11-3-2000

Loss of Cone Molecular Markers in Rhodopsin-Mutant Human Retinas with Retinitis Pigmentosa

Sinoj K. John

Julie E. Smith

Gustavo D. Aguirre
University of Pennsylvania, gda@vet.upenn.edu

Ann H. Milam

PMID: 11063754
http://www.molvis.org/molvis/v6/a28/

Loss of Cone Molecular Markers in Rhodopsin-Mutant Human Retinas with Retinitis Pigmentosa

Abstract

Purpose: To examine the effect of rhodopsin mutations on cone photoreceptors in human retinas with retinitis pigmentosa (RP).

Methods: Four RP retinas with rhodopsin mutations and four normal retinas were examined by immunofluorescence with a battery of cell-specific antibodies against cone and rod cytoplasmic and outer segment membrane proteins. Areas of the retinas were studied that showed maximal preservation of photoreceptor structure.

Results: All four RP retinas showed loss of rods, ranging from mild (T-17-M), to more severe (P-23-H), to advanced degeneration (Q-64-ter and G-106-R). The majority of cones in the T-17-M and P-23-H retinas were cytologically normal but showed loss of immunoreactivity for the cytoplasmic proteins 7G6, calbindin, and X-arrestin. The cone outer segments (OS) remained positive for cone opsins and peripherin-2 (rds/peripherin). All remaining cones in the Q-64-ter and G-106-R retinas were degenerate, with short to absent OS, but had strong reactivity for these cytoplasmic and OS membrane markers. Cones in the maculas of the RP retinas were degenerate, with short to absent OS, but retained strong labeling for the cytoplasmic and OS proteins.

Conclusions: Even before cones show cytologic changes in response to rod cell degeneration, they lose immunoreactivity for certain cytoplasmic proteins. These cones later show shortening and loss of OS, although their OS membrane proteins remain well labeled. Cones may down regulate expression of both cytoplasmic and outer segment membrane proteins in response to mutant rod cell dysfunction and/or cell death in human RP retinas. Such cytologic and immunocytochemical changes in the cones may presage death of these critical cells in the later stages of RP.

Disciplines

Eye Diseases | Medicine and Health Sciences | Ophthalmology

Comments

PMID: 11063754

http://www.molvis.org/molvis/v6/a28/

Molecular Vision 2000; 6:204-15 <http://www.molvis.org/molvis/v6/a28>
Received 15 September 2000 | Accepted 31 October 2000 | Published 3 November 2000

Loss of cone molecular markers in rhodopsin-mutant human retinas with retinitis pigmentosa

Sinoj K. John,[1] Julie E. Smith,[1] Gustavo D. Aguirre,[1,2] Ann H. Milam[1]

[1]Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA; [2]James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY

Purpose: To examine the effect of rhodopsin mutations on cone photoreceptors in human retinas with retinitis pigmentosa (RP).

Methods: Four RP retinas with rhodopsin mutations and four normal retinas were examined by immunofluorescence with a battery of cell-specific antibodies against cone and rod cytoplasmic and outer segment membrane proteins. Areas of the retinas were studied that showed maximal preservation of photoreceptor structure.

Results: All four RP retinas showed loss of rods, ranging from mild (T-17-M), to more severe (P-23-H), to advanced degeneration (Q-64-ter and G-106-R). The majority of cones in the T-17-M and P-23-H retinas were cytologically normal but showed loss of immunoreactivity for the cytoplasmic proteins 7G6, calbindin, and X-arrestin. The cone outer segments (OS) remained positive for cone opsins and peripherin-2 (rds/peripherin). All remaining cones in the Q-64-ter and G-106-R retinas were degenerate, with short to absent OS, but had strong reactivity for these cytoplasmic and OS membrane markers. Cones in the maculas of the RP retinas were degenerate, with short to absent OS, but retained strong labeling for the cytoplasmic and OS proteins.

Conclusions: Even before cones show cytologic changes in response to rod cell degeneration, they lose immunoreactivity for certain cytoplasmic proteins. These cones later show shortening and loss of OS, although their OS membrane proteins remain well labeled. Cones may down regulate expression of both cytoplasmic and outer segment membrane proteins in response to mutant rod cell dysfunction and/or cell death in human RP retinas. Such cytologic and immunocytochemical changes in the cones may presage death of these critical cells in the later stages of RP.

Retinitis pigmentosa (RP) is a group of inherited diseases that causes primary degeneration of rod photoreceptors in the human retina [1]. The disease is heterogeneous and caused by mutations in at least 30 different genes (RetNet). The first RP gene identified was *rhodopsin* (*RHO*) [2-6]. More than 80 different *RHO* mutations are now believed to cause autosomal dominant (ad) RP [7-9]. The first symptoms of RP, often noted in adolescence, are night vision disturbances due to rod dysfunction and cell death. Later in the disease, the critical cone photoreceptors lose function and degenerate, significantly decreasing the quality of life for the RP patient.

Many gaps remain in our understanding of how *RHO* mutations lead to primary rod cell death and secondary cone cell pathology [10,11]. It is unknown why defective and/or dying rod cells cause eventual loss of cones. This occurs in patients with *RHO* mutations as well as in patients with mutations in other rod-specific genes (e.g., the cGMP-phosphodiesterase α- and β-subunits [*PDE6A*, *PDE6B*] and the cyclic GMP gated channel protein α subunit [*CNGA1*]) [12]. It has been hypothesized that dying rods release substances toxic to the adjacent cones [13], or that cones are dependent on trophic factor(s) normally derived from the rods [14-18]. Increased information on molecular changes in cones in retinas with rod-specific mutations is essential for understanding the pathophysiology of RP and developing rational therapies for this disease. To gain insight into the process of rod and cone cell degeneration in RP, we have used immunocytochemistry to evaluate the retinas of four RP patients with ad RP caused by different *RHO* mutations. We report the labeling patterns produced by a battery of antibodies against cone and rod specific proteins, and present new evidence on the sequence of molecular changes occurring in cones that appear structurally normal or show degenerative changes in RP retinas.

Correspondence to: Ann H. Milam, Ph.D., Scheie Eye Institute, 51 North 39th Street, Philadelphia, PA, 19104; Phone: (215) 662-8102; FAX: (215) 662-5879; email: annmilam@mail.med.upenn.edu

METHODS

Tissue Preparation: Post mortem adult human eyes were obtained through the donor programs of the Foundation Fighting Blindness (FFB, Hunt Valley, MD) and the University of Washington Lions Eye Bank (Seattle, WA). The research followed the tenets of the Declaration of Helsinki and informed consent was obtained from all donors ante mortem. The research was approved by the institutional human subjects review board of the University of Pennsylvania. Four RP and four normal retinas were evaluated (Table 1). All globes were fixed for several weeks to months in 4% paraformaldehyde, with or without 0.5% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.3, and stored thereafter in 2% paraformaldehyde in the same buffer. The RP retinas had the following rhodopsin mutations: threonine-17-methionine (T-17-M; FFB-316 [19]), glutamine-64-ter (Q-64-ter; FFB-424 [20]), proline-23-

histidine (P-23-H; FFB-360), and glycine-106-arginine (G-106-R; FFB-517 [10]). Clinical information on three of the patients has been published [10,19,20]. The fourth patient (P-23-H mutation; FFB-360) was from a family with ad RP affecting 10 family members in six generations. He had been night blind since early childhood, lost peripheral vision in his 30's, and macular vision in his 50's.

Immunocytochemistry: Retinal samples were sectioned with a cryostat at 12 µm and processed for immunofluorescence by published techniques [21]. The secondary antibodies (goat anti-rabbit or anti-mouse IgG) were labeled with Alexa Fluor (green; Molecular Probes, Eugene, OR), Cy-2 (green) or Cy-3 (red; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Nuclei were stained (blue) with DAPI (1 µg/ml; Molecular Probes). Control sections were treated in the same way with omission of primary antibody.

Cell specific antibodies prepared in rabbits or mice were used for immunofluorescence study of the RP and normal human retinas (Table 2). The immunolabeled retinal sections were examined with a Leica DMR microscope (Deerfield, IL)

Table 1. Characteristics of human retinas used in study

Case	Age/Gender	PMI (h)	Fixative	Diagnosis
FFB-316	68 M	8.25	P	ad RP*
FFB-424	50 F	6.5	P + G	ad RP**
FFB-360	84 M	24.	P + G	ad RP#
FFB-517	99 F	5.25	P + G	ad RP##
FFB-525	65 M	6.0	P + G	Normal
0164-00	75 M	4.25	P + G	Normal
0403-93	51 F	3.0	P	Normal
99-11-23	53 M	0.	P	Normal

PMI = post mortem interval
P = 4% paraformaldehyde
G = 0.5% glutaraldehyde
*rhodopsin thr-17-met mutation
**rhodopsin glu-64-ter mutation
#rhodopsin pro-23-his mutation
##rhodopsin gly-106-arg mutation

Normal and RP eyes were fixed and processed by similar methods.

Table 2. Cell-specific markers used in study

Cell	Marker	Specificity	Species	Dilution	Source
Cones	7G6	unknown	mouse	1:250	P. MacLeish
Cones	-	X-arrestin	mouse	1:100	G. Inana
Cones	JH492	red/green cone	rabbit opsin	1:5,000	J. Nathans
Cones	UW-16	red/green cone opsin	rabbit	1:200	J. Saari
Cones	JH455	blue cone opsin	rabbit	1:5,000	J. Nathans
Cones	PNA-rhodamine (RL1072)	cone sheaths	plant lectin	1:100	Vector Labs
Interphotoreceptor matrix	-	interphotoreceptor retinoid-binding protein	rabbit	1:100	J. Saari
Cones and rods	Per 3Bg/5H2	peripherin-2	mouse	1:30	R. Molday
Cones, rods bipolar cells	P26	recoverin	rabbit	1:1000	A. Dizhoor
Cones, Horizontal, bipolar and amacrine cells	C8666	calbindin	mouse	1:200	Sigma
Cones, rods, inner retinal cells	A6403	cytochrome C oxidase	mouse	1:1000	Molecular Probes
Rods	4D2	rhodopsin	mouse	1:40	R. Molday
Rods	-	rhodopsin	rabbit	1:3000	E. Kean
Rods (S-antigen)	A9C6	rod arrestin	mouse	1:1000	L. Donoso
Astrocytes and reactive Müller cells	ZO334	GFAP	rabbit	1:750	Dako

Cell specific antibodies were used to assess cone immunoreactivity in retinas with RHO mutations.

using epifluorescence and Nomarski differential interference contrast (DIC) microscopy. The sections were photographed with Kodak (Rochester, NY) Elite Chrome film, ASA 400. After each immunofluorescence exposure, the same region was photographed by Nomarski DIC microscopy. Images were digitized with a flat bed scanner (Saphir HiRes, Heidelberg CPS GmbH, Bad Homburg, Germany) using LinoColor Elite 5.1 software (Heidelberg CPS GmbH), imported into a graphics program (PhotoShop 5.0, Adobe, San Jose, CA) and dye-sublimation prints were generated.

RESULTS

Normal Retinas: In the normal human retinas, all photoreceptors in the macula and periphery showed the expected patterns of labeling with the cell-specific markers used in this study. All cones had cytoplasmic and outer segment (OS) labeling with 7G6 (Figure 1A), and X-arrestin (Figure 1B), and the cytoplasm of their inner segments, cell bodies and synapses was positive with anti-calbindin (Figure 1C), which also labeled horizontal cells and some bipolar and amacrine cells. The cytoplasm and OS of all cones and rods were positive with anti-recoverin, which also labeled a population of cone

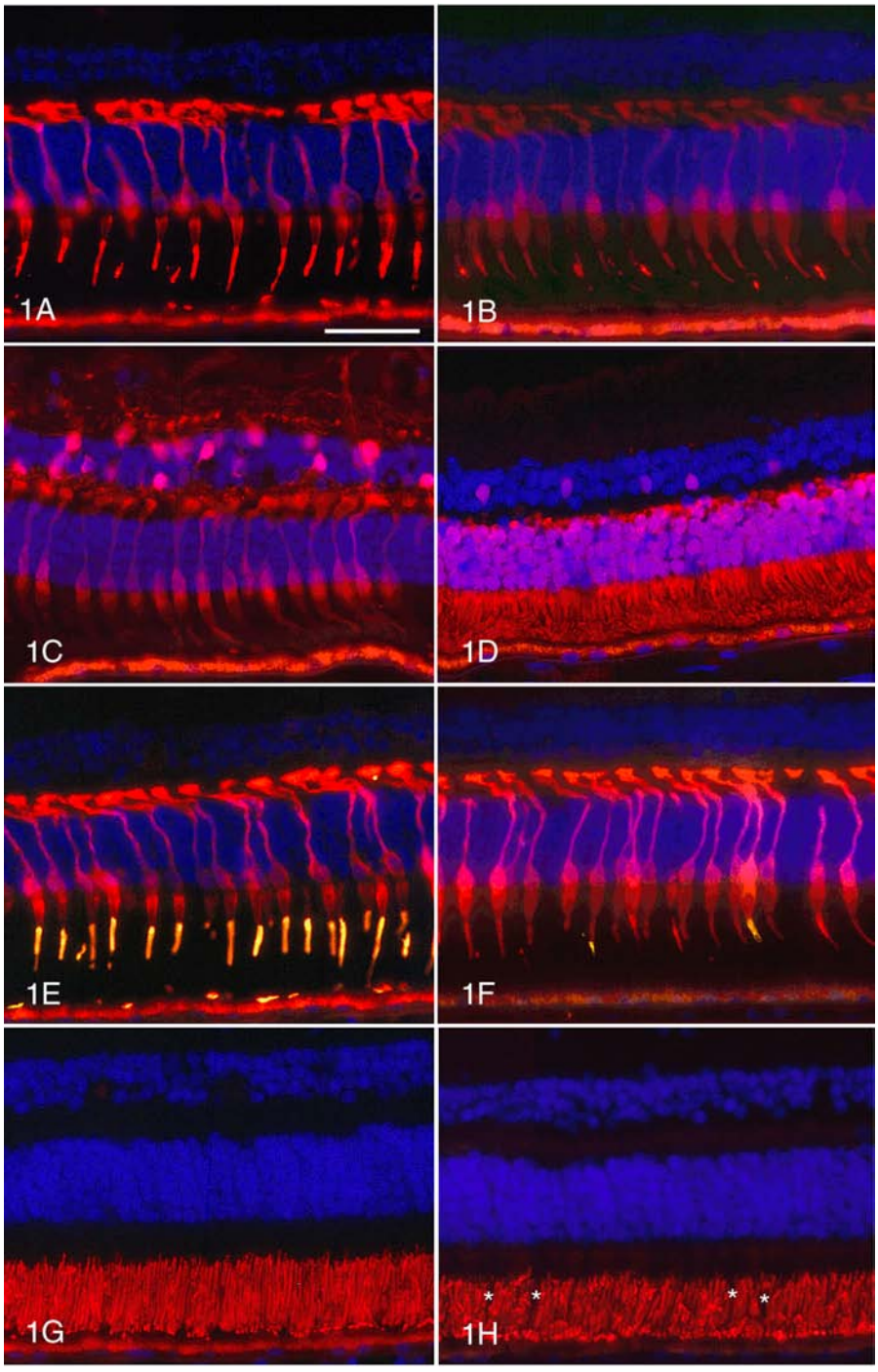

Figure 1. Immunocytochemical labeling of normal human retinas. **A**. Antibody 7G6 produces labeling of the outer segments and cytoplasm of the inner segments, cell bodies and synapses of all cone photoreceptors. **B**. Anti-X-arrestin labels the outer segments and the cytoplasm of the cell bodies and synapses of all cones. **C**. Anti-calbindin labels the cytoplasm of the cone inner segments, cell bodies and synapses. The horizontal cells and some bipolar and amacrine cells are also labeled. **D**. The outer segments and cytoplasm of the cones and rods are positive with anti-recoverin. A population of cone bipolar cells is also recoverin-positive. **E**. All cones are positive with antibody 7G6 (red) and the majority of cone outer segments are positive (gold) for red/green cone opsin. **F**. All cones are 7G6-positive (red) and a few cone outer segments (gold) are blue cone opsin-positive. **G**. The cone and rod outer segments are positive with anti-peripherin-2. **H**. The rod outer segments are rhodopsin-positive but the cones (*) appear as negative images.

bipolar cells (Figure 1D). Most cone OS were labeled with anti-red/green cone opsin (Figure 1E) and the remaining cone OS were positive for blue cone opsin (Figure 1F). The cone and rod OS were labeled with anti-peripherin-2 (rds/peripherin, Figure 1G) and the rod OS were reactive for rhodopsin (Figure 1H). The cone sheaths were uniform in structure and labeled with PNA-rhodamine (Figure 1I) and the interphotoreceptor matrix (IPM) was positive for IRBP (Figure 1J). The rod OS were strongly labeled and a few rod cell bodies and synapses were weakly labeled with anti-rod arrestin (Figure 1K). Mitochondria in the ellipsoids of cones and rods were strongly positive for cytochrome C oxidase (Figure 1L). Mitochondria in the outer and inner plexiform layers, and in some inner nuclear layer cells, were also labeled with anti-cytochrome C oxidase (Figure 1L). Immunolabeling with anti-GFAP was restricted to astrocytes and Müller end feet (Figure 1M). Treatment of the sections with no primary antibody produced no specific labeling but the retinal pigment epithelium (RPE) was filled with autofluorescence lipofuscin granules (Figure 1N).

Threonine-17-Methionine RHO *Mutation (FFB-316):* As reported previously [19], the inferior half of this retina was severely degenerate and contained no photoreceptors. Samples for the current study were taken from the superior and nasal

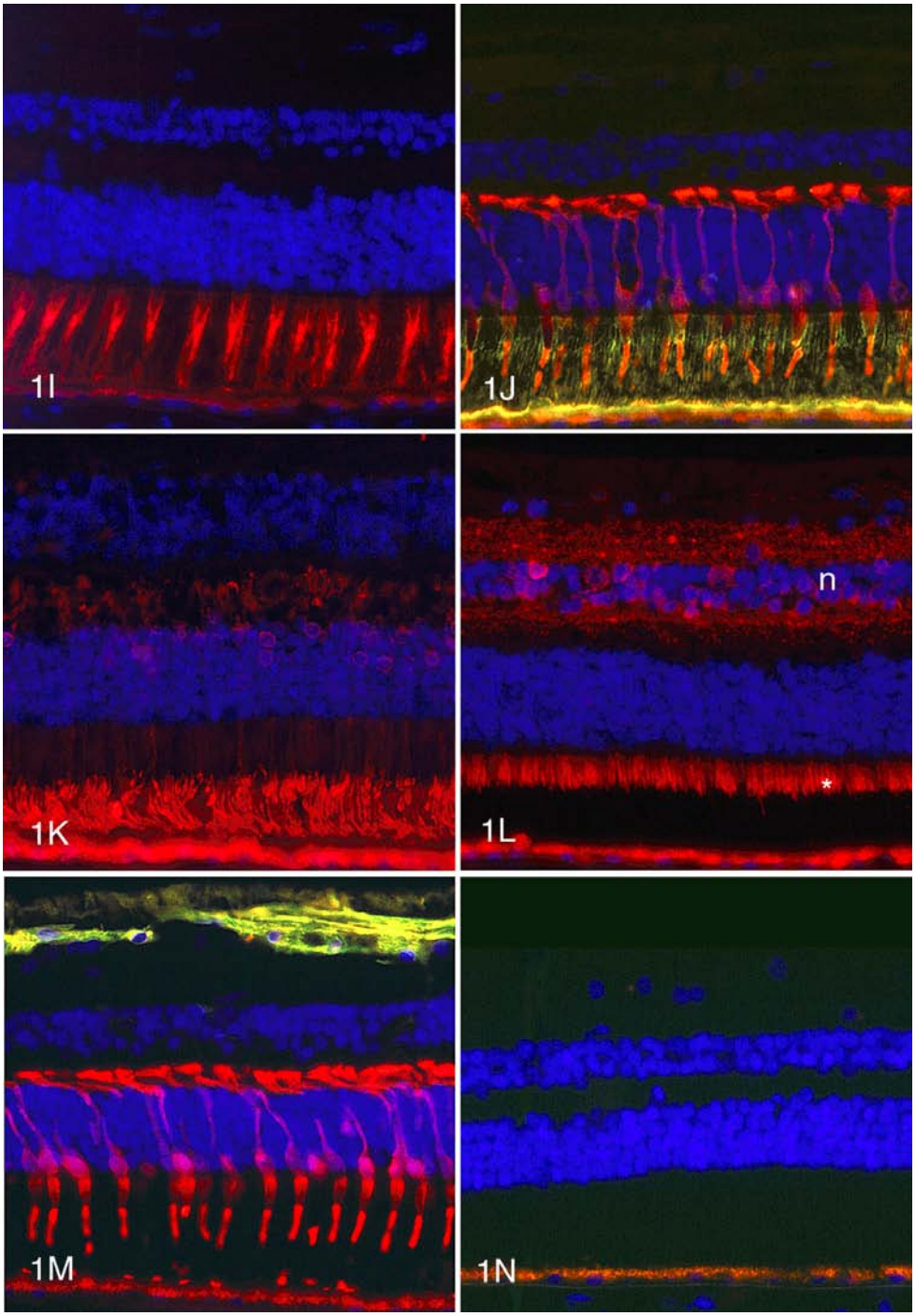

Figure 1, continued. **I**. The extracellular cone sheaths are uniform in structure and PNA-positive (red). **J**. The inter-photoreceptor matrix is positive for IRBP (green). All cones are labeled (red) with 7G6. **K**. The rod outer segments are strongly labeled (red) with anti-rod arrestin. Some rod cell bodies and synapses are weakly positive. **L**. Mitochondria in the cone and rod inner segments (*) are positive for cytochrome C oxidase. The inner and outer plexiform layers and some cells in the inner nuclear layer (n) are also labeled. **M**. Labeling with anti-GFAP (green) is restricted to astrocytes and Müller end feet. The cones are labeled (red) with 7G6. **N**. Control section treated with no primary antibody shows only auto-fluorescent lipofuscin granules in the RPE. Autofluorescent lipofuscin is present in the retinal pigment epithelium at the bottom of the panels. The scale bar represents 50 μm.

mid and far periphery, which contained both cones and rods, and the macula, which contained only scattered cones that lacked OS. When observed by DIC, cones and rods in the superior regions appeared near normal in numbers and cytology except for some OS shortening, particularly in the rods. The superior retina retained 40-50% of outer nuclear layer (ONL) nuclei in the mid periphery and 50-75% of ONL nuclei in the far periphery.

Approximately 75% of the cones in the superior retina had marked loss of 7G6 immunoreactivity in their inner segments, cell bodies and synapses (Figure 2A) even though these cones were cytologically intact by DIC microscopy (Figure 2B). Cones that had lost 7G6 immunoreactivity could not be distinguished from neighboring 7G6-positive cones by DIC (Figure 2B). All cone OS were labeled with anti-peripherin-2, and most were positive for red/green cone opsin (Figure 2C); only a few cone OS were labeled with anti-blue cone opsin.

The pattern of labeling with anti-X-arrestin, anti-recoverin, and anti-calbindin was very similar to that with 7G6. Many cones had lost OS and cytoplasmic labeling for X-arrestin (Figure 2D) and double labeling with anti-X-arrestin and 7G6 showed that the same cones had lost immunoreactivity for both proteins (Figure 2D-F). Immunoreactivity for calbindin was also absent in the majority of the cones, although

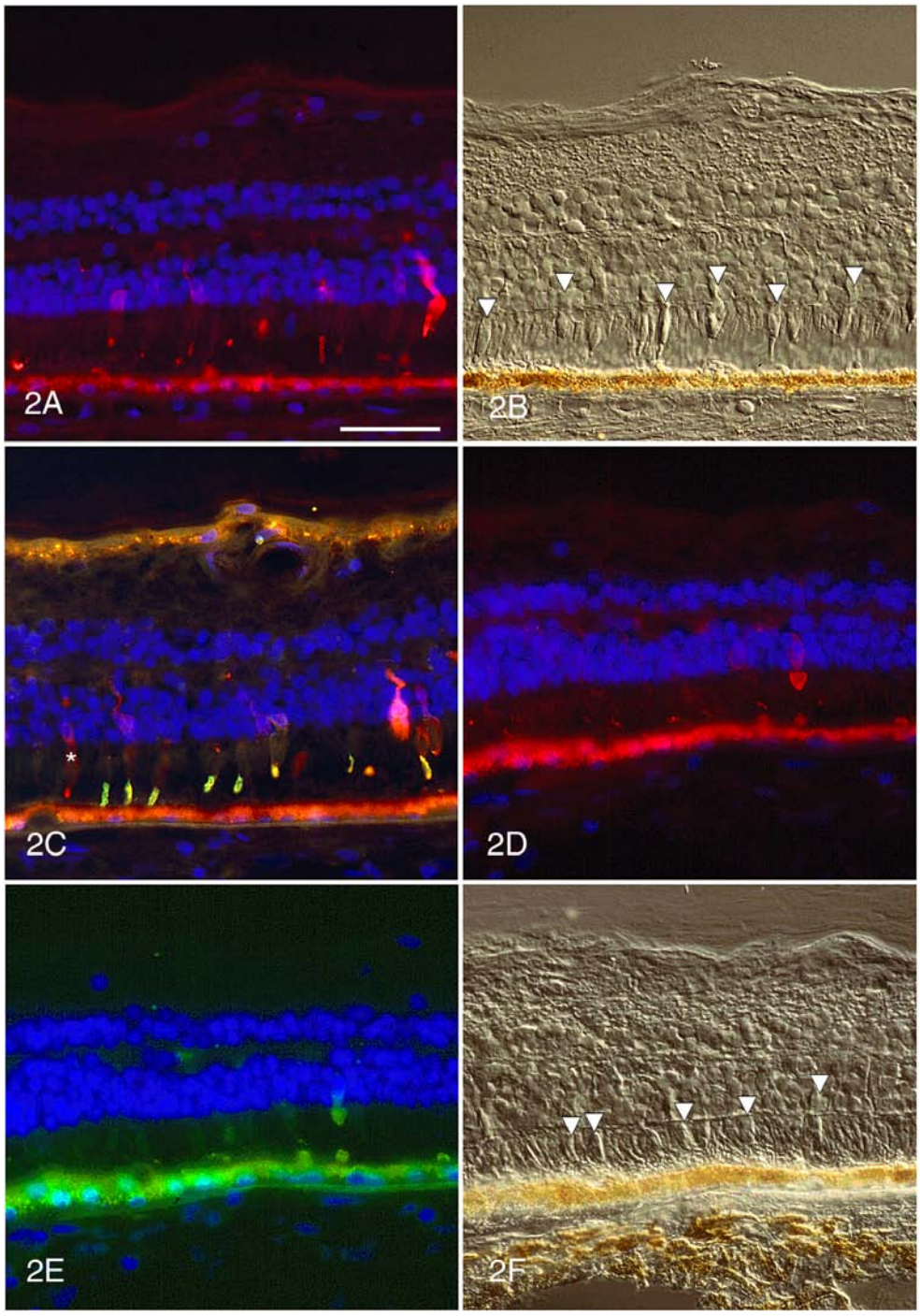

Figure 2. Immunocytochemistry of retina with T-17-M *RHO* mutation. **A**. 7G6 labels some cone outer and inner segments, cell bodies and synapses. The remainder of the cones have lost 7G6 immunoreactivity in their inner segments, cell bodies and synapses. **B**. DIC microscopy of the same field shown in **A**, illustrating the near normal cytologic appearance of the cones (arrowheads). **C**. Only a few cones are labeled with 7G6 (red) but most cone outer segments are labeled with anti-red/green cone opsin (green). The cone (*) with a 7G6-positive but red/green cone opsin-negative outer segment is presumably a blue cone. **D**. Labeling with anti-X-arrestin (red) shows that many cones have lost reactivity for this protein. **E**. Double labeling of the field in **D** with 7G6 (green) illustrates that the same cones have lost immunoreactivity for both arrestin and 7G6. **F**. Nomarski image of field shown in **D** and **E**. The cones are indicated by arrowheads.

the horizontal, bipolar and amacrine cells in the same sections continued to show strong labeling with anti-calbindin (Figure 2G). The extracellular domains of cones and rods in the IPM were examined with PNA lectin and anti-IRBP. The cone matrix sheaths were shortened and weakly PNA-positive. IRBP in the IPM was very weakly labeled (Figure 2H). In the macula, the cone matrix sheaths were strongly PNA-positive but very short (Figure 2I) and IRBP labeling was absent.

The rod OS were shortened but showed normal intensity of rhodopsin labeling in the superior retina, as observed in the controls. Rhodopsin labeling was also delocalized to a few rod cell bodies and synapses (Figure 2J) but no rod neurite sprouting was observed. Double labeling with 7G6 and anti-rhodopsin revealed that rods were well labeled with anti-rhodopsin in the areas where 7G6 cone labeling was markedly reduced. Rod arrestin was strongly labeled in the rod OS, inner segments, cell bodies and synapses (Figure 2K). This labeling pattern was different from the control retinas, where labeling with anti-arrestin was heaviest in the OS (Figure 1K). Mitochondria in the rod and cone inner segments were well labeled with anti-cytochrome C oxidase (Figure 2L). Treatment with anti-GFAP produced a few labeled Müller cell processes extending from the inner retina to the external limiting membrane, although no correlation could be found between

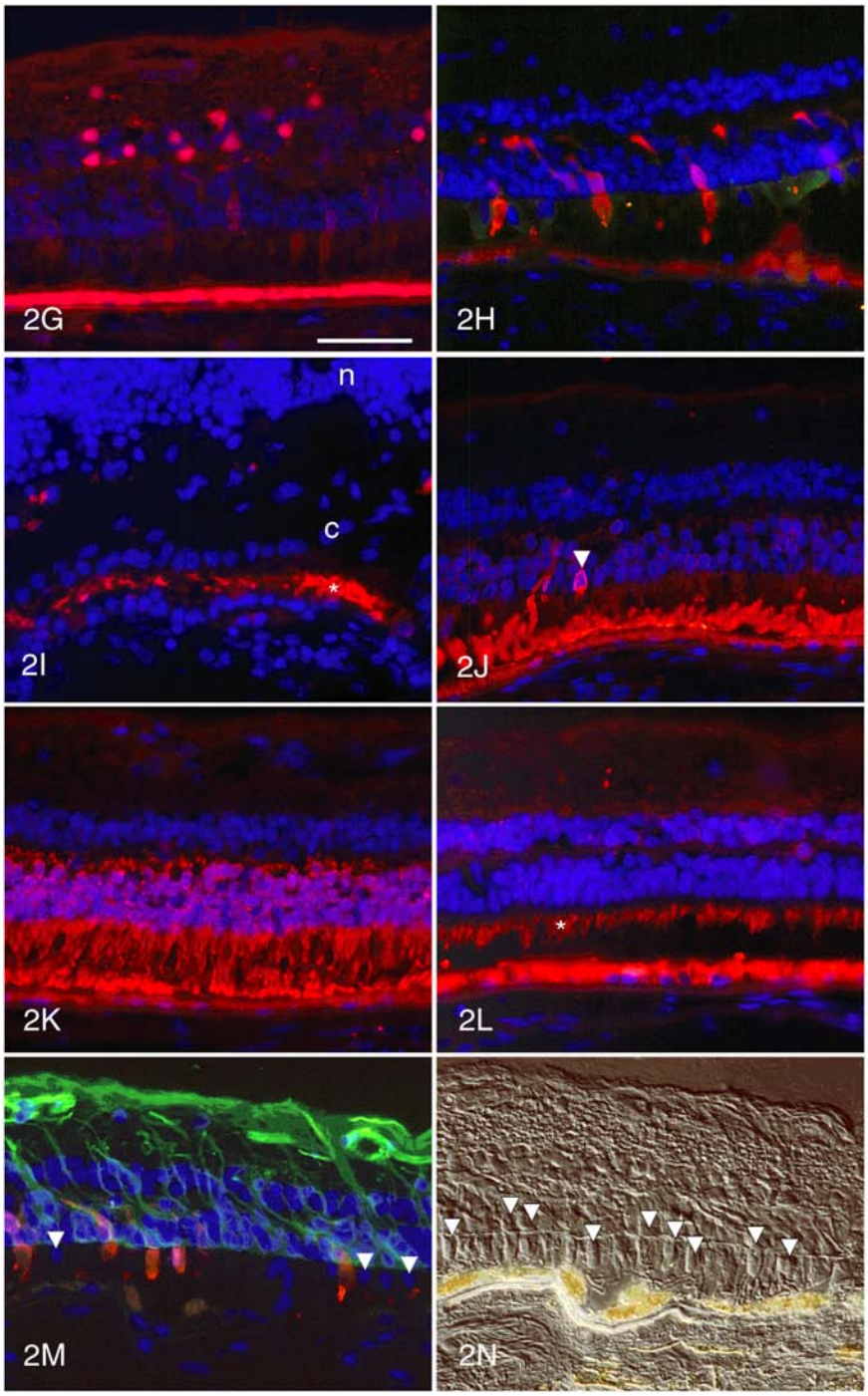

Figure 2, continued. **G**. Labeling with anti-calbindin illustrates loss of reactivity for this protein in most cones, although the horizontal, bipolar and amacrine cells continue to show strong labeling with the antibody. **H**. Immunolabeling with anti-IRBP reveals very weak reactivity for this protein (green) in the interphotoreceptor matrix. The cones are labeled (red) with 7G6. **I**. In the macula, the cone matrix sheaths (red, *) are very short but strongly PNA-positive. c, monolayer of cone nuclei; n, inner nuclear layer. **J**. In the periphery, the rod outer segments are shortened but show normal intensity of labeling with anti-rhodopsin. One rod (arrowhead) shows cell body labeling with anti-rhodopsin. **K**. Rod arrestin is localized in the rod outer and inner segments, cell bodies and synapses. **L**. The rod and cone inner segments (*) are well labeled for the mitochondrial protein, cytochrome C oxidase. **M**. GFAP labeling is increased in Müller processes in an area where some cones have lost 7G6 reactivity and have ectopic nuclei (arrowheads) in their inner segments. **N**. DIC of field in **M** illustrating the cytologic features of the cones (arrowheads). Autofluorescent lipofuscin is present in the retinal pigment epithelium at the bottom of the panels. The scale bar represents 50 μm.

GFAP-positive Müller processes and abnormalities in the rods or RPE. GFAP-labeling was increased in Müller processes in areas where cones had lost 7G6 reactivity and had nuclei displaced into their inner segments (Figure 2M). DIC microscopy revealed that near normal numbers of cones were retained in these regions (Figure 2N).

Glutamine-64-ter RHO *Mutation (FFB-424):* Retinal samples were taken from the temporal far periphery and the macula. This retina showed advanced disease and the ONL was reduced to 1-3 layers of cells in the peripheral region examined. All photoreceptors had absent or very short outer segments. All remaining cones, as identified by DIC, were strongly labeled with 7G6 (Figure 3A), anti-X-arrestin, anti-recoverin and anti-calbindin. The shortened cone OS were positive for peripherin-2 and red/green cone opsin (Figure 3B) but not for blue cone opsin. The cone matrix sheaths were very short and PNA-positive but IRBP-labeling was absent. The macular cones were reduced to a single layer of cell bodies that showed uniformly strong labeling with 7G6 (Figure 3C) and anti-X-arrestin. The cone OS were tiny but well labeled with 7G6 (Figure 3C), anti-peripherin-2 and anti-red/green cone opsin. Their matrix sheaths were shortened but strongly PNA-positive (Figure 3D). Scattered rhodopsin-positive rod cell bodies were retained in the macula (not shown).

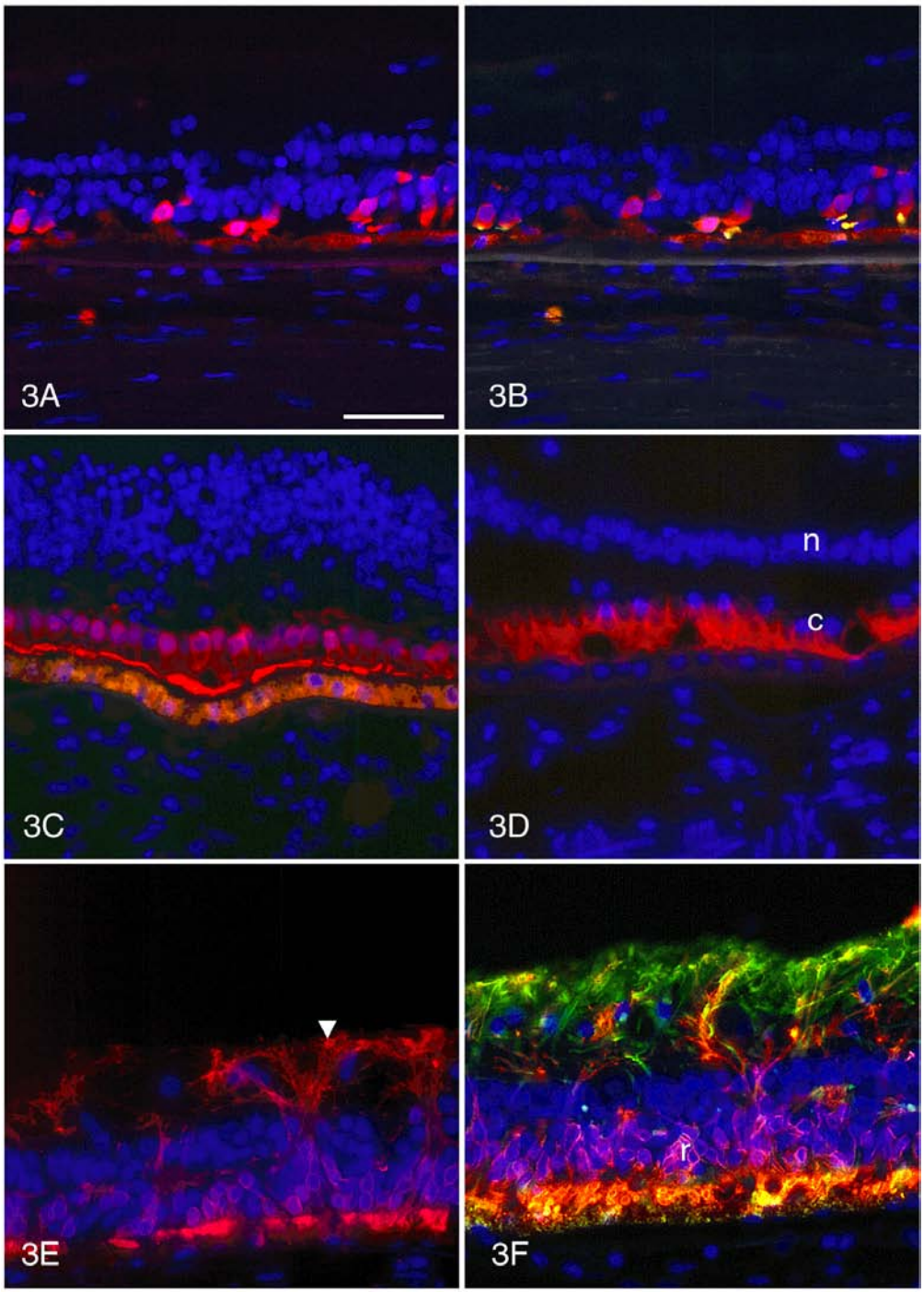

Figure 3. Immunocytochemistry of retina with Q-64-ter *RHO* mutation. **A**. All remaining cones are strongly labeled with 7G6. Their outer segments are very short. **B**. The shortened cone outer segments (gold) are positive for red/green cone opsin and 7G6 (red). **C**. The macular cones are reduced to a single layer of cell bodies that are strongly labeled with 7G6. Their outer segments have formed a layer of 7G6-positive debris in the subretinal space. **D**. The matrix sheaths of the macular cones are shortened but strongly PNA-positive (red). c, cone nuclei; n, inner nuclear layer. **E**. As revealed by labeling with anti-rhodopsin, the rods have very short or absent outer segments and rhodopsin is delocalized to their cell bodies and long neurites extending into the inner retina. Arrowhead indicates inner limiting membrane. **F**. GFAP-positive Müller processes (green) are hypertrophied. Rhodopsin in the rod cell bodies (r) and neurites is labeled red. Autofluorescent lipofuscin is present in the retinal pigment epithelium at the bottom of the panels. The scale bar represents 50 μm.

Rods in the peripheral retina had very short OS strongly reactive for rhodopsin, which was also present in the surface membranes of the inner segments and cell bodies. Many rods had rhodopsin-positive neurites extending into the inner retina as far as the inner limiting membrane (Figure 3E). Labeling with anti-peripherin-2 was restricted to the shortened rod OS. Arrestin and recoverin were well labeled in the rod inner segments and cell bodies. Mitochondria in the rod and cone inner segments were very weakly reactive for cytochrome C oxidase. Müller cell processes were hypertrophied and strongly positive for GFAP (Figure 3F).

Proline-23-Histidine RHO *Mutation (FFB-360):* Retinal samples were taken from the nasal and temporal mid and far periphery. Macular samples were not available. The peripheral regions showed retention of 1-4 rows of ONL nuclei. Examination of the peripheral regions by DIC revealed shortening and loss of rod and cone OS. Approximately 30% of the remaining cones had reduced immunoreactivity for 7G6, X-arrestin and calbindin. The rest of the cones showed normal labeling with antibodies to these proteins. Most cones were well labeled with 7G6 (Figure 4A,B). The shortened cone OS were reactive for peripherin-2 and red/green (Figure 4C) but

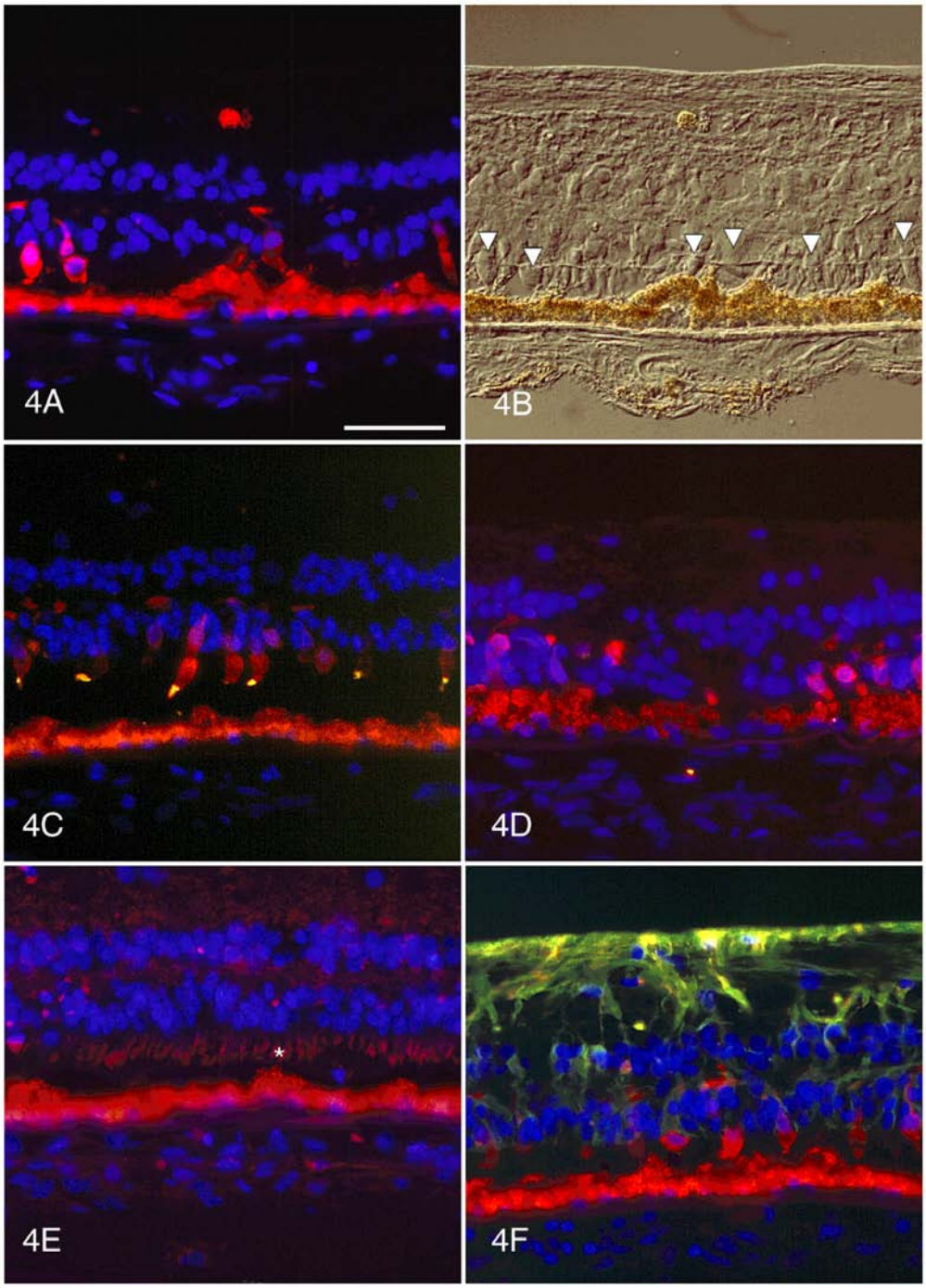

Figure 4. Immunocytochemistry of retina with P-23-H *RHO* mutation. **A**. Most remaining cones are well labeled with 7G6. **B**. DIC microscopy of same field as Figure 4A. The cones are indicated by the arrowheads. **C**. Some cones are well labeled with 7G6 (red) and most cone outer segments are positive (gold) for red/green cone opsin. **D**. IRBP immunolabeling (green) is absent from the interphotoreceptor matrix. The cones are 7G6-positive (red). The retinal pigment epithelium cells are enlarged and engorged with autofluorescent (red) lipofuscin granules. **E**. Cytochrome C oxidase labeling is weak in the shortened photoreceptor inner segments (*). **F**. GFAP labeling is strong in the hypertrophied Müller cell processes. Some cones, whose outer segments are tiny, are 7G6-positive (red). Autofluorescent lipofuscin is present in the retinal pigment epithelium at the bottom of the panels. The scale bar represents 50 μm.

not blue cone opsin. The cone matrix sheaths were very short and PNA-positive but IRBP labeling was absent (Figure 4D).

The rods were markedly reduced in number in all retinal regions. The rod OS were shortened and rhodopsin was delocalized to the inner segments, cell bodies and synapses. Rod arrestin was localized to the rod OS, inner segments and cell bodies. Mitochondria in the rod and cone inner segments were weakly labeled with anti-cytochrome C oxidase (Figure 4E). GFAP was increased in the hypertrophied Müller cell processes (Figure 4F).

Glycine-106-Arginine RHO *Mutation (FFB-517):* Retinal samples were taken from the nasal and temporal far periphery and the macula. Like FFB-424 (Q-64-ter), this retina had advanced disease, and only 1-3 layers of cones and rods with very short outer segments were present in the peripheral regions examined. All remaining cones, identified by DIC microscopy, were well labeled with 7G6 (Figure 5A,B) anti-X-arrestin, anti-calbindin, and anti-recoverin. The short cone OS were positive for peripherin-2, and red/green (Figure 5C,D) but not blue cone opsin. The cone matrix sheaths were very short and weakly PNA-positive but IRBP labeling was absent. The macula of this retina showed geographic atrophy and choroidal neovascularization, and was not used in the present study.

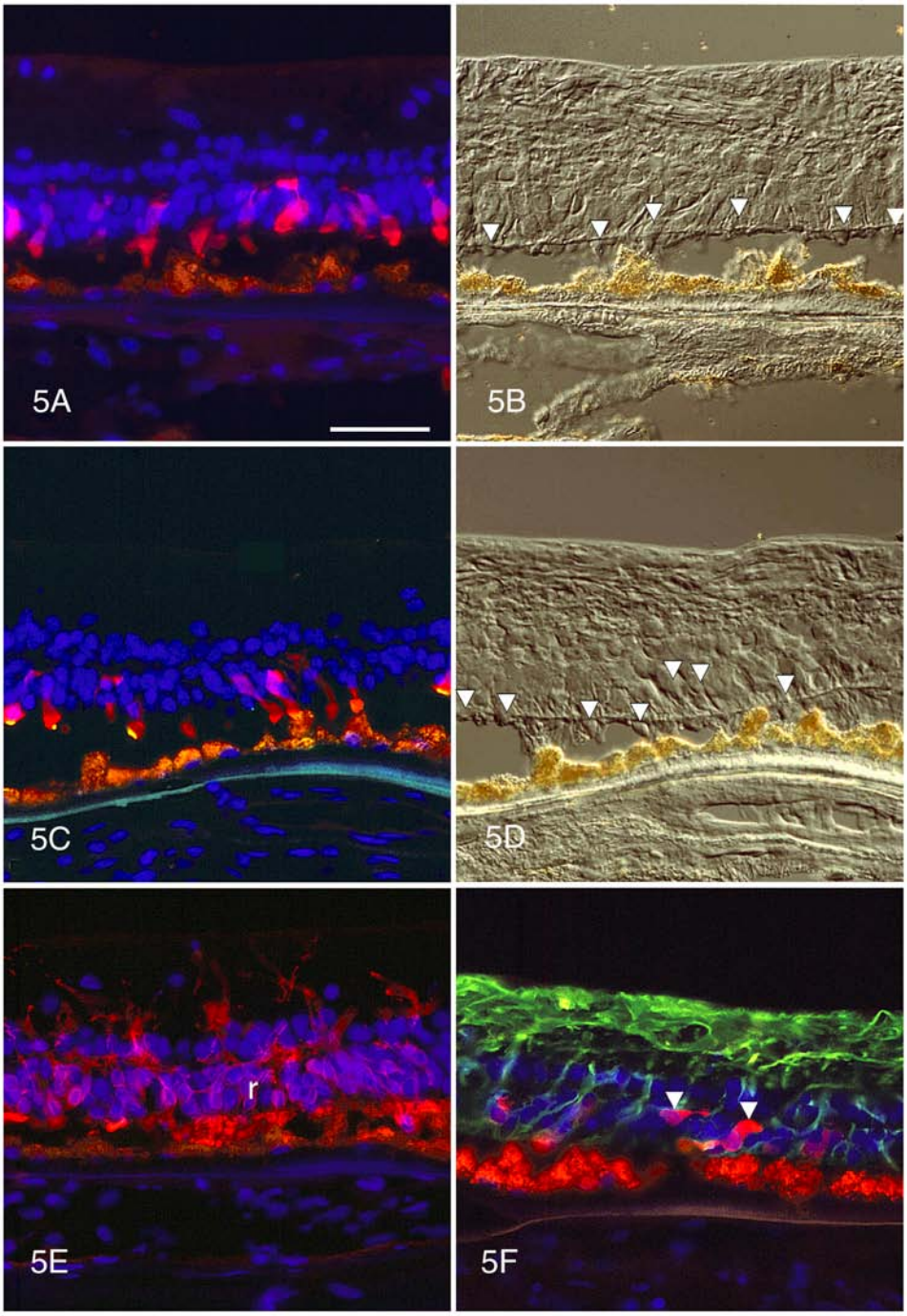

Figure 5. Immunocytochemistry of retina with G-106-R *RHO* mutation. **A**. All remaining cones are strongly positive with 7G6. Their outer segments are short to absent. **B**. The cones (arrowheads) are documented by DIC microscopy in the same field shown in Figure 5A. **C**. The shortened cone outer segments are positive for red/green cone opsin (gold). The cone cytoplasm is 7G6-positive (red). **D**. Nomarski image of field in Figure 5C illustrates the cones (arrowheads). **E**. As revealed by anti-rhodopsin labeling, the rods have tiny or absent outer segments and rhodopsin (red) is localized to the cell bodies (r) and neurites. **F**. Hypertrophied Müller processes are strongly positive for GFAP (green), and the few remaining cones (arrowheads) are well labeled with 7G6 (red). Autofluorescent lipofuscin is present in the retinal pigment epithelium at the bottom of the panels. The scale bar represents 50 μm.

Rods in this retina had tiny or absent OS and rhodopsin was delocalized to their cell bodies and extensive neurites that projected into the inner retina (Figure 5E). The few remaining rod OS were peripherin-2-positive. Most rod inner segments and cell bodies were weakly labeled with anti-arrestin. All remaining rods were strongly recoverin-positive. The rod and cone inner segments were shortened and were not reactive for cytochrome C oxidase. Hypertrophied Müller cell processes were strongly reactive for GFAP (Figure 5F).

DISCUSSION

During development of the primate retina, the structural proteins of cone outer segments, the cone opsins and peripherin-2, are expressed before the cytoplasmic proteins [22-24]. Specifically, blue cone opsin is expressed before red/green cone opsin, and the cone visual transduction proteins (alpha-transducin, phosphodiesterase and rhodopsin kinase) are expressed even later [22]. Although the function of 7G6 is not known, this cytoplasmic protein is also expressed after the opsins in some human cones [25; Personal communication, P. K. Swain]. In the RP retinas evaluated in this study, it appears that cones have reversed the sequence of normal development and regressed to a more primitive state, retaining OS structural proteins but lacking their specialized cytoplasmic proteins. As these cone cells are stressed and possibly dysfunctional in response to loss or dysfunction of neighboring rods, they may down regulate expression of the proteins that characterize their differentiated state and retain only their structural proteins. It is also possible that cones degrade cytoplasmic proteins as an early step in apoptosis. Little is known about the rate of turnover of normal cone OS structural proteins, the opsins and peripherin-2 [26,27]. This process may be very slow and require relatively little energy expenditure by the dedifferentiated cones to maintain their diminutive OS. Protein turnover experiments in cones of animal models for human RP would help to elucidate this process.

The loss of cone immunoreactivity is most clearly illustrated in the T-17-M *RHO* retina. The cone OS, although slightly shortened, showed qualitatively normal labeling with anti-peripherin-2 and anti-red/green cone opsin. However, reactivity for several cytoplasmic proteins was decreased in many of the otherwise normal appearing cones surrounded by T-17-M mutant rods. It should be noted that although these cones appeared normal at the level of DIC microscopy, further studies to assess the cone cytology are needed using electron microscopy.

In a given area, the same cones showed loss of labeling with 7G6, anti-X-arrestin, anti-calbindin, and anti-recoverin. Very similar observations were made on cones surrounded by mutant rods with shortened OS in rhodopsin-transgenic pigs [28]. The present results may also correlate with the physiologic demonstration of cone dysfunction spatially following the expanding “front” of rod dysfunction in autosomal dominant RP due to Class B1 *RHO* mutations, which include T-17-M [10]. Loss of immunoreactivity for cytoplasmic proteins in cones may correspond to the time when neighboring rods are losing up to 75% of their OS. Consistent with relatively normal morphology of these cones, cone dysfunction is not detectable until there is greater than 75% loss of rod OS [10].

In advanced disease with severe loss of rods, as seen in the Q-64-ter and G-106-R retinas, the few remaining cones showed strong reactivity for all cytoplasmic proteins examined. It is possible that the cones lacking cytoplasmic proteins had died but that these cones survived because they still expressed these proteins, although this argument is somewhat circular. Little is known about the control of expression of photoreceptor proteins, but this observation offers a clue that not all cones depend on adjacent rods for their survival. In the RP retinas, the maculas usually show loss of nearly all rods and many of the cones, with reduction of the remaining cones to a monolayer of cells with wide cell bodies and tiny or absent outer segments [1]. These cones can provide reasonably good visual acuity [29] and closely resemble those in the maculas of newborn human infants [30], again suggesting that the RP disease progress results in regressed differentiation of the cones.

Several lines of evidence indicate that death of mutant photoreceptor cells can be delayed by treatment with various growth/survival factors [15,31], some of which are intrinsic to human retina [32]. However, the mechanism(s) of rescue are not known and there is evidence that some neurotrophic factors activate signaling pathways in Müller cells and other cells of the inner retina, but not in photoreceptors themselves [33,34]. The relationship between Müller cells and cones in degenerate retinas is not well understood, although a recent study demonstrated that hyperplastic Müller processes are preferentially associated with cones in experimentally detached cat retinas [35]. Interestingly, in the present study Müller cell processes had accumulated GFAP in areas where cone cells showed loss of cytoplasmic protein reactivity and ectopic nuclei, although cytologically these cones were otherwise unremarkable.

Cytochrome C oxidase was decreased in the inner segments of cones in the severely degenerate retinas with the Q-64-ter and G-106-R *RHO*mutations. A similar reduction in cytochrome C oxidase was noted in the inner segments of photoreceptors in detached cat retinas [36]. Maintaining the cats in hyperoxia prevented the loss of cytochrome C oxidase immunoreactivity in the photoreceptors [36]. It is not known if similar loss of cytochrome C oxidase occurs in animal models of RP, as observed here in the human RP retinas, or if supplemental oxygen would prevent or reverse this pathologic change.

We found that the mutant rods showed a different pattern of immunolabeling with anti-arrestin than the controls. As noted previously in light adapted bovine retinas [37], in the normal human retinas the labeling with anti-arrestin was most intense in the OS, with weaker labeling in the cell bodies and synapses. In the RP retinas examined here (Figure 2K, for example), labeling with anti-arrestin was strong in the rod cell bodies and synapses as well as the OS, more closely resembling the pattern found in dark adapted rods. These observa-

tions need to be extended to additional RP retinas, but would appear to be inconsistent with the equivalent-light hypothesis for rod cell degeneration in RP [38].

Although this study documented loss of immunoreactivity for cytoplasmic proteins and did not measure mRNA levels, the results are consistent with down regulated expression of these proteins. If this is the case, the results suggest that protein renewal has been markedly reduced in cones in response to mutant rod cell dysfunction or death. Decreased protein renewal is also consistent with the severe OS shortening observed in the surviving cones. Other studies have suggested that normal rods have a trophic influence on neighboring cones [16-18], but the putative survival agent has not yet been identified. As noted previously [20], IRBP immunoreactivity was decreased in all four RP examined in the present study, suggesting that loss of this rod-derived protein might lead to cone OS shortening and final cone cell death.

Results from the present study suggest that loss of an unknown rod factor leads to decreased protein synthesis and, finally, to degeneration of the neighboring cones. However, we found loss of cytoplasmic protein reactivity in otherwise normal appearing cones surrounded by mutant but also normal appearing rods with shortened OS in the T-17-M *RHO* mutant retina. This suggests that a putative down regulation of cone protein expression is a response to loss of relatively few rod cells, not an end result of massive rod cell death. If this is true, initial rod cell death may affect neighboring cones in a dominant manner, rather than by simply reducing the putative trophic factor derived from rods. This could be secondary to the release of a toxic factor or from reduced input by rods into the neural network that interconnects rods and cones in the outer plexiform layer. It is important to obtain further information on this mechanism, as cones are critical cells essential for daytime vision in the RP patients.

ACKNOWLEDGEMENTS

The authors thank the scientists listed in the Methods section for providing antibodies, the Foundation Fighting Blindness and the University of Washington Lions Eye Bank for providing human tissues, Dr. A. Cideciyan for scientific input and Mr. J. Bouy for technical help. Supported by the Foundation Fighting Blindness, Hunt Valley, MD; the Paul and Evanina Bell Mackall Foundation Trust, NY, NY; NIH Grants EY06855 and EY13132, Bethesda, MD; and Research to Prevent Blindness, Inc., NY, NY.

REFERENCES

1. Milam AH, Li ZY, Fariss RN. Histopathology of the human retina in retinitis pigmentosa. Prog Retin Eye Res 1998; 17:175-205.
2. Dryja TP, McGee TL, Reichel E, Hahn LB, Cowley GS, Yandell DW, Sandberg MA, Berson EL. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. Nature 1990; 343:364-6.
3. Dryja TP, McGee TL, Hahn LB, Cowley GS, Olsson JE, Reichel E, Sandberg MA, Berson EL. Mutations within the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa. N Engl J Med 1990; 323:1302-7.
4. Dryja TP, Hahn LB, Cowley GS, McGee TL, Berson EL. Mutation spectrum of the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa. Proc Natl Acad Sci U S A 1991; 88:9370-4.
5. Farrar GJ, McWilliam P, Bradley DG, Kenna P, Lawler M, Sharp EM, Humphries MM, Eiberg H, Conneally PM, Trofatter JA, et al. Autosomal dominant retinitis pigmentosa: linkage to rhodopsin and evidence for genetic heterogeneity. Genomics 1990; 8:35-40.
6. Sung CH, Davenport CM, Hennessey JC, Maumenee IH, Jacobson SG, Heckenlively JR, Nowakowski R, Fishman G, Gouras P, Nathans J. Rhodopsin mutations in autosomal dominant retinitis pigmentosa. Proc Natl Acad Sci U S A 1991; 88:6481-5.
7. Gal A, Apfelstedt-Sylla E, Janecke AR, Zrenner E. Rhodopsin mutations in inherited retinal dystrophies and dysfunctions. Prog Retin Eye Res 1997; 16:51-79.
8. Dryja TP, McEvoy JA, McGee TL, Berson EL. Novel rhodopsin mutations Gly114Val and Gln184Pro in dominant retinitis pigmentosa. Invest Ophthalmol Vis Sci 2000; 41:3124-7.
9. Inglehearn CF, Tarttelin EE, Plant C, Peacock RE, al-Maghtheh M, Vithana E, Bird AC, Bhattacharya SS. A linkage survey of 20 dominant retinitis pigmentosa families: frequencies of the nine known loci and evidence for further heterogeneity. J Med Genet 1998; 35:1-5.
10. Cideciyan AV, Hood DC, Huang Y, Banin E, Li ZY, Stone EM, Milam AH, Jacobson SG. Disease sequence from mutant rhodopsin allele to rod and cone photoreceptor degeneration in man. Proc Natl Acad Sci U S A 1998; 95:7103-8.
11. Sung CH, Tai AW. Rhodopsin trafficking and its role in retinal dystrophies. Int Rev Cytol 2000; 195:215-67.
12. Rattner A, Sun H, Nathans J. Molecular genetics of human retinal disease. Annu Rev Genet 1999; 33:89-131.
13. Bird AC. Investigation of disease mechanisms in retinitis pigmentosa. Ophthalmic Paediatr Genet 1992; 13:57-66.
14. Milam AH. Strategies for rescue of retinal photoreceptor cells. Curr Opin Neurobiol 1993; 3:797-804.
15. Steinberg RH. Survival factors in retinal degenerations. Curr Opin Neurobiol 1994; 4:515-24.
16. Mohand-Said S, Hicks D, Dreyfus H, Sahel JA. Selective transplantation of rods delays cone loss in a retinitis pigmentosa model. Arch Ophthalmol 2000; 118:807-11.
17. Hicks D, Sahel J. The implications of rod-dependent cone survival for basic and clinical research. Invest Ophthalmol Vis Sci 1999; 40:3071-4.
18. Curcio CA, Owsley C, Jackson GR. Spare the rods, save the cones in aging and age-related maculopathy. Invest Ophthalmol Vis Sci 2000; 41:2015-8.
19. Li ZY, Jacobson SG, Milam AH. Autosomal dominant retinitis pigmentosa caused by the threonine-17-methionine rhodopsin mutation: retinal histopathology and immunocytochemistry. Exp Eye Res 1994; 58:397-408.
20. Milam AH, Li ZY, Cideciyan AV, Jacobson SG. Clinicopathologic effects of the Q64ter rhodopsin mutation in retinitis pigmentosa. Invest Ophthalmol Vis Sci 1996; 37:753-65.
21. Li ZY, Kljavin IJ, Milam AH. Rod photoreceptor neurite sprouting in retinitis pigmentosa. J Neurosci 1995; 15:5429-38.
22. Sears S, Erickson A, Hendrickson A. The spatial and temporal expression of outer segment proteins during development of Macaca monkey cones. Invest Ophthalmol Vis Sci 2000; 41:971-9.
23. Bumsted K, Jasoni C, Szel A, Hendrickson A. Spatial and temporal expression of cone opsins during monkey retinal develop-

ment. J Comp Neurol 1997; 378:117-34.
24. Xiao M, Hendrickson A. Spatial and temporal expression of short, long/medium, or both opsins in human fetal cones. J Comp Neurol 2000; 425:545-59.
25. Wikler KC, Rakic P, Bhattacharyya N, Macleish PR. Early emergence of photoreceptor mosaicism in the primate retina revealed by a novel cone-specific monoclonal antibody. J Comp Neurol 1997; 377:500-8.
26. Guerin CJ, Lewis GP, Fisher SK, Anderson DH. Recovery of photoreceptor outer segment length and analysis of membrane assembly rates in regenerating primate photoreceptor outer segments. Invest Ophthalmol Vis Sci 1993; 34:175-83.
27. Young RW. The renewal of rod and cone outer segments in the rhesus monkey. J Cell Biol 1971; 49:303-18.
28. Li ZY, Wong F, Chang JH, Possin DE, Hao Y, Petters RM, Milam AH. Rhodopsin transgenic pigs as a model for human retinitis pigmentosa. Invest Ophthalmol Vis Sci 1998; 39:808-19.
29. Geller AM, Sieving PA. Assessment of foveal cone photoreceptors in Stargardt's macular dystrophy using a small dot detection task. Vision Res 1993; 33:1509-24.
30. Hendrickson AE, Yuodelis C. The morphological development of the human fovea. Ophthalmology 1984; 91:603-12.
31. LaVail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, Steinberg RH. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. Proc Natl Acad Sci U S A 1992; 89:11249-53.
32. Li ZY, Chang JH, Milam AH. A gradient of basic fibroblast growth factor in rod photoreceptors in the normal human retina. Vis Neurosci 1997; 14:671-9.
33. Wahlin KJ, Campochiaro PA, Zack DJ, Adler R. Neurotrophic factors cause activation of intracellular signaling pathways in Muller cells and other cells of the inner retina, but not photoreceptors. Invest Ophthalmol Vis Sci 2000; 41:927-36.
34. Zack DJ. Neurotrophic rescue of photoreceptors: are Muller cells the mediators of survival? Neuron 2000; 26:285-6.
35. Lewis GP, Fisher SK. Muller cell outgrowth after retinal detachment: association with cone photoreceptors. Invest Ophthalmol Vis Sci 2000; 41:1542-5.
36. Mervin K, Valter K, Maslim J, Lewis G, Fisher S, Stone J. Limiting photoreceptor death and deconstruction during experimental retinal detachment: the value of oxygen supplementation. Am J Ophthalmol 1999; 128:155-64.
37. Smith WC, Milam AH, Dugger D, Arendt A, Hargrave PA, Palczewski K. A splice variant of arrestin. Molecular cloning and localization in bovine retina. J Biol Chem 1994; 269:15407-10.
38. Fain GL, Lisman JE. Light Ca2+, and photoreceptor death: new evidence for the equivalent-light hypothesis from arrestin knockout mice. Invest Ophthalmol Vis Sci 1999; 40:2770-2.