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Defective phototransductive disk membrane morphogenesis in transgenic mice expressing opsin with a mutated N-terminal domain

Xinran Liu[1], Ting-Huai Wu[2], Sally Stowe[3], Atsuko Matsushita[4], Kentaro Arikawa[4], Muna I. Naash[2] and David S. Williams[1,*]

[1]Departments of Pharmacology and Neurosciences, UCSD School of Medicine, La Jolla, California 92093-0983, USA
[2]Department of Ophthalmology and Visual Science, University of Illinois, Chicago, Illinois 60612, USA
[3]Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia
[4]Department of Biology, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236, Japan

*Author for correspondence (e-mail: dswilliams@ucsd.edu)

SUMMARY

Retinitis pigmentosa is a heterogeneous group of inherited retinal disorders in which the photoreceptor cells degenerate. A line of transgenic mice expresses a mutant opsin gene that encodes three missense mutations near the amino terminus, including P23H, which is the basis for a common form of dominant retinitis pigmentosa. By studying the photoreceptor cells of these mice and their normal littermates, we found that: (1) opsin was routed correctly, (2) the concentration of opsin in the disk membranes appeared normal by freeze fracture analysis, (3) the amount of disk membrane shedding was normal, but (4) the basal disks of the outer segments were disorganized, indicating defective disk membrane morphogenesis. Defective disk membrane morphogenesis appears to result in the formation of fewer mature disks, thus accounting for observed gradual shortening of the photoreceptor outer segments with age. We suggest that abnormal disk membrane morphogenesis is the primary cellular defect that leads to blindness, and that it arises from the inability of nascent disk membranes, containing normal and mutant opsin, to interact normally with each other.

Key words: Retinitis pigmentosa, Opsin, Photoreceptor cell, Rod outer segment, Membrane turnover, Transgenic mice

INTRODUCTION

Retinitis pigmentosa (RP) is a heterogeneous group of inherited retinal disorders that cause progressive photoreceptor degeneration and thus eventually blindness. Mutations in the opsin gene are estimated to account for about 10% of RP cases in the United States (Berson, 1996). The first mutation to be identified and the most common amongst RP patients in the United States is a point mutation, P23H, which causes dominant RP (Dryja et al., 1990). Expression of P23H opsin in COS cells has shown that this mutant is like a number of other opsin missense mutations in that it binds 11-*cis*-retinal poorly and is not transported from the endoplasmic reticulum to the plasma membrane due to misfolding (Sung et al., 1991; Kaushal and Khorana, 1994). Most recently, Liu et al. (1996) have found that the structure of misfolded P23H opsin is less compact than that of normal opsin.

The distribution of mutant opsin also appears to be defective in vivo. In transgenic mice expressing Q344ter mutant opsin (which lacks the last five amino acids; Sung et al., 1994) and in *Drosophila* expressing some 13 different opsin mutations (although not including P23H; Colley et al., 1995), the mutant opsin accumulates at the site of synthesis, as in COS cells. In mice expressing human P23H opsin, it appears to be routed to the synaptic terminal, in addition to accumulation in the inner segment and routing to the outer segment (Olsson et al., 1992; Roof et al., 1994).

In the present study, we examined transgenic mice expressing mutant mouse opsin (Naash et al., 1993). These mice are not an exact model for the P23H mutation, for in addition to this mutation they contain V20G and P27L mutations (and are thus termed VPP mice). However, V20G and P27L probably are not deleterious. First, these two mutations have not been detected in patients with RP. Second, in contrast to P23, neither V20 nor P27 is conserved amongst different opsins (cf. Dryja et al., 1990). Third, it is known that retinal degeneration does not occur in mice carrying the V20G mutation (Al-Ubaidi et al., 1990). Importantly, the VPP mice express equal amounts of mutant and normal opsin, and undergo slow progressive photoreceptor degeneration (Naash et al., 1993), with many of the pathophysiological changes found in RP patients (Goto et al., 1995, 1996).

Our aim was to identify the primary cellular defect in the photoreceptor cells of the VPP mice. First, we examined by immunofluorescence and immunoelectron microscopy whether there were any signs of incorrect routing of opsin. Second, we determined the packing density of opsin in the disk membranes by immunoelectron microscopy and freeze fracture analysis. As P23H opsin is a less compact molecule (Liu et al., 1996), a resulting defect might be a lowered concentration of opsin in

the membrane, with a consequential effect on the stability of the membranous disks. Finally, we analyzed photoreceptor ultrastructure in mice before the onset of degeneration. Our observations suggest that perturbed disk membrane morphogenesis is the primary cellular defect.

MATERIALS AND METHODS

Animals

The transgenic mice have been described previously by Naash et al. (1993). They are from the C57BL/6 strain and express VPP mutant opsin under the control of the mouse opsin promoter. The transgene consisted of a mutated 15 kb mouse opsin genomic fragment, including 6 kb of the upstream sequence and 3.5 kb of the downstream sequence. Transgenic (heterozygous) and nontransgenic litter mates were obtained by crossing heterozygous transgenic mice with wild-type C57BL mice. Transgenic mice were identified after PCR amplification of exon 1, followed by *Nco*I digestion; the transgene contains an RFLP that results in the deletion of one *Nco*I site, so that an additional fragment is generated by *Nco*I digestion (Naash et al., 1993). Nontransgenic littermates served as controls for the transgenic mice. Animals were kept on a 12 hour light/12 hour dark cycle. During the light cycle, cool fluorescent lighting provided a maximum illuminance of 50-60 lux around the cages.

Determination of opsin synthesis

Mice were killed at 18 days of age by exposure to 100% carbon dioxide. At this age, under the lighting conditions used, VPP mice have photoreceptor outer segments that are no more than 10% shorter than control mice. The neural retinas were removed immediately after death of the mouse, and incubated for 2 or 4 hours in methionine- and cysteine-free DMEM medium (ICN Biomedicals, Inc.) to which 50 μCi of [^{35}S]methionine and [^{35}S]cysteine (1100 Ci/mmol, NEN DuPont) had been added. They were then rinsed with PBS and frozen at −70°C.

Rod outer segment membranes were purified in discontinuous sucrose density gradients, as described by Fung (1983). Retinas were placed in buffer A (20 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 10 mM MOPS, pH 7.5) with 26% (w/v) sucrose. Rod outer segment membranes were released from retinas by repeated passage of the tissue through the orifice of a pipette. To each 11 mm × 60 mm centrifuge tube, 0.5 ml of the resulting suspension was layered gently on top of a discontinuous gradient containing 0.7 ml buffer A with 26% (w/v) sucrose on top of 3 ml buffer A with 40% (w/v) sucrose. After centrifugation for 30 minutes at 140,000 ***g*** at 4°C (SW 60 rotor; Beckman Instruments, Inc.), rod outer segment membranes were collected from the interface of the 26% and 40% sucrose solutions. They were then washed and suspended in buffer A. It seems that perturbed disk membranes in the transgenic mice (see Results) were isolated with the intact disk membranes in this procedure. If the perturbed disk membranes were not included, the specific radioactivity of the opsin should have been much lower than that obtained from the VPP mice. Also, the density of these membranes is probably similar to that of normal disk membranes, so that they should equilibrate with the normal disk membranes in the sucrose density gradient. Protein concentration was determined by the method of Lowry.

Rod outer segment membrane proteins were separated by 10% SDS-PAGE and either exposed for autoradiography or transferred to nitrocellulose. Gels for autoradiographs contained ~100 ng protein per lane. Gels for western blots contained ~20 ng protein per lane. Western blots were blocked with buffer containing 5% (w/v) nonfat milk and incubated with a 1:1,000 dilution of a mAb 1D4. This mAb, which is specific for the C terminus of rod opsin (Hodges et al., 1988), was provided by Dr R. Molday. It has been used previously to label normal opsin and opsins with mutations on the extracellular/intradiskal surface (e.g. Olsson et al., 1992; Liu et al., 1996). Opsin was visualized, following incubation with anti-mouse IgG conjugated to HRP, and enhanced chemiluminescence (ECL kit, Amersham). The blot was incubated in detection solution for 1 minute and then exposed to a BIOMAX film (Kodak) for 2 minutes. The intensity of opsin labeling was determined by densitometry (BioImage Intelligent Quantifier).

Data were obtained from three separate experiments, each using both retinas from three control and three VPP mice.

Tissues for microscopy

Mice were anaesthetized with urethane during the early part of the light period of the light/dark cycle. One eye was removed and immersed in fixative A (0.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The animal was then perfused with saline followed by fixative B (2% glutaraldehyde and 1.2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) through the left ventricle of the heart. The remaining eye was removed, immersed in the same fixative, and the anterior part of the eye was removed. After immersion in appropriate fixative for 2-4 hours, the posterior half of each eye was divided into quadrants. Tissues fixed in fixative A were processed either for cryosectioning, or for embedment in L.R. White followed by ultrathin sectioning, as described previously (Williams et al., 1990; Arikawa et al., 1992). Semithin (500 nm) cryosections were labeled for immunofluorescence microscopy, using opsin mAb 1D4 and then goat anti-mouse IgG conjugated to Texas Red. Ultrathin sections were labeled with the same opsin antibody and goat anti-mouse IgG conjugated to 15 nm gold particles. Tissues fixed in fixative B were processed for conventional transmission electron microscopy or freeze fracture analysis. For the former, retinal quadrants were washed 4 times in 0.137 M cacodylate buffer (pH 7.4), postfixed in 1.3% OsO_4 for 2 hours, dehydrated in an acetone series and embedded in Spurrs resin. Ultrathin sections were collected and examined by transmission electron microscopy. For freeze fracture analysis, retinas were infiltrated with 30% glycerol in fixative and cut into three portions. Pieces were placed on copper specimen holders and frozen in Freon 22. They were fractured in a Balzers 301 freeze-fracture apparatus at a vacuum of 10^{-6} to 10^{-7} mbar, and a temperature of −115°C, and allowed to warm to −100°C for 1-2 minutes before shadowing with Pt/C at an angle of 45 degrees and backing with rotary-shadowed carbon at an angle of 85 degrees. Replicas were cleaned in chromic acid, mounted on Formvar-coated slot grids, and viewed by transmission electron microscopy at 75kV.

Quantification

The relative concentration of opsin in the outer segment, ellipsoid, and myoid domains of transgenic and control rod photoreceptor cells was determined from the density of gold particles on ultrathin sections that had been immunogold-labeled with opsin mAb 1D4. Data were obtained from three transgenic mice and three control mice, all from the same litter at 40 days after birth. Data were collected from six different areas and at least 15 different rod cells of each retina.

The relative concentration of opsin in transgenic and control rod outer segment disk membranes was determined from the P-face intramembrane particles evident on freeze fracture replicas. The density and size of the particles were measured from electron micrographs printed at a magnification of 200,000 times. Data were obtained from two transgenic mice and three control mice, all from the same litter at 35-40 days after birth.

The degree of disorganization of the basal disks of rod outer segments was assessed by examining ultrathin sections of retinas fixed by perfusion (see above) in an electron microscope. Retinas of mice were examined from three different litters, each containing control and transgenic mice. The mice were either 16, 21, or 35-40 days old. The region of the retina examined was restricted to the mid-periphery of the inferior nasal quadrant. This region was scanned and rod photoreceptor cells with their connecting cilia evident in longitudinal section were assessed. A minimum of 40 such photoreceptor cells per retina were assessed. As illustrated in the Results section, their basal disks were described either as normal, or with a particular degree of

disorganization according to the amount of membrane vesiculation observed (stages 1, 2 or 3). For each animal, the percentage of cells ascribed to each stage was determined. The mean ± s.e.m. was then calculated for each group of retinas of the same age and type.

The amount of disk membrane shedding was determined by counting the number of phagosomes per length of retinal pigment epithelium in 1-μm sections of the mid-periphery of the inferior nasal quadrant. Phagosomes that appeared in section with a diameter ≥0.5 μm (and thus could be distinguished readily from pigment granules and smaller membrane debris, see Results) were counted. Data were confirmed by electron microscopy analysis of some ultrathin sections. Phagosome concentrations in control and transgenic littermates fixed within half an hour of each other were compared. Mice from three different litters were examined. Ages of the different mice were 16 days, 21 days, and 35-40 days. Retinas were from mice that were fixed by perfusion (see above) 1-4 hours after the time of light onset.

A one-tailed Student *t*-test was used to determine the probability of no significant difference between control and transgenic groups.

RESULTS

Opsin synthesis

Previous work showed that in VPP mice, VPP opsin mRNA and normal opsin mRNA are present in the same quantity, and that the amount of total opsin mRNA is similar in the retinas of 10- to 15-day-old VPP and control mice (Naash et al., 1993; Cheng and Naash, 1995). Here, we tested if the rate of total opsin synthesis differed between VPP mice and their normal littermates. Isolated retinas were incubated in culture medium containing [^{35}S]methionine and [^{35}S]cysteine for 2 or 4 hours. Rod outer segment membranes were purified from the retinas and prepared for western blotting and autoradiography. The western blots were labeled with mAb 1D4. VPP mouse retinas yielded an amount of opsin that was not significantly different from control retinas. As illustrated in Fig. 1A, all the opsin from VPP retinas appeared to have the same molecular mass in SDS-PAGE as normal mouse opsin. Moreover, the rate of ^{35}S-labeled methionine and cysteine incorporation into opsin, and therefore total opsin synthesis, was similar in VPP and control mice (Fig. 1B and C).

Opsin localization

When P23H mutant opsin is expressed in COS cells it remains in the endoplasmic reticulum, whereas normal opsin is routed to the plasma membrane (Sung et al., 1991; Kaushal and Khorana, 1994). Similarly, P23H mutant human opsin has an abnormal distribution in the photoreceptor cells of transgenic mice (Olsson et al., 1992; Roof et al., 1994). We investigated the distribution of opsin in normal and transgenic mice by immunolocalization with mAb 1D4. We found no evidence of accumulation of opsin anywhere in the photoreceptor cells outside of the outer segments in animals aged from 16 days old up to 40 days old. In immunofluorescence images, label was evident only in the outer segment layer of both control and transgenic retinal sections (Fig. 2). Likewise, in immunogold-labeled electron micrographs, the outer segments were the only well-labeled structures in both controls and transgenics (Fig. 3). On sections of predegenerate photoreceptor cells from three control and three transgenic mice, aged 35-40 days, there was no significant difference (P≥0.15) in gold particle density between control outer segments, 135±21 particles per μm^2 (s.e.m., n=3), and transgenic outer segments, 185±39. Some gold particles were evident in the myoid (arrowhead in Fig. 3B) and the ellipsoid of the inner segment. The myoid contains most of the cell's endoplasmic reticulum and Golgi, and opsin must pass through the ellipsoid en route to the outer segment. However, there was no more label in these domains of transgenic mouse photoreceptor cells than in that of controls. Fig. 4 illustrates the relative opsin concentration in ellipsoid and myoid domains, as determined by immunogold labeling density. The subcellular distribution of opsin in transgenic mice is the same as that in control mice. No immunogold label was evident in the nuclear or synaptic regions of the photoreceptor cells (not shown).

Freeze fracture analysis of outer segment disk membranes

P23H opsin is a less compact molecule than normal opsin (Liu et al., 1996), so that its presence in the disk membrane might result in a lower opsin packing density. A significant dilution

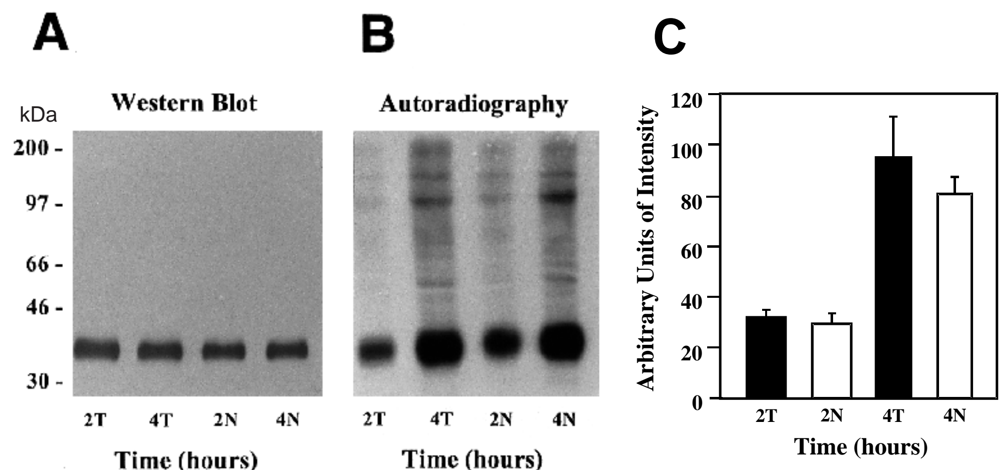

Fig. 1. Opsin synthesis in VPP and normal mice. Western blot labeled with opsin mAb 1D4 (A) and autoradiograph (B) of rod outer segment proteins after incubation of retinas with ^{35}S-labeled amino acids from a single experiment. The retinas were from VPP and normal littermates that were 18 days old. Relative ^{35}S incorporation into opsin was determined from densitometric scanning of the autoradiograph and western blot. In the experiment shown in A and B, less newly synthesized opsin was measured in the transgenic VPP retinas than in control retinas after 2 hours. However, as illustrated in C, the mean specific activity of opsin (± s.e.m.), obtained from three separate experiments, performed in the same way, was similar in VPP and control retinas after both 2 and 4 hours incubation. The amount of ^{35}S incorporation after 2 hours was less than half that after 4 hours due to a lag for the ^{35}S-labeled amino acids to enter the cells. T, transgenic VPP; N, normal littermates.

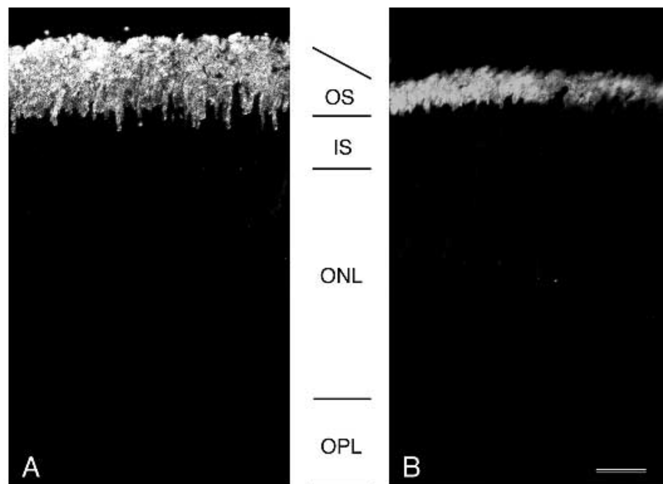

Fig. 2. Immunofluorescence microscopy of semithin cryosections of (A) control and (B) transgenic mouse retinas labeled with opsin mAb 1D4. The control and transgenic mice were littermates and 25 days old. The photoreceptor outer segments (OS), photoreceptor inner segments (IS), the photoreceptor nuclear layer (ONL), and the retinal outer plexiform layer (OPL), which contains the photoreceptor synaptic terminals, are indicated. Immunolabel is present only in the outer segments. The outer segments of the transgenic mice, reared under the lighting conditions used, are about 40% shorter at this age. A and B are of the same magnification. Bar, 20 μm.

of opsin should be evident by a lowered concentration of intramembrane particles on freeze fracture replicas of outer segment disk membranes. Electron micrographs of replicas from a control mouse and a transgenic mouse are shown in Fig. 5. The face that is rich in intramembrane particles corresponds to the cytoplasmic half of the lipid bilayer (P-face) (Besharse and Pfenninger, 1980). The density of intramembrane particles on the P-face was determined from three control mice (measurements were made from 36 different areas) and from two transgenic mice (measurements were made from 35 different areas). In all mice, a few areas were found that were completely free of intramembrane particles (cf. Besharse and Pfenninger, 1980); they were excluded from measurements. The mean number of particles per μm^2 was 2,906±91 (s.e.m.) for control mice and 2961±79 for transgenic mice, indicating that the packing density of opsin in the disk membranes of transgenic mice is not significantly different from that of control mice.

Photoreceptor cell ultrastructure

The earliest abnormality described in the VPP mice is a shortening of the rod outer segments (Naash et al., 1993). We observed that outer segment length in the VPP mice became detectably less (about 10%) than that in controls by 18 days after birth. In the study by Naash et al. (1993), outer segment length decreased at a more rapid rate, probably because the mice in this study were exposed to higher light intensities than those used in the present study.

The ultrastructure of rod photoreceptor cells in transgenic mice was examined at 16 days, 21 days, and 35-40 days after birth. At 16 days, the photoreceptor cells appeared normal. By 21 days, when the rod outer segments have reached their mature length in control mice, the only other evident structural defect, besides shortened outer segments, was disorganization of the membrane at the very base of the rod outer segments; disk membranes in the rest of the outer segment appeared normal. The base of the outer segment is the site where new disk membranes form, so that the disks are nascent and still 'open'; that is, formation of their rims is incomplete (Steinberg et al., 1980; Arikawa et al., 1992). Nascent disks are more likely to be fixed poorly than fully mature disks (Steinberg et al., 1980; Arikawa et al., 1992). Nevertheless, comparison with

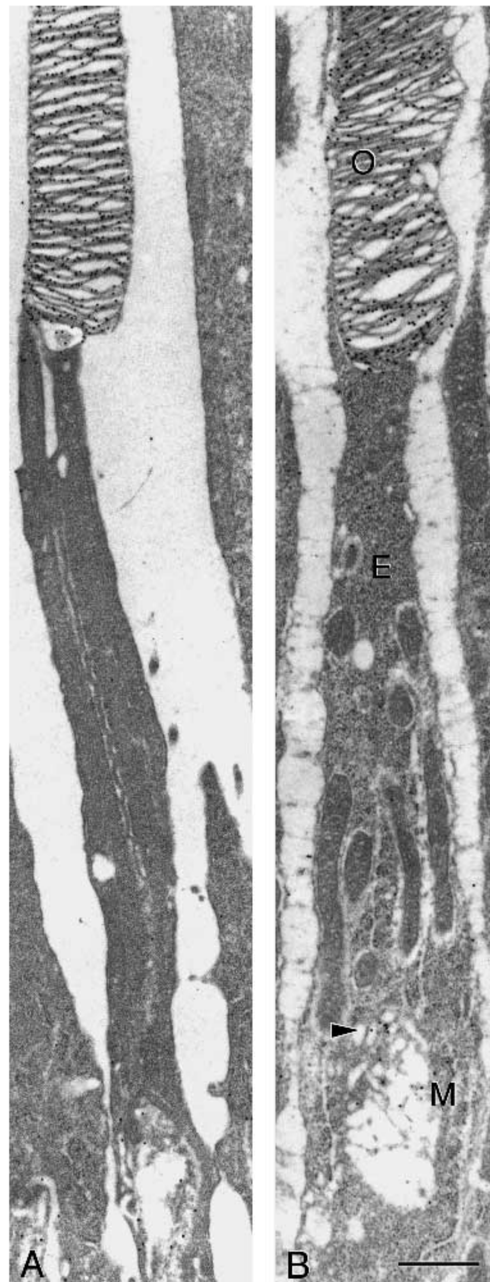

Fig. 3. Electron micrographs of (A) control and (B) transgenic mouse rod photoreceptor cells immunogold-labeled with opsin mAb 1D4. Both control and transgenic mice were littermates and 35 days old. The outer segment (O), ellipsoid of the inner segment (E), and myoid of the inner segment (M) are indicated. Arrowhead in B indicates immunogold label in the myoid. A and B are of the same magnification. Bar, 1 μm.

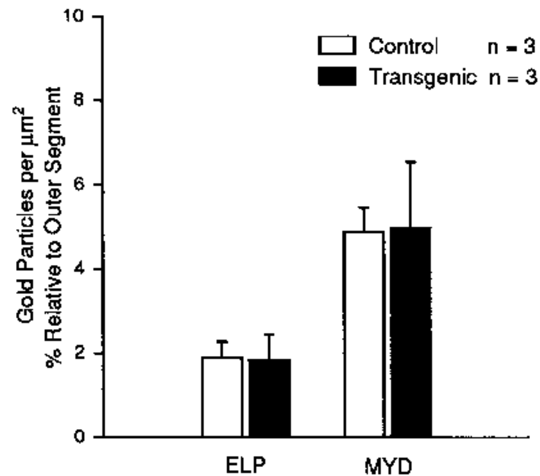

Fig. 4. Histogram illustrating the concentration of opsin mAb 1D4 immunogold labeling in the ellipsoid (ELP) and myoid (MYD) of control (open bars) and transgenic (filled bars) mouse rod photoreceptor cells. The number of gold particles per μm^2 was counted, and it is expressed relative to that in the outer segment. Error bars indicate ± s.e.m.

retinas from nontransgenic littermates that were fixed and processed in the same way at the same time indicated an abnormality in the transgenic mice, aged 21 days or 35-40 days. The membrane at the base of the outer segments had tubular and vesicular profiles rather than the flattened form of a disk. Different degrees of this perturbation were observed as illustrated in Fig. 6.

The extent of disorganization of the basal outer segment membrane was assessed as described in Materials and Methods. Rod photoreceptor cells were scored as normal (stage 0), stage 1, stage 2, or stage 3, depending on whether they most closely resembled the one shown in Fig. 6A,B,C or D, respectively. Cells scored as stage 1, 2, or 3, all appeared normal, except for a shortened outer segment and disorganized membrane at the base of the outer segment. At 16 days, there was no significant difference detected between control and transgenic mice (Fig. 7A). However, by 21 days, 32% of the photoreceptor cells in transgenic mice were scored as stage 1, significantly above the background level of 1% in control mice ($P<0.05$) (Fig. 7B). By 35-40 days of age, over half the transgenic photoreceptor cells contained disorganized membranes at the base of their outer segment (Fig. 7C). A few photoreceptor cells had basal disks as extremely disorganized as the one illustrated in Fig. 6D. By this age, some photoreceptor cells had already died, for there were slightly fewer nuclei in the photoreceptor cell nuclear layer and some pyknotic cells were observed. Ultrastructural changes occurring after the disorganization of the basal outer segment membrane and prior to cell death were not analyzed in the present study.

Disk membrane shedding

As perturbation of the basal outer segment membranes indicates a defect in disk membrane morphogenesis (see Discussion), we tested whether disk membrane shedding was also abnormal in the transgenic mice. Disk membranes are shed mostly during the first few hours after the onset of light, and are evident as phagosomes in the retinal pigment epithelium (Young and Bok, 1969; LaVail, 1976). Comparison of transgenic and control mice fixed within 20 minutes of each other, during the period 1-4 hours after light onset, showed that the concentration of phagosomes in the retinal pigment epithelium of transgenic retinas was not different from that in control retinas. At 16-21 days, it was 111%±5 (s.e.m; n=4) of that in controls; at 35-40 days, it was 98%±5 (n=8).

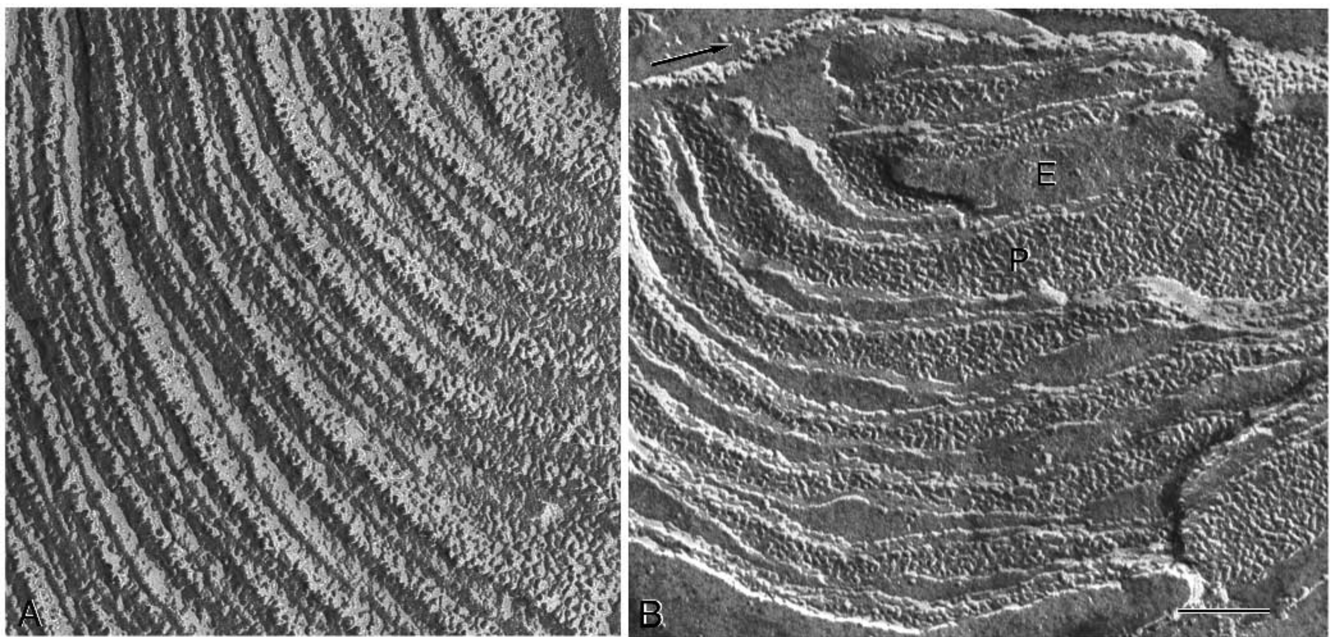

Fig. 5. Electron micrographs of freeze fracture replicas of rod outer segment membranes from (A) control and (B) transgenic mouse retinas. Both mice were 35 days old. Intramembrane particles are evident on the P-faces (P); E, E-face. Direction of shadowing is the same in both and is indicated by arrow in top left of B. In A, the fracture plane has passed through the disk membranes at a more oblique angle than that in B. A and B are of the same magnification. Bar, 100 nm.

In addition to phagosomes, smaller fragments of condensed membrane were observed in the retinal pigment epithelium of VPP mice, but not control mice, of both age groups. These fragments were often clustered together, enclosed by a membrane, suggesting that they had been engulfed by the retinal pigment epithelium (e.g. Fig. 8). They perhaps represent membrane that has been sloughed off from the base of the outer segment as a result of failure to be incorporated into a disk membrane.

DISCUSSION

The present results indicate that: (1) opsin synthesis in VPP mice is normal; (2) VPP mutant opsin does not accumulate in cellular compartments other than the outer segment; (3) the VPP mutation does not affect the concentration of opsin in the outer segment disk membranes; (4) nor does it affect the amount of disk membrane shedding; but (5) it results in perturbation of the nascent disks. There is no direct proof that the

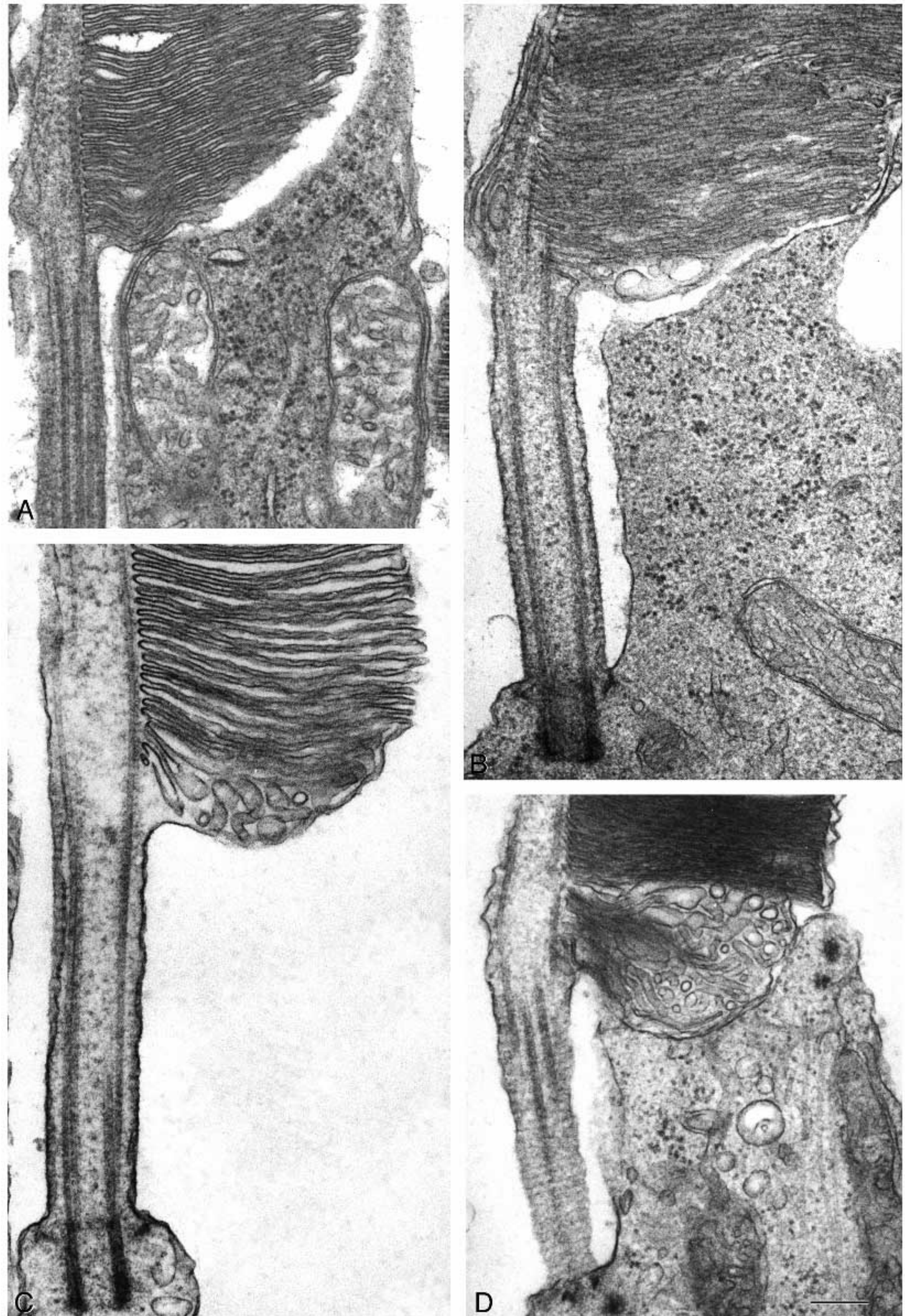

Fig. 6. Electron micrographs of the basal region of the outer segment of individual rod photoreceptor cells, illustrating the various degrees of membrane disorganization. (A) Normal photoreceptor cell from 40-day-old control mouse. The shorter most basal disks are nascent. Note that although in this particular example the disks have not been preserved perfectly (they appear wavy) they do not appear vesiculated or otherwise distended. (B) Photoreceptor cell from 21-day-old transgenic mouse. The nascent disks are distended and appear vesiculated. (C) Photoreceptor cell from 40-day-old transgenic mouse. Disorganization of the nascent disks is more extensive than in B. (D) Photoreceptor cell from 40-day-old transgenic mouse, demonstrating extremely extensive membrane disorganization at the base of the outer segment. A, B, C, and D are of similar magnification. Bar, 200 nm.

VPP mutant opsin is actually synthesized by the transgenic mouse photoreceptor cells. Attempts to obtain an antibody specific for VPP opsin have not been successful. Nevertheless, considerable evidence indicates that it is synthesized, most likely in approximately the same amount as normal opsin. First, VPP opsin mRNA is present at the same level as normal opsin mRNA in VPP mice, and total opsin mRNA is similar in VPP transgenic and control mice at 10 and 15 days after birth (Naash et al., 1993; Cheng and Naash, 1995). These data indicate that the amount of normal opsin in VPP mice is probably only 50% of that in control mice, but, together, VPP opsin and normal opsin result in a total opsin amount that is similar to that in control mice. Second, total opsin synthesis is similar in transgenic and control retinas in short-term culture (Fig. 1). Third, mRNA for human P23H opsin is translated in transgenic mice (Olsson et al., 1992). By generating mice expressing human mutant opsin rather than mouse mutant opsin, Olsson et al. were able to detect specifically the mutant protein with a selective human opsin antibody. Fourth, the perturbations observed in the outer segment disks, which contain a normal concentration of opsin and are present in an otherwise normal-looking cell, suggest the presence of defective molecules in the outer segment. Finally, a 50% reduction in the amount of normal opsin is not in itself sufficient to cause degeneration of photoreceptor cells, for the photoreceptor cells of humans that are heterozygous for a null opsin mutation do not degenerate (Rosenfeld et al., 1992).

That there was no accumulation of opsin other than in the outer segments of predegenerate photoreceptor cells contrasts with the mislocation of P23H mutant opsin in COS cells (Sung et al., 1991; Kaushal and Khorana, 1994) and P23H mutant human opsin in transgenic mice (Olsson et al., 1992; Roof et al., 1994). Even in mice expressing equal amounts of normal mouse and mutant human opsin (which are probably similar to the VPP mice, in terms of the ratio of mutant to normal opsin), some mutant opsin was routed aberrantly to the synaptic terminal (Olsson et al., 1992; Roof et al., 1994). Roof et al. noted that this mislocation at the synaptic terminal occurs well before a general redistribution of opsin around the entire cell,

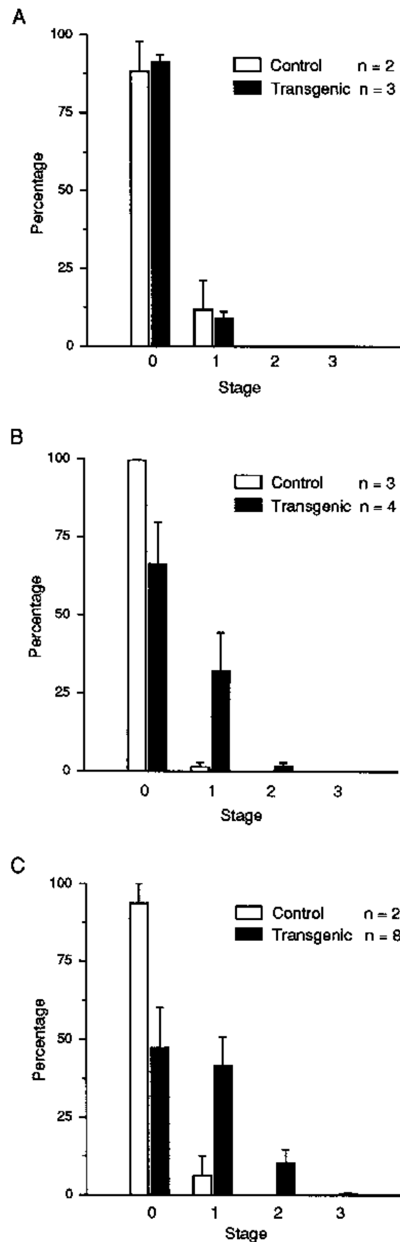

Fig. 7. Histograms illustrating the percentage of rod photoreceptor cells with disorganized basal outer segment membranes in control (open bars) and transgenic (filled bars) mice at (A) 16 days, (B) 21 days, or (C) 35-40 days after birth. Cells were scored as either normal (stage 0), stage 1, stage 2, or stage 3, depending on whether they were most similar to that shown in Fig. 6A,B,C or D, respectively. Error bars indicate ± s.e.m.

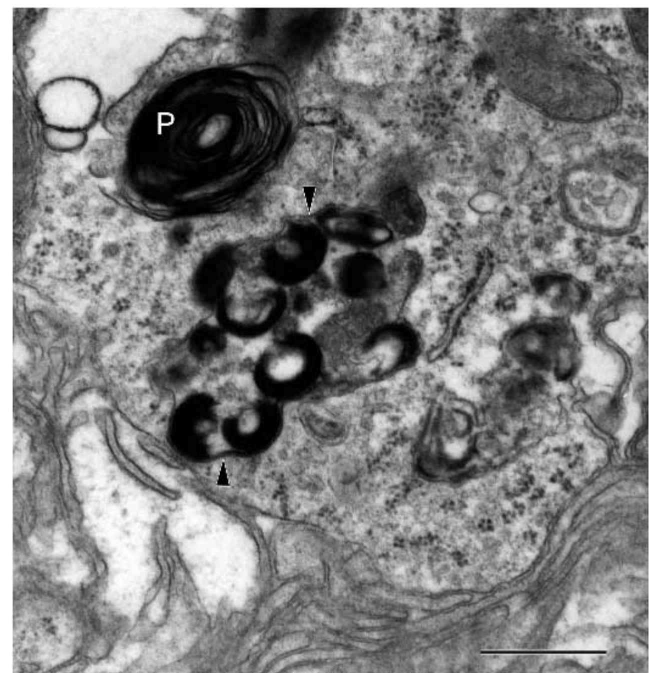

Fig. 8. Electron micrograph of the retinal pigment epithelium of a 21-day-old VPP mouse. A cluster of condensed membrane fragments (many are crescent-shaped) are evident in the center. They are enclosed by a membrane (arrowheads), suggesting that they have been engulfed by the epithelial cell. P, a phagosome, containing membrane that appears to have been shed normally from the distal end of the outer segment. Bar, 500 nm.

which occurs prior to cell death in many types of photoreceptor degeneration (Nir et al., 1987, 1989; Roof et al., 1994). A reason for the difference in routing of human P23H opsin might be because some of the human P23H opsin, unlike mouse VPP mouse opsin, is truncated at its N terminus in transgenic mice (Jin et al., 1995; cf. Fig. 1, present paper).

The freeze fracture analysis indicated that the density of opsin in the outer segment disk membranes was unaffected in transgenic mice, despite the likelihood that the mutant opsin in COS cells has a less compact structure (cf. Liu et al., 1996). It is generally assumed that the intramembrane particles on the P-faces of replicas of fractured photoreceptor disk membranes represent opsin molecules. First, opsin accounts for more than 95% of the integral disk membrane protein (Krebs and Kühn, 1977). Second, freeze fracture replicas of lipid bilayers containing reconstituted opsin look similar to those from disk membranes (Chabre et al., 1972; Chen and Hubbell, 1973; Hong and Hubbell, 1972). The freezing and fracturing of photoreceptor outer segment membranes appears to result in aggregation of opsin molecules (Chen and Hubbell, 1973; Clark and Branton, 1968; Corless et al., 1976). However, under a given set of freeze fracture conditions the degree of aggregation appears to be fairly constant (e.g. Besharse and Pfenninger, 1980).

The ultrastructural observations of disorganized basal disks were made in animals at an age when their outer segments become noticeably shorter than the outer segments of control animals (cf. Naash et al., 1993). Outer segment length is maintained by a balance of the addition of new disks at the base of the outer segment and the shedding of old disks from the distal end (Young and Bok, 1969; Besharse et al., 1977). A decrease in outer segment length would therefore result from a decrease in the former or an increase in the latter. Disk membrane shedding was found to be normal, but the disorganization of the basal disks points to a defect in disk membrane renewal, indicating that the decrease in outer segment length arises from the formation of fewer mature disks. As opsin synthesis was unimpaired, and there was no accumulation of opsin in the inner segment, the defect appears to lie solely in the morphogenesis of the disk membranes. This defect is not so severe as to affect the morphogenesis of every disk. Indeed, we observed that most disks were formed properly during the first few postnatal weeks. But, beyond 3 weeks of age, disorganized membrane became more evident, indicating that fewer disks were being formed. The fate of membrane that fails to be incorporated into disk membranes is unclear. It does not appear to be retained in the outer segment, for disorganized membrane was only observed at the base of the outer segment. Membrane debris was not observed in the extracellular space surrounding the outer segments. However, the presence of phagocytosed membrane fragments in the retinal pigment epithelium of VPP mice (Fig. 8) suggests it might be scavenged from this space by the epithelial cells.

The disorganization of the basal outer segment membranes resembles that described in tunicamycin-treated retinas. In these retinas, normal glycosylation of the N-terminal of opsin was blocked, membrane with vesicular and tubular profiles accumulated in the place of nascent disk membranes, and the photoreceptor cells eventually died (Fliesler et al., 1984, 1985). These observations, together with the present results, emphasize the importance of the structure of the N terminus of opsin in disk membrane morphogenesis. Interestingly, however, nonglycosylated opsin still appears to fold correctly (Kaushal et al., 1994), in contrast to P23H opsin (Kaushal and Khorana, 1994). In a current model of disk membrane morphogenesis, a nascent disk adheres to its more mature neighbor as it grows out, perhaps using this neighboring nascent disk as a template (Steinberg et al., 1980; Fliesler et al., 1986; Williams et al., 1988). The presence of opsin with an altered extracellular (later to become intradiskal) structure, or opsin lacking its sugar groups, probably interferes with this interaction between two nascent disks (cf. Fliesler et al., 1986). Consequently, some membrane may fail to form a proper nascent disk shape, instead producing disorganized vesicles and tubules that do not become incorporated into the outer segment. Liu et al. (1996) have suggested that most, if not all, of the point mutations in the extracellular domain of opsin that lead to dominant RP cause misfolding of opsin. If VPP mutant opsin is misfolded in vivo, this may be the molecular basis for why the nascent disk membranes fail to interact properly with each other in VPP mice.

During the present study, Li et al. (1996) published a study on transgenic mice expressing human opsin with a missense mutation, P347S, near the C terminus (which is cytoplasmic). This mutation results in a relatively severe form of dominant RP (Berson et al., 1991). Interestingly, Li et al. suggest from electron microscopy analysis that this mutation also results in aberrant disk membrane morphogenesis. As in VPP mice, an early defect of the P347S mice is a shortening of the rod outer segments. However, in the P347S mice, large numbers of opsin-containing multivesicular bodies accumulate around the rod outer segments (Li et al., 1996), whereas, in the VPP mice, we observed only perturbed basal outer segment disks, with no apparent accumulation of discarded membrane in the extracellular space. There are a number of differences between the study of Li et al. and the present one, including procedural differences, such as methods of tissue fixation. But the most important difference probably results from the sites of mutation; the P347S mutation does not appear to affect protein folding (Kaushal and Khorana, 1994), whereas the VPP mutation probably does. The simplest conclusion is that a variety of opsin mutations may perturb disk membrane morphogenesis, but that mutations at different sites may have different effects on the default organization and fate of the membrane that fails to form proper disks.

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