Deficiency in Light-Dependent Opsin Phosphorylation in Irish Setters With Rod-Cone Dysplasia

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A deficiency in light-dependent opsin phosphorylation and a slight reduction in opsin synthesis were observed during photoreceptor cell development (22–26 days) preceding photoreceptor cell loss in Irish setters with rod-cone dysplasia. In addition to opsin, two other phosphoprotein bands were found associated with the photoreceptor cell layer; synthesis and phosphorylation of one of these (band 3; 44–48 Kd) appeared reduced, while synthesis and phosphorylation of the other (band 1; 29–31 Kd) was within the normal range in 25-day-old affected setters. The deficiency in light-dependent opsin phosphorylation in affected setters was not due to a deficiency in opsin kinase, since soluble proteins from affected or normal outer segments catalyzed equally well opsin phosphorylation in partially kinase-depleted outer segment membranes from normal, while both kinase preparations failed to promote light-dependent opsin phosphorylation in those from affected setters. A deficiency in light-dependent opsin phosphorylation was also observed in rd/rd mice at all ages studied. In contrast, in Royal College of Surgeons (RCS) rats, light-dependent opsin phosphorylation was within the normal range prior to photoreceptor loss, and became nondetectable only after 50% or more of the photoreceptors had degenerated. Invest Ophthalmol Vis Sci 27:1551–1559, 1986

Rod-cone dysplasia, an autosomal recessive hereditary retinal degeneration in Irish setters characterized by a failure of normal photoreceptor cell development and early onset photoreceptor cell degeneration,1,2 becomes evident after the 26th postnatal day.2,3 Biochemical studies have indicated a deficiency in the activity of an outer segment cyclic GMP phosphodiesterase (PDE), with consequent accumulation of cyclic GMP reaching peak values between 22–26 days.1 Although in vitro incorporation of amino acids into opsin appeared to be within the normal range in retinas of 16-day-old affected setters,1 a failure of new membrane assembly at the base of the outer segments was noted after intraocular injection of labeled amino acids in 20-day-old affected setters.2 Opsi synthesis was shown to be reduced in 28–42-day-old affected setters, and at later stages of photoreceptor cell degeneration.4

The present studies were done to evaluate opsin synthesis and phosphorylation in photoreceptor cells of affected setters during early periods of outer segment development (22–26 days of age) prior to the onset of photoreceptor cell death. Since light-dependent opsin phosphorylation has been demonstrated in numerous species,5–8 in the present studies particular attention was given to evaluate light vs dark differences in opsin phosphorylation in photoreceptors and in isolated outer segment preparations from 25–26-day-old affected setters. Studies of light-dependent opsin phosphorylation were extended to affected Irish setters at different stages of photoreceptor cell degeneration, to rd/rd (retinal degeneration) mice with an early onset photoreceptor cell degeneration,9–11 and to Royal College of Surgeons (RCS) rats with late onset photoreceptor cell degeneration secondary to an abnormality in the pigment epithelium.12

Materials and Methods

Materials

Radioactive [14C] amino acids were obtained from Amersham (Arlington Heights, IL). Inorganic 32P, (or-
dependent opsin phosphorylation in mice homozygous for the retinal degeneration gene (rd/rd) and Royal College of Surgeons (RCS) rats. Affected mice and pigmented RCS rats (RCSp+/+) were bred in our own colony from breeders initially obtained from Dr. R. L. Sidman. Control mice (C57 black) and normal rats (pigmented Long Evans) were also raised in our colony from breeders obtained from Jackson and Charles River Breeding Laboratories, respectively. The animals were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School, the ARVO Resolution on the Use of Animals in Research, and the guidelines prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW (DHHS) publication No. (NIH) 78–23, revised 1978]). Mice and rats were studied between 7–25 days and 18–240 days of age, respectively. Progression of photoreceptor cell loss in rd/rd mice and RCS rats was monitored by analysis of retinal DNA content, as previously described.14 The loss of photoreceptor cells in affected settings was determined from counts of rows of photoreceptor cell nuclei at various stages of photoreceptor cell development and degeneration.3 In some experiments, some of the dissected and/or incubated retinas were fixed, embedded, sectioned, and examined under the microscope to ascertain that the outer segment layer had remained attached to the retina, and to compare the thickness of the outer nuclear layer in retinas of normal and affected animals.

**SDS-PAGE**

Retinas or microdissected retinal layers were solubilized in a sample buffer (Tris buffer, 20 mM, pH 6.8; sodium dodecyl sulfate, 2.0%; ethylenediamine tetraacetic acid (EDTA), 0.4 mM; phenylmethylsulfonyl fluoride (PMSF), 2 mM) as previously described.15 The proteins were solubilized at room temperature or dehydrated by incubation at 90°C for 5 min, and were then immediately applied either to tube gels (8% acrylamide) or to gradient slab gels (5–15% or 5–18% acrylamide) according to the method of Laemmli.15 Protein equivalent to 150–250 µg was loaded on tube gels and 100–150 µg per lane on slab gels. The gels were stained with 0.1% Coomassie blue and destained in a mixture of 10% acetic acid and either 10% isopropl or 10% methyl alcohol. After 5–6 days of destaining with frequent changes of destaining solution, tube gels were photographed and sliced into 1.25 mm slices, which were solubilized with Protosol and counted in the presence of Econofluor in a Mark III liquid scintillation counter (RadioAnalytic, Tampa, FL); slab gels were dried down and exposed to Kodak (Rochester, NY) AR-5 X-ray autoradiography film. The protein nature of the Coomassie stained bands was verified in control experiments; 15 min of digestion with proteinase...
ase K (prior to electrophoresis) resulted in a complete disappearance of all bands. Acetone extraction of lipids from homogenates prior to electrophoresis did not alter the banding patterns of these retinal proteins.

Quantitation of Radioactive Labeling

The following measures were taken to standardize quantitation of $^{32}\text{P}$ and $^{14}\text{C}$ radioactivity within opsin and other phosphoproteins along each given gel: 1) radioactivity in each gel slice was expressed in terms of percentage of the total radioactivity in that gel; 2) initial analyses focused on quantitating $^{32}\text{P}$ radioactivity (under double label counting conditions), and, at a later time (when the $^{32}\text{P}$ had decayed to a fraction of the $^{14}\text{C}$ radioactivity within the opsin gel slices), the gel slices were recounted under identical settings to determine the distribution of $^{14}\text{C}$ radioactivity along each gel; 3) the extent of light-dependent opsin phosphorylation was evaluated in each experiment by obtaining a light vs dark (L/D) ratio for $^{32}\text{P}$ radioactivity of the 35 Kd band in retinas of littermate animals, or within different sections of the same dog retina which had been incubated in light or dark; and 4) in each case, the value for the 35 Kd protein band was obtained from the sum of two gel slices (i.e., a total of 2.5 mm gel length).

The amounts of $^{14}\text{C}$ amino acids and $^{32}\text{P}$ accumulated and incorporated into protein during these incubations were determined from the radioactivity of the retinal homogenates and from the radioactivity of hot (90°C) 5% trichloroacetic acid (TCA) precipitates, respectively, as previously described. Protein concentrations in retinal homogenates were measured by the method of Lowry et al.

Immunohistochemical Detection of Opsin

The presence of opsin in retinas was analyzed with immunohistochemical methods using a well-characterized polyclonal (IgG) antibody to bovine opsin. In brief, retinas were sonicated on ice in 50 mM Tris buffer, pH 6.8, containing PMSF, 1 mM; DTT, 10 mM; EDTA, 2 mM; and Kallikrein inhibitor (Trasyol, 100 units/ml). The membranes were pelleted by ultracentrifugation (65,000 × g; 30 min); the proteins in both supernatant and pellet were solubilized in a sample buffer containing SDS, 2%; β-mercaptoethanol, 5%; and bromphenol blue, 0.025%, and resolved by SDS-PAGE on 10% acrylamide slab gels according to Laemmli. Proteins on gels were transferred electrophoretically to phenyliothioether (APT) derivatized paper. The 2‘amino blots were incubated with primary antibody (i.e., rabbit and anti-bovine opsin) diluted 1:20, then with secondary antibody (goat anti-rabbit IgG, Fc specific). The antibody complex was localized on the blots with the horseradish peroxidase anti-peroxidase (PAP) method. For visualization, the PAP reaction product was developed with 4-chloro-1-naphthol. In some experiments, blots from $^{32}\text{P}$ labeled normal and affected retinas were exposed to X-ray film prior to immunochemical localization to confirm the correspondence between the $^{32}\text{P}$ and antibody labeling of opsin.

Opsin Kinase

The activity of opsin kinase was evaluated in crude outer segment preparations from two 25-day-old affected setters and a 26- and a 28-day-old normal setter in two different experiments. Dark-adapted outer segments were isolated in dim red light by vortexing together the two retinas of each dog in 36% sucrose; after pelleting the debris (200 × g; 1 min), the supernatant was layered on 45% sucrose and centrifuged (15,000 × g; 20 min). Outer segments, collected from the interface, were diluted two-fold with Puck’s balanced salt solution and pelleted (25,000 × g; 20 min). The presence of intact outer segments was confirmed by phase contrast microscopy. Outer segment pellets were briefly rinsed and suspended in Puck’s saline and aliquots (corresponding to 60–80 μg protein) were used to measure opsin phosphorylation during 10 min incubation in light with $^{32}\text{P}$-ATP (100 μM; 10$^5$ cpm) in a total volume of 100 μl. To dissociate the soluble opsin kinase from the outer segment membranes, aliquots of the same dark-adapted outer segment suspensions were sonicated (15 sec, Branson sonifier) in dim red light and then pelleted (25,000 × g; 20 min). The phosphorylation of opsin in the partially kinase-depleted outer segment membranes was measured during 10 min incubation in light with $^{32}\text{P}$-ATP as substrate in the presence or absence of the kinase-containing supernatant. Mixing experiments were performed, and the opsin kinase activity of supernatant from normal or affected setters was evaluated separately with partially kinase-depleted outer segment membranes from either normal or affected Irish setters. Gel electrophoretic patterns of $^{32}\text{P}$ incorporation into proteins of complete outer segment membranes and reconstituted outer segment preparations of both normal and affected setters in dark and light were also evaluated by PAGE using slab gels.

Results

Identification and Quantitation of Protein Bands

Figure 1 shows the distribution of $^{32}\text{P}$ and $^{14}\text{C}$ radioactivity along representative SDS polyacrylamide gels from retinas of a 26-day-old normal and 25-day-old affected Irish setter incubated in light with $^{14}\text{C}$
Fig. 1. Representative gel electrophoretic radioactivity profiles of [\(^{32}\)P] (-----) and [\(^{14}\)C] (----) labeled proteins in gel slices of retinas from a 26-day-old normal and a 25-day-old affected Irish setter after incubation for 30 min in light in media containing [\(^{14}\)C] amino acids and [\(^{32}\)P]. The radioactivity for each 1.25 mm slice is expressed as a percentage of total [\(^{14}\)C] or [\(^{32}\)P] radioactivity in that gel. Ten major \(^{32}\)P containing protein regions were defined in normal and affected retinas in the molecular weight range of 29–210 Kd, and these are designated as bands 1–10. Molecular weights were determined from the migration of protein standards.

Affecting amino acids and \(^{32}\)Pi. In retinas of both normal and affected setters, ten discrete \(^{32}\)P-labeled regions were found (designated as bands 1–10). In the normal retina, band 2 (opsin, molecular weight of 34–37 kilodaltons; 35 Kd) was the only band that showed a several-fold light-dependent increase in \(^{32}\)P labeling. Incorporation of \(^{32}\)P into all other protein bands was similar in dark or light. In retinas of 25-day-old affected setters, the light-dependent increase in \(^{32}\)P labeling of opsin (band 2) appeared reduced compared to normal, band 3 was barely detectable, while band 1 appeared to be present in amounts comparable to normal (Fig. 1). Analysis of radioactivity profiles within the microdissected photoreceptor layers from normal and affected dogs showed that, in the normal dog, the light-dependent increase in \(^{32}\)P labeling of opsin (35 Kd) was associated with the photoreceptor cell layer. Within this layer, a 5–6-fold light-dependent increase in \(^{32}\)P and a 1.2–1.5-fold increase in [\(^{14}\)C] radioactivity on gels was associated with the opsin band in light, compared with ~5% for either label in dark. In contrast, light vs dark (L/D) ratios for both processes were close to one in the photoreceptor layer of affected setters, and only ~5% of the total \(^{32}\)P or \(^{14}\)C radioactivity was associated with this band in dark or light. This 35 Kd protein band was identified as opsin based on its molecular weight, comigration on SDS gels with purified bovine rhodopsin, association with outer segments, and its light-dependent phosphorylation.

The deficiency in opsin phosphorylation within photoreceptors of affected setters was evident prior to photoreceptor loss at a time when the thickness of the outer nuclear layer was comparable to normal, and when outer segment length was only slightly reduced compared with the normal (Fig. 3).

Figure 4 demonstrates the presence of opsin in membrane pellets of both normal and affected retinas (lanes 5 and 7). The single 35 Kd immunoreactive band in membrane pellets of retinas from normal and affected setters (lanes 5 and 7) corresponds to the immunoreactive band in purified bovine rod outer segments (lane 9) and to the \(^{32}\)P labeled 35 Kd band. The antibody to opsin specifically binds to opsin, and does not react with any other protein in either membrane or supernatant fractions from normal or affected setters.
Fig. 2. Photographs of representative Coomassie stained polyacrylamide tube gels after electrophoretic separation of proteins in extracts of whole retina (WR), microdissected photoreceptor cell layer (PR), and inner retina (IR) of a normal 26-day-old Irish setter, and the PR and IR from a 25-day-old affected Irish setter. The protein bands corresponding to each of the ten phosphoprotein peaks in Figure 1 are designated 1–10. Three of these phosphoproteins are shown to be concentrated in the photoreceptor cell layer in both normal and affected retinas; they are band 1 (arrow a; 29–31 Kd doublet band), band 2 (arrow R; represents the opsin moiety of rhodopsin; 34–37 Kd) and band 3 (arrow b; 44–46 Kd). Band 4 (arrow c; 54–56 Kd) is shown to be concentrated within the inner retina.

Exposure of blots to the X-ray film indicates that opsin is the major $^32$P-containing band in the membrane pellet of the normal light-incubated dog retina (lane 3), while, in the affected, only a slight $^32$P-labeling of opsin is detected (lane 1). Immunoreactive opsin was also identified in retinas of 35-day-old affected setters, which still retained outer segments but could not be detected in 45-day-old or older affected setters with reduced levels of outer segments and 40% or greater loss of photoreceptor cells.

Fig. 3. Representative light micrographs of retinas from a 25-day-old affected (A) and a 26-day-old normal (B) setter after 30 min incubation in Puck's balanced salt solution. The arrow points to the outer segment layer, which is slightly thinner in the affected than in the normal retina. The thickness of the layer of photoreceptor cell nuclei (ON) in the affected is comparable to that in the normal retina (bar; 50 μm).
 Opsin Kinase

Table 1 shows that, in outer segments of affected setters incorporation of $\gamma^{32}$P-ATP into protein was 62% lower in light than in normal outer segments, while, in dark, the values were similar to normal. Analysis of $[^{32}P]$ radioactivity profiles of outer segments of normal dogs showed that, in light, opsin represented the major peak (containing 60–70% of the total $[^{32}P]$ radioactivity) and that two lesser peaks were also present with molecular weights of ~70 and 140 kilodaltons (Fig. 5). In normal outer segments in dark, $[^{32}P]$ radioactivity of the opsin peak was greatly reduced (100–200 vs 550–650 cpm/slice; in dark vs light), while the radioactivity of the other two peaks was slightly reduced (40–60 vs 50–90 cpm/slice). In affected outer segments, only a small amount of $[^{32}P]$ was incorporated into opsin in light or dark, and autoradiogram patterns in either dark or light were comparable to normal patterns in dark.

With the removal of soluble proteins (i.e., with partial kinase-depletion of outer segment membranes), the incorporation of $[^{32}P]$ from $\gamma^{32}$P-ATP was reduced by 40% in the normal, but remained unchanged in the affected membranes (Table 1). Adding back the soluble proteins from either normal or affected membranes restored the capacity for light-dependent opsin phosphorylation in normal membranes, but neither kinase preparation had an effect in outer segment membranes of affected setters. This shows that opsin kinase activity was present in affected outer segment preparations, but neither the normal nor the affected kinase preparation was effective in phosphorylating opsin in affected outer segment membranes. Gel electrophoretic patterns of reconstituted outer segments from normal or affected setters, after incubation in light with $\gamma^{32}$P-ATP were similar, respectively, to those shown in Figure 5; in the normal, 60–70%, and, in the affected, 25–35% of the $[^{32}P]$ was associated with the opsin peak.

Comparison of Light-Dependent Opisin Phosphorylation in Species With Hereditary Retinal Degenerations

The developmental pattern of light-stimulated opsin phosphorylation was compared in three different species with photoreceptor cell degeneration (affected setters, rd/rd mice, and RCS rats, Fig. 6). In normal retinas, light-dependent opsin phosphorylation reached adult values in parallel with the development of adult photoreceptor function (45, 18, and 23 days in normal

Table 1. Incorporation of $[^{32}P]$ in outer segment membranes of normal and affected Irish setters

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Normal</th>
<th>Affected</th>
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</thead>
<tbody>
<tr>
<td>Crude outer segment preparations</td>
<td>100 ± 19</td>
<td>38 ± 8</td>
</tr>
<tr>
<td>Partially kinase depleted membranes</td>
<td>60 ± 11</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>Added back kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>from normal</td>
<td>105 ± 20</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>from affected</td>
<td>98 ± 19</td>
<td>37 ± 8</td>
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Isolated outer segments from normal and affected dogs were incubated for 10 min with $\gamma^{32}$P-ATP under conditions identical to those in Figure 5. The radioactivity incorporated into protein was analyzed by TCA precipitation. Total $[^{32}P]$ incorporated into light outer segment proteins in the normal dog (300–400 cpm/µg protein in two different experiments) was normalized to 100 ± 19 cpm/µg protein (in dark, the corresponding value was 32 ± 6 cpm/µg protein).

The data for $[^{32}P]$ incorporation in partially kinase-depleted normal and affected outer segment membranes are expressed as percentages of $[^{32}P]$ incorporated in the normal dog outer segments in light. The values represent the mean ± SD for triplicates in each of two different experiments.
In affected setters and rd/rd mice, a failure of the development of light-dependent opsin phosphorylation was noted, and L/D ratios remained close to one at all ages studied. This despite the fact that, in both affected setters and rd/rd mice, opsin was immunochemically detectable prior to extensive photoreceptor degeneration and loss of outer segments (after 45 days in affected setters or after 14 days in rd/rd mice).

In RCS rats, the capacity for light-dependent opsin phosphorylation developed normally between 18-23 days, coincident with the normal development of photoreceptor cells. Thereafter, the capacity for light-dependent opsin phosphorylation declined with the loss of photoreceptor cells and became nondetectable by 65 days of age after 70% of the photoreceptor cells had degenerated.

**Discussion**

This study shows a deficiency in light-dependent opsin phosphorylation in the retinas of Irish setters with
rod-cone dysplasia at early stages of photoreceptor cell development (22–26 days) preceding the onset of photoreceptor cell loss. In normal setters, the capacity for light-dependent opsin phosphorylation began to develop gradually after ~20–22 days of age, and reached adult levels at 45 days of age in parallel with the attainment of normal adult structure and ERG function of photoreceptor cells. In the affected setters, although the number of photoreceptor cell nuclei between 10–26 days was within the normal range and although opsin was present, it failed to show light-dependent phosphorylation. The absence of opsin phosphorylation did not appear to be due to a deficiency in opsin kinase, since soluble kinase from affected Irish setter outer segments catalyzed the light-dependent phosphorylation of opsin in partially kinase-depleted outer segment membranes from normal dogs, while the addition of kinase from normal or affected dogs failed to enhance opsin phosphorylation in affected outer segment membranes (Table 1).

Several different processes, however, may explain the observed deficiency in opsin phosphorylation in Irish setters with rod-cone dysplasia: 1) high concentrations of cyclic GMP known to be present in or around the photoreceptor cells may have been inhibitory to the activity of opsin kinase; 2) reduced availability of phosphorylation sites on opsin due either to reduced amounts of opsin or to abnormalities in the conformation of opsin; and 3) failure of dephosphorylation in vivo during the period of dark-adaptation that preceded the experiments, such that some or all of the sites were phosphorylated already when light stimulation and [32P] were presented in vitro. Although none of these possibilities can be excluded at present, the first hypothesis requires particular consideration in view of a recent finding that cyclic GMP can inhibit opsin phosphorylation in isolated rat outer segments. Although, in the present study, no attempts were made to measure the levels of cyclic GMP, it can be deduced from previous reports that supernatant fractions from both dark-adapted normal and affected outer segments contained high enough levels of cyclic GMP to be inhibitory to opsin phosphorylation in the affected membranes deficient in cyclic GMP phosphodiesterase. In the normal membranes on the other hand, the light-activated phosphodiesterase may have hydrolyzed all the cyclic GMP immediately at the start of the phosphorylation study in light; therefore, no inhibition of opsin phosphorylation occurred.

In addition to opsin, two other phosphoproteins are shown to be concentrated within the photoreceptor cell layer, a lower molecular weight protein (doublet band 1, 29–31 Kd) and a higher molecular weight protein (band 3, 44–46 Kd) (Fig. 2). In the affected synthesis and phosphorylation of two of these proteins, opsin and band 3, appeared to be reduced at a time (25–26 days) when band 1 and total protein synthesis and phosphorylation were within the normal ranges, and when the thickness of the outer nuclear layer was comparable to that of normal setters. Proteins of similar molecular weights (i.e., 29–31 and 44–46 Kd) have been previously observed in outer segment membranes of bovine retinas. In retinas of mice homozygous for the rd mutation (rd/rd), opsin and a 43 Kd protein band were also found to be missing at late stages of the disease process. Whether or not any correspondence exists between proteins identified in bovine outer segments and those in mice and dogs with retinal degeneration remains to be established.

These studies suggest that there may be a complex mechanism whereby the accumulation of high levels of cyclic GMP in two animal models of hereditary retinal degeneration (Irish setters and rd/rd mice) leads to the loss of rod photoreceptor cells. Previously, toxic effects of high concentrations of cyclic GMP on rod photoreceptor cells have been described in cultured eye rudiments of X. laevis and in the normal human retina. In the latter, incubation with cyclic GMP and/or isobutylmethylxanthine resulted in a selective inhibition of protein synthesis within rod photoreceptor cells. The present observations suggest that the deficiency in light-dependent opsin phosphorylation in Irish setters with rod-cone dysplasia and rd/rd mice may represent a consequence of cyclic GMP accumulation. In rd/rd mice, such a relationship has been clearly demonstrated. In contrast, in RCS rats in which no cyclic GMP accumulation occurs, opsin phosphorylation remained detectable even after 50% or more of the photoreceptor cells had degenerated.

Key words: opsin phosphorylation, opsin kinase, photoreceptor degeneration, Irish setters with rod-cone dysplasia, rd mice, RCS rats

References