Clinicopathologic Effects of the Q64ter Rhodopsin Mutation in Retinitis Pigmentosa

Ann H. Milam,* Zong-Yi Li,* Artur V. Cideciyan,†† and Samuel G. Jacobson††

Purpose. To correlate retinal histopathology with functional changes caused by the rhodopsin Q64ter mutation.

Methods. A 50-year-old female heterozygote was evaluated clinically and with psychophysical and electroretinographic measurements of rod and cone function. The retinas obtained after death were examined microscopically, including immunolabeling with antibodies against the C- and N-termini of rhodopsin.

Results. On clinical examination 4 months before death, patient’s acuity was 20/60, and she had midperipheral scotomas with retained function centrally and in the far periphery. The rod electroretinogram (ERG) was undetectable, and the cone ERG was reduced in amplitude with abnormal receptor and postreceptoral responses. A previous study of the phenotype of mildly affected family members of the donor suggested that the rod outer segments (ROS) were shortened and that only wild-type rhodopsin was functional. The retinas contained only scattered cones in the midperiphery; the maculas and far peripheral regions contained reduced numbers of rods and cones with short to absent outer segments. The ROS appeared to contain wild-type, but not mutant, rhodopsin, and many peripheral rods had sprouted long rhodopsin-positive neurites that projected into the inner retina. Many cone synapses were abnormal, and the axons of some peripheral cones reached the inner plexiform layer.

Conclusions. Microscopic changes in the donor retinas correlated well with the abnormalities in visual function in the patient donor and other family members. Postreceptoral ERG defects may relate to the abnormal photoreceptor processes found in the inner retina. Invest Ophthalmol Vis Sci. 1996;37:753-765.

Autosomal dominant (ad) retinitis pigmentosa (RP) is now known to be caused by more than 70 different mutations in the rhodopsin gene.1-6 Most of the mutations result in an amino acid substitution, but a few stop codon mutations have been identified in adRP that cause abnormal truncation of the rhodopsin molecule.7-9 One of these, the glutamine-64-to-ter (Q64ter) mutation, was found recently to cosegregate with RP in a large ad kindred,10 and the phenotypes of 14 family members heterozygous for this mutation were characterized using noninvasive tests of visual function.11 Based on the test results in mildly affected family members, it was concluded that the rod outer segments (ROS) contained rhodopsin capable of functioning normally in phototransduction. Rhodopsin levels and rod photoreceptor response maximum amplitudes were decreased, but the time course of dark adaptation was not prolonged, suggesting that these patients’ ROS were shortened and that only wild-type rhodopsin was functional.11

Noninvasive studies of visual function in patients with adRP carrying this mutation provide the only insight to date into the disease mechanisms for this putative null mutation in the rhodopsin gene. Whereas there are in vitro preparations and transgenic animals for studying other rhodopsin mutations known to cause RP,8-12-17 comparable studies have not been reported for this mutation. To increase the understanding of the pathophysiology of the retinal degeneration associated with the Q64ter rhodopsin mutation, we examined the retinas obtained after death from a pa-
tant donor heterozygous for the Q64ter mutation who had been followed with clinical and visual function tests for 7 years. We performed histopathologic studies on the retinas, including immunocytochemistry at the light and electron microscopic levels, and present clinicopathologic correlations based on our observations.

METHODS

Description of the Donor Patient

The patient donor was a 50-year-old woman with a 25-year history of night vision disturbances and several years of problems with peripheral vision. She was diagnosed with RP at age 40. We examined the patient clinically and with tests of retinal function in 1987, 1992, and 1994. Breast cancer was diagnosed in 1987; she was treated by mastectomy in 1987 and thereafter with Tamoxifen (Stuart, Wilmington, DE) for 2 to 3 years of problems with peripheral vision. She was diagnosed with RP at age 40. We examined the patient clinically and with tests of retinal function in 1987, 1992, and 1994. Breast cancer was diagnosed in 1987; she was treated by mastectomy in 1987 and thereafter with Tamoxifen (Stuart, Wilmington, DE) for 2 to 3 years. There was no evidence that the neoplastic disease or chemotherapy affected her vision. She died of metastatic disease in July 1994.

Visual Function Tests

Kinetic perimetry was performed with V-4e and I-4e test targets. Static perimetry was used to measure sensitivity at 75 loci across the visual field in the dark-adapted state with 500- and 650-nm stimuli and in the light-adapted state with a 600-nm stimulus (target size V). Sensitivity losses were calculated for rods and cones by comparison to mean normal values at each of the test loci. Details of this methodology have been published.18

Full-field electroretinograms (ERGs) were performed according to methods previously described.11 Four conventional responses were elicited: a rod ERG (blue flash of −0.1 log scot-td’s, dark adapted); a mixed cone and rod ERG (white flash of 5.4 cd·s·m⁻², dark adapted); a 1-Hz cone ERG (white flash of 5.4 cd·s·m⁻², on a white background of 34 cd·m⁻²); and a 29-Hz cone flicker ERG (white flashes of 5.4 cd·s·m⁻², on a white background of 6.9 cd·m⁻²). Oscillatory potentials were analyzed from cone ERGs to a 1-Hz white flash (9 cd·s·m⁻²) on a background light (34 cd·m⁻²) by digitally filtering with a bandpass of 100 to 300 Hz.19 Cone ERG energy series were recorded for 1-Hz (0.02 to 9 cd·s·m⁻² white flashes on a 34 cd·m⁻² white background) and for 29 Hz (0.02 to 5.4 cd·s·m⁻² white flashes on a 6.9 cd·m⁻² white background). “On” and “off” components of the cone ERG were elicited with white stimuli (1500 cd·m⁻²) of 400-msec duration on a white background light (34 cd·m⁻²) using a published method.19

Rod and cone photoreceptor responses were elicited using blue (Wratten 47A) and red (Wratten 26) high-energy (up to 4.6 log scot-td.s) flash stimuli in the dark-adapted state. Isolation of the receptor responses was achieved by a double subtraction technique. Details of the recording methods have been published.11,19 The dark-adapted rod and cone photoreceptor response series was quantified using a newly developed model of phototransduction20 based on the delayed Gaussian model of phototransduction21 and its application to the human rod19,22–24 and cone a-waves.25

Histopathology

Donor eyes (Foundation Fighting Blindness [Baltimore, MD] #424) were fixed at 6.5 hours postmortem in 4% paraformaldehyde and 0.5% glutaraldehyde. The eye from a 51-year-old normal female donor (UW0403-93) fixed in the same manner at 3 hours postmortem served as a control for the light microscopic studies. For examination by immunofluorescence,36,27 retinal samples were cryosectioned at 12 μm and processed using secondary antibodies labeled with fluorescein isothiocyanate, rhodamine, or Cy-3 (Jackson ImmunoResearch, West Grove, PA). The following rod specific antibodies were used: anti-rhodopsin (mAbs 4D2 [N-terminus] and 1D4 [C-terminus], Dr. R. Molday, University of British Columbia, Canada); -arrestin (Dr. H. Ishii, Wayne State University, Detroit, MI); -rhodopsin kinase (Dr. K. Palczewski, University of Washington, Seattle, WA); -rod transducin-α (Drs. C. and K. Lerea, New York Medical College, Valhalla, NY); -ROM-1 (Drs. R. McLennan and R. Bascom, Hospital for Sick Children, Toronto, Canada); and -cyclic guanosine monophosphate-gated channel (mAb 1D1, Dr. Molday). Antibodies against other retinal proteins were: anti-recoverin (Dr. A. Dizhoor, University of Washington, Seattle, WA); -retis/periipherin (mAb 5H2, Dr. Molday); -interphotoreceptor retinoid binding protein (IRBP; Dr. John Saari, University of Washington, Seattle, WA). Cone-specific antibodies were anti-red/green and anti-blue cone opsin and anti-cone transducin-α (Drs. Lerea). Synapse-specific antibodies were anti-synaptophysin (Sigma, St. Louis, MO) and -SV2 protein (Dr. K. Buckley, Harvard Medical School, Boston, MA); and the marker for reactive Müller cells was anti-glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA). Control sections processed in the same way with omission of the primary antibody showed only weak autofluorescence of the retina, including the photoreceptor inner segments, and strong autofluorescence of lipofuscin granules in the retinal pigment epithelium.

Some retinal samples were embedded in Medcast; 1-μm sections were stained with Richardson’s mixture, and 90-nm sections were contrasted with uranyl acetate and lead citrate for electron microscopy. Other
FIGURE 1. Pedigree of family with adRP caused by the Q64ter rhodopsin mutation. The filled symbols represent affected family members, and the solid arrow indicates the 50-year-old patient donor.

Retinal samples were embedded in LR-White resin for 1-μm and 90-nm sections or in Sorvall JB-4 resin for 4-μm sections. The sections were processed for immunocytochemistry using anti-rhodopsin (mAbs 4D2 and 1D4), -IRBP, and -GFAP, and secondary antibodies labeled with 1-nm gold particles, followed by silver enhancement.26 Control sections processed without primary antibody contained only a few randomly scattered gold particles.

Quantitative electron microscopy was performed on LR-White sections processed with mAbs 4D2 and 1D4. The numbers of particles per ROS unit area were determined from electron micrographs of two normal retinas (UW836-87, 57-year-old man, 1.5 hours postmortem; UW0780-91, 85-year-old woman, 3 hours postmortem); the Q64ter rhodopsin mutant retina, and the retina from a donor with X-linked RP (Foundation Fighting Blindness #215; 46-year-old man, 1 hour postmortem). The data from the two RP retinas were expressed as the ratio of counts with mAb 4D2 to mAb 1D4, divided by the mean of the same ratios from the two normal retinas. The data were analyzed using the standard two-sample t-test.

Human Subjects
The research followed the tenets of the Declaration of Helsinki, informed consent was obtained after the nature and possible consequences of the study were explained, and the research was approved by the institutional human experimentation committee. Legal requirements for use of human donor tissues after death were met (University of Washington Human Subjects Approval #25-034-E, dated 02/01/95).

RESULTS
Clinical Examination
The patient donor was part of a large family (Fig. 1) previously studied with molecular genetic techniques and found to have adRP caused by the Q64ter rhodopsin mutation.10 Details of the donor's branch of the family have not been published; findings on her clinical examination at age 43 were reported previously (patient 8 in1'). The donor's best-corrected visual acuities were 20/30 in 1987, 20/50 in 1992, and 20/60 in 1994. In 1987, color vision tested with a Farnsworth D-15 panel showed no abnormalities, but in 1992, both eyes had a tritan axis of confusion. When the patient was last examined in 1994, 4 months before her death, there were small posterior subcapsular cataracts and cells in the vitreous. The retinas had sparse bone spicule-like pigment in the midperiphery, and very small white dots in the midperiphery and far periphery.

Visual Function Tests
Figure 2 shows results of electroretinography and static perimetry from an examination of the patient donor in 1987 and serial kinetic perimetry from 1987 to 1994. There were no detectable rod ERG b-waves to a dim blue flash, dark adapted; small signals to white flashes in the dark-adapted state were likely to be cone mediated because of their similarity in waveform appearance to those recorded in the light-adapted state. Cone flicker ERGs were reduced in amplitude and delayed in timing (Fig. 2, upper left).

Dark-adapted static perimetry showed that rod sensitivity was reduced by at least 4 log units throughout the visual field. Cone function with light-adapted perimetry was measurable at most loci but was reduced in sensitivity by approximately 1 to 1.5 log units (Fig. 2, lower left). Kinetic perimetry performed in 1987 showed a relatively full field with the V-4e test target but only a central island of function with the I-4e target. By 1992, there were absolute scotomas in the midperipheral field, and in 1994, there was a complete annular midperipheral scotoma with only islands of function centrally and peripherally (Fig. 2, right).

Photoreceptor responses derived from dark-adapted ERGs are shown in Figure 3 for the patient donor and a normal subject. In the normal subject, the response to the maximal blue flash (Bl) was mostly rod-mediated, with a smaller cone component removed by double subtraction (R-B2). The mean normal rod photoreceptor response maximal amplitude is 458 μV with a SD of 50 μV. The maximal blue flash in the patient donor, however, was of cone origin; the photopically matched red stimulus (R) elicited exactly the same waveform (Fig. 3, upper), and the subtraction led to no measurable rod photoreceptor response (Fig. 3, middle). Quantitation of the cone response using a model of phototransduction showed that the maximum amplitude (Rmax = 22.5 μV; normal mean...
ELECTRORETINOGRAPHY

1987
NORMAL

PATIENT

Rod
Mixed
Cones-Rod

1987, 1992, 1994

12°
2°
36°
48°

ECCENTRICITY [deg]

36 41 61 72

FIGURE 2. Electroretinography and perimetry in the patient donor. (upper left panel) Rod, mixed cone-rod, and cone electroretinograms performed in a representative normal subject and the patient donor in 1987. Arrows indicate stimulus onset; calibration bars are to the right of the waveforms. (lower left panel) Rod and cone static perimetry performed in 1987 for the patient donor displayed as gray scales of sensitivity loss. Gray scales have 16 levels: white = 0-dB sensitivity loss; black = 54-dB loss for rods and 25-dB loss for cones; intermediate levels of gray divide the range of sensitivity loss equally. The physiological blind spot is shown as a black square at 12° in the temporal field. (right panel) Serial kinetic perimetry with the V-4e and I-4e test targets performed in 1987, 1992, and 1994.

= 87.4 µV; SD = 2.7 µV; n = 6) and the sensitivity
(σln = 2.29 log phot·td⁻¹·s⁻³; normal mean = 2.67 ± 0.08 log phot·td⁻¹·s⁻³) were abnormally reduced; the time constant of transduction was normal (Fig. 3, lower). Of interest, the maximal blue (B1) and red (R) responses of the patient donor appeared to lack oscillatory potentials and b-waves.

The question of whether there was postreceptor dysfunction in addition to receptoral abnormalities is addressed in Figure 4 by analyses of implicit time-energy functions, oscillatory potentials, and on-off components. For the major positive peak elicited by flashes of increasing energy at 1 Hz and 29 Hz, there were delays in the responses from the patient donor compared to those of normal subjects (Fig. 4, upper). Receptor sensitivity reduction has been suggested to result in a slowing of implicit time. The cone a-wave sensitivity reduction of 0.4 log units in our patient donor’s data, however, did not account completely for the observed timing delay. When the 1-Hz and 29-Hz cone ERG b-wave data were shifted horizontally by 0.4 log units, an additional 15 msec was needed to register the patient’s results with those of normal subjects (Fig. 4, upper). Amplitudes of the cone ERG b-waves (data not shown) were reduced to approximately 10% of mean normal.

Oscillatory potentials also were lacking in the patient (Fig. 4, lower left). Responses to a stimulus of long duration showed that the normal response to the onset had a negative-going a-wave (65 ± 8 µV; 25.8 ± 0.4 msec) followed by a positive-going on-wave (56 ± 21 µV; 42.8 ± 2.3 msec). The normal response to stimulus offset is a positive going off-wave (64 ± 11 µV; 44.7 ± 1.2 msec). The patient had reduced and delayed a-waves (16.1 µV; 44 msec), on-waves (5 µV; 64 msec), and off-waves (10 µV; 60 msec). The extent of abnormality appeared to be significantly greater for the on-wave than the off-wave.

Gross Pathology of Donor Globes

Both anterior segments were normal for age, but there were small posterior subcapsular cataracts. Optic nerve heads were pale and waxy and had attenuated retinal blood vessels. Retinas appeared thin with
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Figure 3. Photoreceptor responses in the dark-adapted state in a representative normal subject and the patient donor. (upper panels) Dark-adapted electroretinographic responses elicited by the maximal blue stimulus (B1), red stimulus (R) photopically matched to B1, and lower energy blue stimulus (B2) scotopically matched to R. (middle panels) The initial 10-msec portion of the rod photoreceptor response derived by subtracting the cone component (R-B2) from the maximal blue response. (lower panels) The initial 10-msec portion of the cone photoreceptor response derived by subtracting the lower energy blue response from the red response. A model of phototransduction (solid lines) is fit to the derived cone photoreceptor responses.

sparse bone spicule pigmentation in the midperiphery and far periphery. There was mild postmortem retinal edema, most pronounced in the maculas, and the choroidal vessels were prominent.

Light Microscopy and Immunocytochemistry

Macula. The photoreceptor layer in the normal retina had 8 to 10 rows of cells, but in the Q64ter mutant retina, it was reduced to one to two layers of cones with very short or absent outer segments. No true fovea could be identified. Cone outer segments were immunoreactive for red/green cone opsin but not for blue cone opsin. Cone outer segments and somata were positive for cone transducin-α (Fig. 5A) and recoverin. Scattered rod somata lacking ROS were immunoreactive for rhodopsin (mAbs 1D4 and 4D2) (Fig. 5B), arrestin, rhodopsin kinase, rod transducin-α, and recoverin. As reported previously for other RP retinas, the Müller cells were strongly immunoreactive for GFAP, as were the astrocytes in the nerve fiber layer.

The inner nuclear and ganglion cell layers contained slightly reduced numbers of cells (6 to 8 rows of cells each; the normal amount for each layer is 8 to 10 rows), but the retinal blood vessels were hyalinized. The nerve fiber layer, inner limiting membrane, choriocapillaris, Bruch’s membrane, retinal pigment epithelium, and sclera were normal for age.

Midperipheral Retina. The choroid and Bruch’s membrane were within normal limits, but the photoreceptors were reduced to scattered cones that lacked outer segments and whose subtype could not be identified. The retina was gliotic, including strong expression of GFAP in the reactive Müller cells. A few retinal pigment epithelial cells had migrated to perivascular sites in the inner retina, the microscopic correlate of the bone spicule pigment observed grossly.

Far Peripheral Retina. The choroid and Bruch’s membrane appeared normal, but the retinal pigment
FIGURE 5. Immunofluorescence labeling of the Q64ter rhodopsin mutant and normal retinas. Magnification, ×270. (A) Labeling of macula of retinitis pigmentosa (RP) retina with anti-cone transducin-α demonstrates a monolayer of cone somata (open arrows) that lack outer segments. Cone nuclei appear as negative images. R = retinal pigment epithelium that contains autofluorescent lipofuscin granules. (B) Labeling of RP macula with mAb 1D4 that recognizes only wild-type rhodopsin. Specific label is found in scattered rod somata (open arrows) that lack outer segments. R = retinal pigment epithelium. (C) Labeling of peripheral region of normal retina with anti-rhodopsin (mAb 1D4). Specific labeling is restricted to the rod outer segments (*). The rod inner segments show weak autofluorescence. R = retinal pigment epithelium. (D) Labeling of peripheral region of RP retina with anti-rhodopsin (mAb 1D4). Note labeling of a few very short outer segments (open arrow) and surface labeling of the rod somata and long neurite processes (small arrows) in the inner retina. The same labeling pattern was found with anti-rhodopsin mAb 4D2. R = retinal pigment epithelium.

epithelial cells were double layered in foci, and some had migrated into the inner retina. The inner nuclear layer was reduced to 2 to 3 rows of cells (the normal amount is 3 to 4 rows), and only a few ganglion cells were present.

Photoreceptors were reduced to 1 to 3 rows of rods and cones (the normal amount is 4 to 6 rows), and their outer segments were extremely short to absent. In both the normal (Figs. 5C, 6A) and the RP retina (Figs. 5D, 6B, 6C), the ROS were well labeled with the mAbs against the N- and C-termini of rhodopsin. Rod inner segments and somata in the normal retina were not reactive with anti-rhodopsin (Figs. 5C, 6A). In the RP retina, many rods showed delocalization to the inner segments and somata of rhodopsin that was reactive with both anti-rhodopsin mAbs (Figs. 5D, 6B, 6C). The ROS and rod somata in both the normal and RP retinas were reactive for arrestin, rhodopsin kinase, rod transducin-α, and recoverin.

As recently demonstrated in retinas from donors with different genetic forms of RP, the peripheral rods in the RP retina showed prominent neurite sprouting. Rod neurites extended as far as the inner limiting membrane and were well labeled with both mAbs against rhodopsin (Figs. 5D, 6B, 6C) as well as the antibodies against arrestin, rhodopsin kinase, rod transducin-α, and recoverin. Rod neurites often had beaded varicosities and terminals near the inner limiting membrane that were labeled with antibodies against the synaptic vesicle proteins, synaptophysin and SV2. Although the ROS in both the normal and RP retinas were labeled with anti-rod/peripherin, anti-ROM-1 and anti-cyclic guanosine monophosphate-gated channel, the rod neurites were not labeled with these three antibodies. In the normal retina, anti-GFAP labeled astrocytes and delicate Müller processes in the inner retina (Fig. 7A), whereas in the RP retina, the Müller cells were hypertrophied and strongly reactive for GFAP (Fig. 7B).

The short cone outer segments were reactive for...
FIGURE 6. Dark-field microscopy of normal and retinitis pigmentosa (RP) peripheral retinas processed for localization of rhodopsin by the immunogold procedure with silver intensification. The gold particles and the melanin granules in the retinal pigment epithelium (R) appear as bright white dots. Magnification, ×160. (A) Normal retina labeled with mAb 1D4. Specific labeling is restricted to the rod outer segments (*). The photoreceptor nuclei (P) and neurons in the inner nuclear (N) layer are unlabeled. (B) RP retina labeled with mAb 1D4, which recognizes only wild-type rhodopsin. Note specific labeling of the tiny rod outer segments (small solid arrows), the surface membranes of the rod inner segments (large arrows) and somata (open arrows), and the rod neurites (arrowheads) in the inner retina. (*) Inner limiting membrane. (C) RP retina labeled with mAb 4D2, which recognizes both wild-type and Q64ter rhodopsin. The same labeling pattern is found as with mAb 1D4 (see Fig. 6B). (arrowheads) Rod neurites; (*) inner limiting membrane.

red/green cone opsin but not for blue cone opsin, and the cone outer segments, somata, and axons were reactive for cone transducin-α (Fig. 8A) and recoverin. Some cone axons (~1 to 2 per ~20 in a 40X field) were abnormally elongated and appeared to terminate in the inner plexiform layer, which was visualized by labeling with anti-synaptophysin and anti-SV2 protein (Fig. 8B).

As reported previously, anti-IRBP produced heavy labeling of the interphotoreceptor matrix in all regions of the normal retinas (Fig. 7C). In the RP retina, no IRBP immunoreactivity was present in the macula or the midperipheral and far peripheral (Fig. 7D) regions.

**Electron Microscopy**

The surviving peripheral rods had short outer segments, but most of their inner segments and somata were morphologically normal (Fig. 9A). Occasionally, the surface membranes of the rod inner segments appeared to bud off small packets of cytoplasmic material into the interphotoreceptor space (Fig. 9B). Surface membranes of the buds were labeled with anti-rhodopsin (not shown), and the packets contained dense multivesicular bodies with heterogeneous contents (Fig. 9B).

Most of the rods showed labeling of the surface membranes of their inner segments and somata with anti-rhodopsin (both mAbs), but a few with retained
synapses in the outer plexiform layer appeared flattened and misshapen (Fig. 11D).

Electron microscopic immunogold labeling was performed on the ROS using mAbs directed against the N- and C-termini of rhodopsin. We reasoned that ROS did not show this surface labeling (Fig. 10A). The rod neurites also had surface labeling with antirhodopsin and originated as single processes in the outer plexiform layer (Fig. 10A). As they passed through the inner nuclear and plexiform layers, the neurites often were bundled together to form fascicles (Fig. 10B; also see Figs. 5D, 6B), and they fanned out in the ganglion cell layer as individual processes that terminated near the inner limiting membrane (Fig. 10C).

The neurite terminals contained numerous ~50 nm synaptic vesicles and larger, dense multivesicular bodies, but synaptic ribbons were not identified (Fig. 11A). Most of the rod neurites were closely apposed to hypertrophied Müller cell processes, identified by their content of prominent intermediate filaments. Small regions of specialized membrane contacts were found between the rod neurites and the Müller processes (Figs. 11B, 11C). Conventional synapses were not identified between the rod neurites and neurons in the inner retina. In addition to the abnormally elongated axons of some cones (Fig. 8), many of the cone

**FIGURE 8.** Immunofluorescence labeling of the peripheral region of the RP retina. Magnification, ×340. (A) The short cone outer segments (arrowheads), somata, and axons (arrows) are reactive for cone transducin-α. (B) Double labeling of the section in Figure 8A with anti-SV2 protein. The two elongated cone axons (white arrows) reach the inner plexiform layer (between open arrows), which is labeled with anti-SV2. *= inner limiting membrane.

**FIGURES.** Electron microscopy of peripheral region of the retina. (A) A rod has a normal appearing inner segment (RIS) but a shortened outer segment (*). An adjacent cone inner segment (CIS) does not have an outer segment in this plane of section. S = unidentified membranous structure in the interphotoreceptor space (IPS). (arrow) Striated rootlets; E = external limiting membrane; M = Müller microvilli. Magnification, ×5750. (B) A process budding from an RIS projects into the interphotoreceptor space. Note multivesicular bodies (arrowheads) with heterogeneous contents within the process. Magnification, ×23,000.
if only wild-type rhodopsin were present, the ratio of counts with N- versus C-terminus labeling could be considered as 1. If Q64ter rhodopsin also were present, the N:C ratio should be >1 because each mutant rhodopsin molecule would contribute N- but not C-terminus labeling. The retina from a man with X-linked RP was included as a positive control because the ROS were likely to contain only wild-type rhodopsin (the rhodopsin gene is on chromosome 3^23). Data were expressed as the ratio of N:C counts from an RP retina divided by the mean of the same ratios from the two normal retinas. The calculated ratio of ROS counts for the Q64ter rhodopsin mutant was 0.95 (P = 0.44 (NS); 95% confidence interval = [0.84, 1.08]). For the X-linked RP retina, the calculated ratio was 1.07 (P = 0.96 (NS); 95% confidence interval = [0.92, 1.25]). These results are consistent with the probability that only wild-type rhodopsin is present in the ROS of the patient with X-linked RP, as expected, and also in the ROS of the patient with adRP caused by the Q64ter rhodopsin mutation.
DISCUSSION

This article represents the fifth histopathologic study of a retina from a patient donor with adRP caused by a rhodopsin mutation. The other studies were of retinas from patients carrying the P23H, R135W, and T17M point mutations in the rhodopsin gene. Only the patient donor with the T17M mutation had antemortem visual function test results that permitted detailed correlation with the histopathology findings postmortem. Unlike the earlier studies, the current study offered the opportunity to study the pathobiology of a putative null mutation in which the functional findings suggest a different pattern of visual dysfunction from the others. The P23H and T17M mutations, found near the N terminus of the rhodopsin molecule, are associated with prolonged rod dark adaptation and an atypical form of disease expression. The R135W mutation, lying at the border of helix C and the second cytoplasmic loop of the molecule, is characterized by relatively severe and early-onset rod dysfunction diffusely across the retina.

Several histopathologic features of the Q64ter rhodopsin mutant retinas correlated with the patient’s reduced visual function antemortem. Midperipheral regions corresponding to the absolute scotomas had undergone marked loss of photoreceptors. The lack of detectable rod function by ERG and psychophysical methods was consistent with the loss of many rod photoreceptors and shortening of their outer segments throughout the retina. The abnormal maximum amplitude of the cone ERG a-wave was in keeping with the cone cell losses and decreased cone outer segment lengths in all retinal regions. Abnormal color vision test results suggesting blue cone abnormalities was consistent with the absence of blue cone opsin reactivity of cone outer segments in the macula and peripheral retina.

In the macula, although the photoreceptors were reduced to one to two rows of cones with tiny or absent outer segments, the patient’s visual acuity was 20/60. The exact relationship in retinal disease between central visual function, the number of cones, and the lengths of their outer segments is not understood completely, but markedly reduced numbers of macular photoreceptors have been found in retinas from other patient donors with RP who had reasonably good acuity before death.

Our analyses of the cone ERG in the patient donor indicate that there was not only dysfunction at the receptor level but also involvement at postreceptoral retinal sites. Implicit times of the patient donor’s cone ERG b-waves and oscillatory potentials are the absent oscillatory potentials and the reduced on and off components. The donor retinas offered the opportunity to correlate the histopathologic changes observed in the cone pathway with the dysfunction found in the postreceptoral cone ERG. The observed cone photoreceptor abnormalities included deformed synapses in the outer plexiform layer and elongation of some cone axons, which appeared to project into the inner plexiform layer. It could be speculated that these abnormalities interfered with signal flow from the cone outer segments to the inner nuclear layer. The decreased number of neurons in the inner nuclear layer in both the macula and peripheral retina may have contributed to the observed reductions and delays in the cone b-waves and oscillatory potentials.

It is important to determine why mutations in rhodopsin and other rod-specific proteins lead to death of cones as well as rods. It has been suggested that this is caused by release of toxic factors from degenerating rods or to loss of a rod-derived trophic factor. Regions of rod cell death in RP retinas usually show cone outer segment shortening and ultimately, cone cell death, suggesting that some product of the rods is required for cone outer segment maintenance and cellular viability. The retinoid-binding protein IRBP, a secreted protein in the interphotoreceptor matrix, is primarily a product of the rods, with less contribution from the cones. Coupled with the evidence that vitamin A deficiency leads to photoreceptor outer segment shortening and cell death, it is possible that decreased IRBP secondary to loss of rods contributes to the cone outer segment alterations and cell death that are characteristic of RP.

Patients with RP with the P23H or certain other rhodopsin mutations, as well as transgenic mice with the P23H mutation, show greatly prolonged rod dark adaptation. Because P23H rhodopsin is present in the ROS of transgenic mice, it appears likely that the mutant rhodopsin interferes with the visual cycle and leads to prolonged dark adaptation. In contrast, heterozygotes for the Q64ter rhodopsin mutation tested with dark adaptometry show a nearly nor-
normal time course of recovery of sensitivity. Taken together with the findings in these patients of decreased rhodopsin and reduced maximum amplitudes of the rod response, it was predicted that the ROS would be reduced in size and contain mainly wild-type rhodopsin, which would control the recovery of sensitivity after bleaching. In agreement, we found that ROS in the donor retinas were shortened to absent, and our electron microscopic immunocytochemistry indicated that they probably contained only wild-type rhodopsin. The latter observation is also consistent with the suggestion that the C-terminus of rhodopsin is required for its transport or retention in the ROS.

Recent studies on transgenic mice demonstrated transport to the ROS of P23H, Q344ter, and S334ter mutant forms of rhodopsin. Mice with the Q344ter rhodopsin transgene showed delocalization of mutant, but not wild-type, rhodopsin to the rod inner segments and somata, whereas the rods of the human Q64ter rhodopsin mutant clearly had wild-type rhodopsin in the inner segments, somata, and neurites. Delocalization of wild-type rhodopsin to proximal regions in the rods may be a nonspecific indicator of rod cell degeneration or a consequence of the reduction in ROS membranes.

An antibody specific for Q64ter rhodopsin is unavailable; therefore, direct observations cannot be made of its distribution in the rods of the donor retina. Given the extreme truncation of the molecule, it is possible that the mutant mRNA is unstable or that the mutant protein is translated but undergoes rapid degradation. We looked for, but did not find, increased numbers of rod lysosomes that might function in autophagy of mutant rhodopsin, profiles of rhodopsin-containing endoplasmic reticulum, or osmiophilic Golgi inclusions. In fact, the majority of the rod inner segments and somata in the RP retinas appeared entirely normal by electron microscopy. A few rod inner segments appeared to bud off small packets of cytoplasm, which could represent attempts to eject abnormal cytoplasmic materials not recognized for transport to the ROS. Small surface buds also are found on rod inner segments in dystrophic pcd and rdS mice, but to our knowledge they have not been noted in human RP retinas. Shedding of surface membrane vesicles is also a characteristic feature of cells undergoing apoptosis, a form of cell death that is ongoing in the rods in RP retinas.

Many of the peripheral rods in the RP retinas had sprouted long, rhodopsin-positive neurites, and some of the cones had abnormally elongated axons. As discussed elsewhere, these unusual structures may be formed in response to changes in Müller cell surface molecules or retinal growth factors. Neuronal axons do not normally form synapses on glial cells, and the unusual synapse-like structures between the rod neurites and Müller processes appear to be novel. Rod neurites contained numerous ~50-nm vesicles closely resembling those in normal rod spherules (see also), but the apparent failure of the neurites to form synapses with inner retinal neurons suggests that they may not contribute to vision. Alternatively, if these vesicles contain glutamate, as found in normal rod spherules, the neurites might function like en passant fibers and affect the activity of other neurons by altering the levels of glutamate in the inner retina. Although speculative, if rhodopsin in the rod neurites can be regenerated, perhaps by means of retinaldehyde derived from Müller cells that contain cellular retinaldehyde-binding protein, this non-ROS form of the visual pigment might contribute to measurements of rhodopsin made by fundus reflectometry. This may help to explain previous reports that rhodopsin levels in some RP retinas were higher than accounted for by the patient's rod-mediated vision.

In summary, the abnormalities in photoreceptor function in the patient donor and other family members with the Q64ter rhodopsin mutation correlated well with the microscopic changes in the photoreceptors, including the finding of shortened ROS that appeared to contain only wild-type rhodopsin. The functional significance of the abnormal photoreceptor processes in the inner retina is unknown, but they may relate to the unexpectedly high levels of rhodopsin found in the retinas of some other patients with RP and to postreceptoral ERG alterations found in the patient donor.

Key Words
electroretinography, immunocytochemistry, photoreceptor, retinitis pigmentosa, rhodopsin

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References


21. Lamb TD, Pugh EN Jr. A quantitative account of the activation steps involved in phototransduction in amphi


25. Hood DC, Birch DG. Human cone receptor activity: The leading edge of the a-wave and models of recep


27. Milam AH, Jacobson SG. Photoreceptor rosettes with blue cone opsin immunoreactivity in retinitis pigmento

28. Gouras P, MacKay CJ. Light adaptation of the electro

29. Li Z-Y, Possin DE, Milam AH. Histopathology of bone
cipule pigmentation in retinitis pigmentosa. *Ophthal


33. Berson EL, Rosner B, Sandberg MA, Dryja TP. Ocular findings in patients with autosomal dominant retinitis pigmentosa and a rhodopsin gene defect (Pro-23-


36. Shady S, Hood DS, Birch DG. Rod phototransduction in retinitis pigmentosa: Distinguishing alternative