retinas or rod outer segments (ROS) obtained by a sucrose-density gradient centrifugation method from RCS or control rats at the age of 3 to 8 weeks.

RESULTS. 2D-PAGE showed that retinal proteins of RCS rats were generally less abundant than those of the control animals and that the difference became more evident with aging. However, no significant difference was observed in the protein-mapping patterns in 2D-PAGE between RCS and control rats in any ages tested. Analysis by SDS-PAGE of ROS proteins and by western blot using antibodies against opsin, rhodopsin kinase (RK), recoverin, or arrestin demonstrated that a 20-kDa protein and RK were selectively less abundant in RCS than in control rats. Edman sequence analysis of the proteolytic peptides obtained by in-gel digestion of the corresponding protein band using endoproteinase Lys C identified the 20-kDa protein as αA-crystallin. Reverse transcription-polymerase chain reaction confirmed selective low levels of mRNA expressions of αA-crystallins and RK in RCS rats.

CONCLUSIONS. This study demonstrates that decreased expression of αA-crystallins and RK in RCS rats, may have significant roles in the development of retinal dystrophy. (Invest Ophthalmol Vis Sci. 1999;40:2788–2794)
restin, and recoverin during development of retinal degeneration, because these proteins were most likely affected as described.

**Materials and Methods**

All experimental procedures were designed to conform to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and our institution’s guidelines. The animals used in this study were 3- to 8-week-old inbred RCS (rdy/rdy) rats and congenic control RCS (rdy/+ ) rats originally obtained from Crea, Tokyo, Japan. Unless otherwise stated, all procedures were performed with ice-cold solutions.

**Antibodies**

Anti-recoverin rabbit serum or anti-arrestin rat serum was obtained by immunization of purified recoverin23 or arrestin24 from fresh bovine retinas to rabbit or rat, respectively. IgG was isolated from these sera by use of a protein G Sepharose column, according to the protocol described by the manufacturer. For affinity purification, IgG was applied to a Sepharose 6B column covalently linked with recoverin or arrestin, and IgG binding to the column was eluted by lowering the pH using 0.2 M glycine buffer (pH 2.5). An aliquot (1 ml each) was collected and mixed immediately with 0.1 ml 1 M Tris buffer (pH 8.5) to adjust the pH to 7.5. The purity and protein contents were determined by SDS-PAGE and spectrophotometry, respectively. Anti-human rhodopsin kinase (RK) monoclonal antibodies25 and anti-rhodopsin monoclonal antibodies26 were generous gifts of Krzysztof Palczewski (Department of Ophthalmology, University of Washington, Seattle) and Fumio Tokunaga (Osaka University, Japan). The specificity and titers of all antibodies were examined by western blot and enzyme-linked immunosorbent assay (ELISA), respectively.

**Preparation of Rod Outer Segment Membranes**

Rat ROS membranes were prepared by the method described by Papermaster,27 with some modifications using sucrose-density gradient centrifugation. Briefly, four freshly dissected rat retinas were suspended in 0.3 ml 45% (wt/vol) sucrose in ROS buffer (10 mM HEPES [pH 7.5], 20 mM NaCl, 60 mM KCl, and 1 mM benzamidine), shaken vigorously in a tube (Eppendorf, Fremont, CA) for 20 minutes, and centrifuged for 10 minutes at 13,000 rpm. The supernatant was collected, and the pellet was aspiration of the solution, the gel was incubated with 0.2 ml 100 mM Tris-HCl (pH 9.0) containing 0.1% SDS at 30°C for 1 hour. Thereafter, 1 µg endoproteinase Lys C (Boehringer Mannheim, Mannheim, Germany) was added, and the mixture was incubated at 30°C for 24 hours. The reaction was terminated by adding 20 µl 10% trichloroacetic acid. The solution was collected, and the remaining gel was crushed and washed in 0.2 ml of the same buffer. The combined solution was then loaded onto a reversed-phase C8 column (2.1 × 250 mm; Shiseido, Tokyo, Japan) using a precolumn of DEAE-5PW (4.6 × 10 mm, Tosoh, Tokyo, Japan) to remove the SDS, and the cleaved peptides were purified by using a linear gradient of acetonitrile from 0% to 70% in 0.05% trifluoroacetic acid for 70 minutes at a flow rate of 0.3 ml/min.

**Amino Acid Sequence Analysis**

The peptide sequence was obtained by Edman degradations using an automated gas-phase protein sequencer (model 477; Applied Biosystems, Foster City, CA), as described by Crabb et al.31

**Reverse Transcription–Polymerase Chain Reaction Analysis and Relative Amount of α Crystallins and RK**

For reverse transcription–polymerase chain reaction (RT-PCR) total RNA from retinas was isolated using reagent according to the procedure recommended by the manufacturer (Isogen; Nippon Gene, Tokyo, Japan.). The cDNAs were generated from 5 µg of total RNA in a 12-µl reaction using 1 µl oligo(dT) primer (0.5 mg/ml; Gibco Life Technologies, Rockville, MD). The reaction mix was denatured at 70°C for 2 minutes. Four microliters first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂; Superscript), 2 µl dithiothreitol (0.1 M; DTT), 1 µl dNTP (10 mM), 1 µl RNase inhibitor (40 U/µl), and 1 µl reverse transcriptase (200 U/µl; Superscript II) were added to the mix. The reaction mix was incubated for 42°C for 5 minutes and at 70°C for 15 minutes. The PCR amplifications were performed using 4 µl from the RT reaction, 5 µl 10× PCR buffer (200 mM Tris-HCl and 500 mM KCl), 2 µl MgCl₂ (50 mM), 1 µl dNTP, 5 µl sense and antisense primers (10 pM/µl), and 0.5 µl Taq polymerase (5 U/µl; Gibco). The PCR mix was denatured at 94°C for 4 minutes and then run for 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes.

The primers used for RT-PCR were 5′-ATGGACGTCCATCCAGCA-3′ corresponding to bases 158 to 178 of the αA-Crystallin and Rhodopsin Kinase in RCS Rat  2789 acrylamide/bis-acrylamide. 8 M urea, 2% NP40, and 2% amphotoline producing a pH gradient of 4.0 to 8.0. After a 2-hour prerun at 200 V, electrofocusing was performed successively for 1 hour at 200 V, 16 hours at 300 V, and 1 hour at 500 V, with 20 mM NaOH and 10 mM H₃PO₄ as the cathode and anode solutions, respectively. In the second dimension, IEF gels were removed from the glass tubes, incubated with solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 5% β-mercaptoethanol, and subjected to SDS-PAGE.

**In-Gel Digestion and Peptide Separation by High-Performance Liquid Chromatography**

In-gel digestion was performed by the method described by Ohguro et al.30 Briefly, the protein band stained with Coomassie blue was excised and washed twice with 200 µl 50% acetonitrile and 0.2 M ammonium bicarbonate solution at 30°C for 20 minutes. After aspiration of the solution, the gel was incubated with 0.2 ml 100 mM Tris-HCl (pH 9.0) containing 0.1% SDS at 30°C for 1 hour. Thereafter, 1 µg endoproteinase Lys C (Boehringer Mannheim, Mannheim, Germany) was added, and the mixture was incubated at 30°C for 24 hours. The reaction was terminated by adding 20 µl 10% trichloroacetic acid. The solution was collected, and the remaining gel was crushed and washed in 0.2 ml of the same buffer. The combined solution was then loaded onto a reversed-phase C8 column (2.1 × 250 mm; Shiseido, Tokyo, Japan) using a precolumn of DEAE-5PW (4.6 × 10 mm, Tosoh, Tokyo, Japan) to remove the SDS, and the cleaved peptides were purified by using a linear gradient of acetonitrile from 0% to 70% in 0.05% trifluoroacetic acid for 70 minutes at a flow rate of 0.3 ml/min.
cDNA sequence and 5'-AGCTGGGCTTCTCCTCCCGT-3' corresponding to bases 713–732 of the cDNA sequence for αA-crystallin and αA-crystallin with expected PCR products of 485 bp and 574 bp, respectively; 5'-CCACATGCTGGAGACCCCAA-3' corresponding to bases 371–399 of the cDNA sequence and 5'-AGCTGGGCTTCTCCTCCCGT-3' corresponding to bases 713–732 of the cDNA sequence for αA-crystallin with an expected PCR product of 352 bp; 5'-ATGAGATAGATGGAGACCCCAA-3' corresponding to bases 2683–2703 of the genomic DNA sequence.

**RESULTS**

To understand the pathologic molecular course of retinal dystrophy in the RCS rat, retinal proteins of RCS (rdy/rdy) and control rats (rdy+/rdy+) were analyzed by 2D-PAGE, and the protein mapping patterns were compared during the disease progression. Retinal proteins of RCS (rdy/rdy) were generally less abundant than those of the control (rdy+/rdy+) in 3- to 8-week-old animals, and these differences became apparent with advancing age. However, the distributions of the retinal proteins were almost identical, and specific changes were not detected (data not shown). These results suggest that the amounts of major retinal protein components generally decreased during the course of retinal dystrophy, but their distributions were not significantly changed.

As the next step in the investigation, we isolated rod outer segments (ROS) of 3- to 8-week-old animals, and their protein components were analyzed by SDS-PAGE. As shown in Figure 1 (upper panel), ROS protein components of 3-week-old RCS rats, in which retinal dystrophy was not apparent, were almost identical with those of control rats. However, the amount of each ROS protein component in 7-week-old RCS rats, in which retinal dystrophy had become obvious, was generally decreased compared with those of the control when equal volumes of ROS were applied. In addition, it was noted that the 67-kDa protein, 23-kDa protein and 20kDa protein bands, designated with arrowheads and an arrow, were significantly less abundant than the other proteins. In the case of overloaded SDS-PAGE gel, the most significant difference was noted in the corresponding 20-kDa band (Fig. 1, lower panel).

To identify the 20-kDa protein, the corresponding band in the gel was cut out and subjected to in-gel digestion with endoproteinase Lys C. The resultant peptides were purified on a reversed-phase high-performance liquid chromatography C8 column using a linear gradient of acetonitrile from 0% to 70% during 70 minutes (Fig. 2). The six major peak fractions were then subjected to the Edman sequence analysis. As shown in Figure 3, sequence analysis of the proteolytic peptides identified the 20-kDa protein as αA-crystallin.

In the previous reports, changes in opsin, arrestin, and ROS protein phosphorylation levels have been identified. Our present study identified that the 67-kDa and 23-kDa bands, which have molecular masses similar to those of RK and recoverin, were significantly less abundant in RCS ROS (Fig. 1, upper panel). These observations suggested that the quenching of the phototransduction pathway might be impaired in RCS. To study changes of the protein components in the process of quenching of the phototransduction pathway during the development of retinal dystrophy in RCS rats, western blot analysis was performed using antibodies against opsin, RK, arrestin, and recoverin. As shown in Figure 4, immunoreactive bands were detected in 4- and 6-week-old rats that corresponded to recoverin, arrestin, and opsin, but those in 8-week-old rats were almost missing. The development of the destruction of ROS components at this age may have been the cause of these findings. In contrast, a trace of a band of RK was also found in 4-week-old rats, and no band of RK was detected in 6- and 8-week-old rats. These results indicate that the expressions of αA-crystallins and RK in RCS rat were already low at the beginning stage of retinal dystrophy, and they were specifically less abundant among proteins in ROS.

The following two possibilities were considered as a possible molecular mecha-
nism of these changes in αA-crystallins and RK: mRNA expressions of αA-crystallins and RK are low in RCS rat, and αA-crystallin and RK are specifically proteolyzed.

To answer these questions, mRNA expressions of αA-crystallins and RK were examined by RT-PCR during the development of retinal dystrophy. As shown in Figure 5, mRNA expressions of αB-crystallin and opsin were comparable between RCS and control rats at 4, 5, 6, and 8 weeks of age. mRNA levels of αA-crystallin and its splice variant form (αAins-crystallin) were significantly less and those of RK were relatively less in RCS rats at all ages examined than in control rats.

Lower mRNA expression of RK in RCS rats was confirmed by competitive quantitative PCR, as shown in Figure 6.

**DISCUSSION**

The RCS rat (rdy−/−) is the most extensively studied as a model of inherited retinal dystrophy such as RP. In this strain, it is thought that a primary genetic defect in the RPE causes a deficit in phagocytosis of shed ROS materials by the RPE.⁴⁻⁻⁶ Accumulation of the ROS debris between photoreceptor and RPE causes unknown processes and finally results in photoreceptor degeneration, but the process has not been clarified.

The molecular defect that causes this rd⁴−/− mutation remains to be identified, although it has been reported that several proteins including opsin,¹⁶ arrestin,¹⁷,¹⁸ hsp 70,¹⁸,²⁰ neurotro-

**FIGURE 1.** Analysis of ROS proteins in RCS and control rats by SDS-PAGE. ROS was isolated from four retinas of 3-week-old or 7-week-old RCS (rdy−/−) and control (rdy+/+) rats by a sucrose-density gradient centrifugation method. ROS pellets were each suspended in 100 μl 10 mM HEPES buffer (pH 7.5) containing 100 mM NaCl. An aliquot (10 μl) was mixed with the sample buffer (10 μl) and loaded on an SDS-PAGE gel (top). An aliquot (50 μl) from 7-week-old animals was lyophilized, dissolved in the sample buffer (10 μl), and loaded on an SDS-PAGE gel (bottom). Significant differences between RCS and control are noted in 67-kDa and 23-kDa bands designated by arrowheads and a 20-kDa band designated by an arrow.

**FIGURE 2.** High-performance liquid chromatography (HPLC) separation of proteolytic peptides of 20-kDa protein after in-gel digestion by endoproteinase Lys C. In-gel digestion of 20-kDa proteins from control rats (rdy+/+) and separation of the proteolytic peptides by reversed-phase HPLC C8 column were performed as described in the Materials and Methods section. Major peaks (designated 1–6) were collected and subjected to Edman sequencing analysis.

**FIGURE 3.** Amino acid sequence of 20-kDa proteolytic peptides and their homology with αA-crystallin. The amino acid sequences of six proteolytic peptides from 20-kDa protein obtained by Edman sequencing are indicated (bold letters with underlines) in the rat αA-crystallin sequence. The peptide designations (P1-P6) correspond to those in Figure 2.
In the present study, we performed protein mapping of either whole retina or ROS by electrophoresis and western blot analysis and found that αA-crystallins and RK in ROS of RCS rat were selectively expressed at lower levels than in control rats. It is unclear whether these changes are the causes or the consequence of retinal degeneration. However, it is speculated that these changes are not simply consequences of photoreceptor destruction but may be involved in the pathogenesis of retinal degeneration because of the following reasons: expression of αA-crystallins and RK were already low before retinal destruction was apparent in RCS rat; abundance of other ROS proteins, such as opsin, recoverin, and arrestin also decreased during the development of the photoreceptor destruction, but their decreases were slower in RCS rat than in control; and mRNA expressions of αB-crystallin and opsin in RCS rats were comparable to those in control rats.

α-Crystallins are known to be major components of lens proteins. It has been shown that α-crystallins are expressed in many extralenticular tissues, including retina, heart, lung, spinal cord, skin, muscle, brain, and kidney. Among these chaperon functions, α-crystallins have been shown to bind specifically to post-Golgi membranes and to be involved in the transportation of newly synthesized rhodopsin. Nir et al. have reported that opsin abnormally accumulates within the inner segment plasma membranes of photoreceptors of RCS rat. These observations suggest that α-crystallins may participate in the renewal of photoreceptor outer segment membrane and that immature ROS may not be properly phagocytized by RPE cells because of the absence of α-crystallins. Alternatively, it is speculated that α-crystallins may directly affect phagocytotic processes, be-

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933585/)  
**Figure 4.** Analysis of ROS proteins in RCS and control rats by western blot analysis using antibody against rhodopsin, RK, arrestin, or recoverin. ROS was isolated from four retinas of 4-week-old, 6-week-old, or 8-week-old RCS rats (rdy+/−) or from 8-week-old control rat (rdy+/+) by a sucrose-density gradient centrifugation method. ROS pellets were each suspended in 100 μl 10 mM HEPES buffer (pH 7.5) containing 100 mM NaCl. An aliquot (10 μl) was mixed with the sample buffer (10 μl), loaded on an SDS-PAGE gel, and then electrotransferred to a polyvinylidene difluoride membrane. Western blot analysis was performed using either anti-rhodopsin monoclonal antibody (mAb) (1:2 dilution), anti-RK mAb (1:3000 dilution), affinity purified anti-arrestin polyclonal Ab (1:2000 dilution), or anti-recoverin polyclonal Ab (1:2000 dilution). The details of the western blot are described in the Materials and Methods section.

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933585/)  
**Figure 5.** Expressions of mRNA for αA-crystallins, αB-crystallin, RK, opsin, and β-actin in RCS and control rats. Two micrograms RNA from retinas were reverse transcribed to generate a cDNA pool. Pooled cDNA (2.2 μl from a total pool of 22 μl) was used for PCR with specific primers as described in the Materials and Methods section. PCR products were evaluated by agarose gel electrophoresis and ethidium bromide staining. Two close PCR products of 485 bp and 574 bp indicated by *ubiTE arrous* are from αA-crystallin and αA**+/−**-crystallin, respectively.
cause they exist in RPE cells. Recent observations have also shown that α-crystallins are involved in other degenerative diseases. Vicart et al. reported that a missense mutation in the αB-crystallin gene causes a desmin-related myopathy. Van Noort et al. reported that αB-crystallin is a candidate for an autoantigen in multiple sclerosis. Tezel et al. found serum autoantibodies against α-crystallins in patients with normal-tension glaucoma and suggested that autoimmunity toward α-crystallins may be related to the pathogenesis of the glaucomatous optic neuropathy.

RK is a member of the G-protein-coupled receptor kinase family and plays a pivotal role in desensitization of phototransduction by phosphorylating rhodopsin in a light-dependent manner. In terms of the relationship with retinal diseases, a mutation of RK has been identified as a cause of Oguchi’s disease, a retinal degenerative disease that involves congenital stationary night blindness. It has been suggested that absence of or abnormally high levels of rhodopsin phosphorylation are possible mechanisms of retinal degeneration in RP. In cancer-associated retinopathy, which is caused by defective ingestion of rod outer segments by RCS rats, it has been shown that an autoantibody against recoverin may block its function of regulating rhodopsin phosphorylation in a Ca2+-dependent manner and that this is a possible mechanism of retinal degeneration. Taken together, these observations indicate that abnormal regulation of rhodopsin phosphorylation may commonly be involved in the pathogenesis of photoreceptor degeneration. We do not know why RK expression is low in RCS rats, in addition to the low expression of α-crystallins, compared with that in control. However, if α-crystallins are really involved in the transportation of newly synthesized rhodopsin, low expressions of α-crystallins and RK may both be involved synergistically in the pathogenesis of the retinal degeneration.

In conclusion, we have found selectively low expressions of α-crystallins and RK in ROS of RCS rat, which we believe may be related to the pathogenesis of retinal dystrophy. Further study is required to clarify the mechanism by which these changes of α-crystallins and RK cause retinal degeneration.

Acknowledgments
The authors thank Hitomi Sano, Department of Biochemistry, Sapporo Medical University School of Medicine for generous support of the study.

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