An Extracellular Retinol-binding Glycoprotein in the Eyes of Mutant Rats with Retinal Dystrophy: Development, Localization, and Biosynthesis

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ABSTRACT Interstitial retinol-binding protein (IRBP) is a soluble glycoprotein in the interphotoreceptor matrix of bovine, human, monkey, and rat eyes. It may transport retinol between the retinal pigment epithelium and the neural retina. In light-reared Royal College of Surgeons (RCS) and RCS retinal dystrophy gene (rdy) + rats, the amount of IRBP in the interphotoreceptor matrix increased in corresponding proportion to the amount of total rhodopsin through postnatal day 22 (P22). In the RCS-rdy + rats, the amount increased slightly after P23. However, in the RCS rats there was a rapid fall in the quantity of IRBP as the photoreceptors degenerated between P23 and P29. No IRBP was detected by immunocytochemistry in rats at P28. The amount of rhodopsin fell more slowly. Although retinas from young RCS and RCS-rdy + rats were able to synthesize and secrete IRBP, this ability was lost in retinas from older RCS rats (P51, P88) but not their congenic controls. The photoreceptor cells have degenerated at these ages in the RCS animals, and may therefore be the retinal cells responsible for IRBP synthesis. The putative function of IRBP in the extracellular transport of retinoids during the visual cycle is consistent with a defect in retinol transport in the RCS rat reported by others.

The interphotoreceptor matrix (IPM) is the extracellular material that separates the neural retina from the retinal pigment epithelium (RPE). The IPM is traversed by nutrients and metabolites that are exchanged between these two layers. The IPM differs from other extracellular matrices in that it lacks collagen and fibronectin. The major component of bovine IPM, present at a concentration of 30-100 μM, is interstitial retinol-binding protein (IRBP) (2, 10, 33, 34). We have purified and characterized this protein from human, bovine, and rat IPM (20, 21, 24-26). Bovine IRBP has an apparent M, of 144,000, binds about two molecules of all-trans retinol, and has four to five sialylated, fucosylated biantennary complex oligosaccharide chains. Experiments with labeled sugar and amino acid precursors have demonstrated that IRBP is synthesized and secreted into the extracellular space by the neural retina (9, 20, 21).

Before the demonstration that IRBP carried endogenous retinol, it was suggested that IRBP might function in neural retina-RPE adhesion (1), in a manner comparable to the role of fibronectin in other systems (45). Because the amount of retinol bound to IRBP depends on the degree of rhodopsin bleached in the retina (2, 34), it is now believed that this protein may be implicated in the transport of retinoids between the cells of the neural retina and the RPE, a process that is known to occur during the bleaching and regeneration of rhodopsin in the vertebrate eye (7, 12, 46). However, neither the function of IRBP as a putative transport molecule for retinoids in the IPM, nor the retinal cell type that synthesizes and secretes this protein, has been firmly established.

In the present work, we used rats with hereditary retinal dystrophy to investigate the occurrence, synthesis, and proposed physiological function of IRBP. The mutant used was...
the pink-eyed Royal College of Surgeons (RCS) rat, which exhibits selective degeneration of the photoreceptor cells. In this strain, photoreceptor cell death is first detectable between postnatal days 16 and 20 (P16–P20), and most of these cells have died by P60 (14). Characteristic of the disease is the presence in the IPM of isolated rod–outer-segment membranes that have accumulated because of a genetic defect in the phagocytosis mechanism of the RPE cells (4, 14, 27, 30, 31, 35). At an early stage in the degeneration, the IPM appears to be abnormal when stained with compounds that react with mucosubstances (32, 38). We have studied the emergence, distribution, and biosynthesis of IRBP in the RCS rat. Our findings provide information on the function and possible site of synthesis of this protein.

**MATERIALS AND METHODS**

**Animals:** Breeding pairs of tan-hooded RCS rats homozygous for the retinal dystrophy gene (rdy), and of their congenic controls (RCS-rdy) were obtained from M. M. LaVail. The early history of the RCS strain is described by Sidman and Pearlstein (43), and of the congenic controls by LaVail (29). The animals were maintained on a 12-h light-dark cycle. Illumination was provided by overhead fluorescent lights that gave an illumination level ranging from 2–22 foot-candles.

**Miscellaneous:** SDS PAGE on 5–20% linear gradients, fluorography, sample preparation, and protein determinations were carried out as described (20, 21, 34).

**Preparative Procedures:** Rats were dark-adapted overnight, and subsequent operations were carried out under dim red light. They were decapitated, the eyes were enucleated, the sector anterior to the limbus (together with the lens) was cut away, and the bulk of the vitreous picked out with forceps. Each pair of opened eyes was immersed in ~0.5 ml of phosphate-buffered saline (PBS) and the retina was gently detached with a small glass hook. The retinas and the fluid in which the eyes had been immersed were removed and placed on ice with a subsequent 1.0 ml PBS rinse of the eyecups and an additional 2.5 ml of PBS. The mixture was incubated on ice for 10 min with occasional swirling and centrifuged at 500 g for 2 min. The supernatant that contained the IPM was then centrifuged at 100,000 g for 1 h. The low- and high-speed pellets were combined, homogenized in 4.0 ml PBS (glass homogenizer), and the retina cytosol was obtained by centrifuging at 100,000 g for 1 h. The rhodopsin content of the pellet was determined as described below.

The retinal pigment epithelium and choroid (RPE-Ch) were peeled away from the sclera with forceps under ~1 ml PBS and homogenized; the cytosol was obtained from the 100,000 g supernatant as described above. The pellet was retained and used to determine whether any rhodopsin-containing material was present (see below).

Soluble fractions were dialyzed (Spectrapor Membrane, M, cutoff 12,000–14,000) against distilled water, lyophilized, and then dissolved in dissociation buffer before SDS PAGE (34).

IRBP was detected either immunologically after electrophoretic transfer to nitrocellulose sheets (see below) or determined by scanning its Coomassie Blue-stained band (M, 144,000) on SDS gels with an LKB Zeineh soft laser densitometer. For the RCS and RCS-rdy rats, each lane contained 90% of the total protein from a pair of eyes. We determined that the area under the M, 144,000 band was proportional to the amount of IPM sample loaded on the gel over the full range encountered. The highest loading tested gave a band with an intensity equivalent to 14 μg of pure bovine IRBP.

**In Vitro Biosynthesis of IRBP:** Rats were dark-adapted overnight. All dissections and incubations were performed under red safelights. The retinas were isolated as described above and placed in vials that contained 2 ml of modified Roswell Park Memorial Institute 1640 medium without unlabeled retinol (20, 21) together with 77–90 μCi [4,5-3H]-retinol (Amerham Corp., Arlington Heights, IL; 46 Ci/mmol). One pair of retinas was incubated in each vial. Incubations were carried out at 37°C for 4 h while the vials were continually flushed with water-saturated 95% oxygen, 5% carbon dioxide. At the end of this period, the media and tissues were collected separately. The tissues were homogenized, and the cytosol and pellet fractions prepared as described above. Phenylmethylsulfonyl fluoride was added to all solutions (except during incubation) to a concentration of 0.1 mM. The samples were prepared for SDS PAGE and fluorography as described (20, 21).

**RESULTS**

**IRBP in Rats with Retinal Dystrophy**

The nitrocellulose transfers in Fig. 1 (left) show strong immunoreactive bands due to IRBP in the IPM of normal control rats at three different ages, namely P23 (lane 1), P33 (lane 2), and P59 (lane 3). These bands corresponded to an immunoreactive polypeptide band (~140,000) against distilled water, lyophilized, and then dissolved in dissociation buffer before SDS PAGE (34).

IRBP was detected either immunologically after electrophoretic transfer to nitrocellulose sheets (see below) or determined by scanning its Coomassie Blue-stained band (M, 144,000) on SDS gels with an LKB Zeineh soft laser densitometer. For the RCS and RCS-rdy rats, each lane contained 90% of the total protein from a pair of eyes. We determined that the area under the M, 144,000 band was proportional to the amount of IPM sample loaded on the gel over the full range encountered. The highest loading tested gave a band with an intensity equivalent to 14 μg of pure bovine IRBP.

The amount of rhodopsin in the pellets was determined by homogenizing them in 0.8 ml of extraction buffer (100 mM hydroxylamine, 200 mM L-1690, 100 mM Na phosphate [pH 7.0], and centrifuging at 16,000 g for 0.5 h. This procedure extracted 98% of the rhodopsin in the pellet. L-1690 was obtained from the Kyoto Co. Ltd., Tokyo, Japan. It consists of a mixture of long-chain fatty acid esters of sucrose (see reference 22). The rhodopsin content of the pellet was determined as described.

**Immunological Techniques:** Antiserum against bovine IRBP was raised in rabbits by Bethel Laboratories (Montgomery, TX). 100 μg of the pure antigen were emulsified with Freund's complete adjuvant for the initial injection (intramuscular and subcutaneous) and with the incomplete adjuvant for all subsequent injections (subcutaneous). These were administered once every 2 wk for the first 10 wk, then once every month. Animals were bled monthly.

The immunoglobulin fraction was prepared as described (21), then purified by affinity chromatography on a column of ~200 μg of bovine IRBP immobilized on Affigel 10 (Bio-Rad Laboratories, Richmond, CA). The coupling (84% efficiency) was carried out at pH 7.5 in 100 mM HEPES, 80 mM CaCl2, according to the manufacturer's instructions. The bound immunoglobulin was eluted with 4 M MgCl2.

Electrophoretic transfer of proteins from SDS gels to nitrocellulose paper (Bio-Rad Laboratories) was accomplished by the method of Towbin et al. (44) using the buffer system of Gershoni and Palade (23). Immunological visualization of IRBP was then carried out by incubating the nitrocellulose paper with affinity-purified rabbit antibody followed by peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) as described by the manufacturer.

**Immunocytochemistry:** Immunocytochemical localization of IRBP in the eye by indirect fluorescence was performed by a method modified from that described previously (20). The eyes were fixed overnight at 4°C in 4% paraformaldehyde (Ted Pella, Inc., Tustin, CA), sectioned radially at 16 μm in a Minotome microtome (Damoan/IEC, Needham Heights, MA) and mounted on gelatin-coated slides. An excess of high-potassium PBS (250 mM NaCl, 250 mM KCl, 100 mM Na phosphate [pH 7.2], 0.1% Triton X-100) was added to the sections, which were then incubated overnight at 4°C with a 1:10 dilution of rabbit anti-bovine IRBP serum or with the control pre-immunogen serum. After they had been rinsed extensively, the sections were incubated for 3 h with FITC-conjugated goat anti-rabbit IgG (Miles Laboratories), washed, mounted in 1:1 vol/vol glycerol in PBS and examined under a Zeiss Universal or inverted epifluorescence microscope.

The amount of IRBP was then carried out by incubating the paper with affinity-immobilized rabbit antibody followed by peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories), washed, mounted in 1:1 vol/vol glycerol in PBS and examined under a Zeiss Universal or inverted epifluorescence microscope.

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**RESULTS**

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The disappearance of IRBP in the RCS rat was not due to its transfer from the IPM into the retina or RPE. Fig. 2 shows a faint immunoreactive band in the IPM fraction from a P31 RCS rat (lane 4), but none is evident in the cytosol or pellet fractions from the corresponding RPE and retina. No other immunoreactive polypeptides are apparent. In lane L, which represents the IPM preparation that contained a substantial amount of IRBP from an age-matched control, a few faint low M, bands are visible. We have not established whether these bands were generated by proteolysis during preparation, although the routine addition of phenylmethylsulfonyl fluoride should have minimized this possibility.
FIGURE 1 IRBP in the IPM of the RCS rat. Strong immunoreactive bands due to IRBP are visible in the gel transfers on the left, obtained from IPM samples prepared from eyes of the control animals. A band of intensity comparable to that of the age-matched control (lane 1) is seen for the IPM from the RCS rat at P23 (lane 4), but is absent from that of the older animals. (Lane 1) Control, P23; (lane 2) control, P33; (lane 3) control, P59; (lane 4) RCS, P23; (lane 5) RCS, P40; (lane 6) RCS, P60. The nitrocellulose transfers were initially incubated with immunoglobulin from rabbit antibovine IRBP serum. The loading level corresponded to 20% of one eye. Molecular weight, $x 10^{-3}$.

Immunofluorescent Localization of IRBP in the RCS Rat

Fig. 3 shows the immunocytochemical localization of IRBP in the retinas of RCS-rdy* (Fig. 3, A–D) and RCS rats (Fig. 3, E–H). In the control sections, the immunofluorescence fills the space between the external limiting membrane and the surface of the RPE, and becomes progressively thicker as the animals age. In the RCS rat at P18, the band is much wider than in the age-matched controls, apparently because of rod-outer-segment membrane accumulation (panel F). By P28 (panel G), no immunofluorescence was visible in the RCS rat. A preliminary report describing similar observations has been made by Eisenfeld et al. (15a).

The distribution of immunofluorescence within the IPM of dystrophic and control rats is compared in Fig. 4. In the control animal (P28), the fluorescence is most intense in a 6.5-μm-wide band just apical to the RPE (top pair of arrows) and is least intense between the inner segment/outer segment junction and the external limiting membrane (bottom pair of arrows). In the RCS retina (P18), the immunofluorescence was clearly present in the rod-outer-segment membrane debris layer (asterisk), but the intense band adjacent to the RPE was not evident.

IRBP and Rhodopsin in the RCS Rat

The quantity of IRBP was compared with that of rhodopsin in dark-adapted, light-reared dystrophic and normal rats at various ages, ranging from P5, when the photoreceptor outer segments were beginning to form, to P60. The amount of IRBP was determined by scanning the stained SDS gels and integrating the area under the $M_r$ 144,000 IRBP band.

Fig. 5 shows that in the control rats, both IRBP and rhodopsin increased from P5 to P30. In the dystrophic rats, the amounts of IRBP and rhodopsin corresponded to the amounts in the controls until about P18, and continued to increase with age until they reached maxima. These maxima occurred at different ages, however, IRBP was lost rapidly after P22 and was not detectable at P40 (cf. Fig. 1). The decline of rhodopsin was delayed until P25–P30 and progressed more slowly. As much as 50% of the maximum amount was still present at P40–P46 and traces could still be measured at P60.

Synthesis and Secretion of IRBP by the Retinas of Dystrophic Rats

The retinas from RCS rats and their congenic controls were incubated with [3H]leucine. After 4 h, the retina cytosols and media were prepared and examined by SDS PAGE and fluorography. These experiments showed that the retinas from young rats were able to synthesize IRBP. This ability was lost in the older RCS rats, but not in their age-matched controls.

In each of the fluorograms of Fig. 6, lane 3 represents the medium from the dystrophic retinas and lane 4 represents the medium from the controls. Labeled IRBP is visible in the medium from all the normal control retinas, which ranged in age from P14 to P85. In the dystrophic retina medium, however, labeled IRBP was observed at P16 and P26, but not at P51 and P88.

The above findings were confirmed by measuring the radioactivity in segments corresponding to IRBP that had been cut from similar gels. The cytosol and medium equivalent to 32 μg of retina pellet protein (~6.5% of the total retina) were loaded in each track. At P52, 2,332 disintegrations per minute (dpm) were recorded in the IRBP secreted by the controls (mean of two retinas from two rats) compared with only 398 dpm from P51 dystrophic retinas. The latter value was close to the background of 308 dpm measured in gel segments cut
FIGURE 3 Immunocytochemical visualization of IRBP in the retinas of RCS rats and their congenic controls. (A) Control, P12; (B) control, P18; (C) control, P28; (D) control, P41; (E) RCS, P9; (F) RCS, P18; (G) RCS, P28; (H) RCS, P41. (F) The slanted arrow indicates the apical surface of the RPE, and the horizontal arrow the external limiting membrane. Bar, 100 μm. × 215.
Figure 4: Distribution of immunofluorescence in the retina of the RCS rat during debris accumulation (B and D) compared with that in control animals (A and C). Several zones of different intensity appear in the retina of the control (P28); the band of highest intensity (between top two arrows) is seen juxtaposed to the apical surface of the RPE (top arrow), and much weaker fluorescence is visible between the junction of the photoreceptor inner and outer segments (third arrow from top) and the external limiting membrane (bottom arrow). This zone is also apparent in the RCS retina (P18); the debris layer (*) is strongly immunoreactive, but the ordered structure seen in the control has been disrupted. Bar, 40 μm. x 538.

Figure 5: Rhodopsin (○) and IRBP (△) in control (A) and RCS (B) rats. The amount of rhodopsin was averaged in the normal animals from P30-60, and the amount of IRBP (in arbitrary units) was normalized to this level. The data in inset are replotted from Dowling (14), and show the visual sensitivity measured by the ERG for RCS rats (■) and a group of albino animals that were used as controls (x).

From the same tracks above and below the position of IRBP. Similar results were obtained with older animals: P85 controls incorporated 2,711 dpm into IRBP; for P86-P88 dystrophic retinas, this value was only 563 dpm compared with a background of 508 dpm.

Although retinas obtained from younger animals were found to be variable in the amount of IRBP secreted, it appeared that there was little significant difference between the dystrophic and control tissue at the earlier ages examined (P14-P25).

Discussion

In the rat, the photoreceptors start to develop outer segments between P5 and P10, and the interphotoreceptor space widens at about this time (16). We were able to detect IRBP in RCS-ryd+ rats at P5. As the animals aged, its amount increased in corresponding proportion to the amount of rhodopsin present and hence with the elongation of the rod outer segments (cf. reference 14). The RCS rats displayed a similar relationship during the first 3 wk of postnatal life. Subsequently, as demonstrated by biochemical and immunocytochemical means, the amount of IRBP declined rapidly. This rapid disappearance was initiated shortly after the age when pyknotic photoreceptor cell nuclei are first reported (at P16-P22) (4, 14, 31). Most of the IRBP was lost by P27. In contrast, the amount of rhodopsin in the eye decreased much more gradually.

In part, the loss of IRBP in the RCS rat may be explained by a reduction in its rate of synthesis. We demonstrated that the capacity of the retina to synthesize and secrete IRBP was lost in dystrophic animals by P41, an age at which the rate of opsin synthesis has also severely declined (3). Since most of the photoreceptor inner segments have degenerated at this stage, while the other retinal cells appear to be normal (14, 15), these findings suggest that IRBP may be produced by the
FIGURE 6 Fluorograms showing the in vitro synthesis of IRBP by retinas of RCS rats and their congeneric controls. Incubations were carried out with [3H]leucine. For fluorograms A–D: (Lane 1), RCS retina cytosols; (lane 2), control retina cytosols; (lane 3), RCS retina media; (lane 4), control retina media. (A) P16 RCS, P14 controls; (B) P26 RCS and controls; (C) P51 RCS and P52 controls; (D) P88 RCS and P85 controls.

Photoreceptor inner segments. This conclusion is supported by recent observations on isolated human retinas (28).

The quantity of IRBP in the sub-retinal space is also determined by its rate of removal. Little is known about this process, however. In normal rats injected with radiolabeled sulfate, which is a marker for proteoglycans, labeled material disappears from the IPM with a half-life of 2.5 d (36). IPM constituents may be endocytosed by cells of the RPE (cf. reference 37) or even by the retina, and phagocytosis of shed rod–outer-segment membranes by the RPE is almost certainly associated with some degree of nonspecific internalization (18). Some components may also be removed by extracellular degradation. In the dystrophic animals we do not know whether the same mechanisms are operative. Since phagocytosis does not occur in the RCS rat, removal of IPM by this route is unlikely and we found no evidence for the accumulation of IRBP or immunoreactive fragments in the retina and RPE.

The present findings may have a bearing on the suggested function of IRBP in the extracellular transport of retinoids during the visual cycle (2, 8, 20, 21, 34). In RCS rats, Delmelle et al. (11) noted that from P17 onwards there is a progressive retardation in the rate of retinol transfer from bleached rhodopsin into the RPE coupled with a slowdown in rhodopsin regeneration. These effects occur over the period of IRBP loss seen in the present work, and at a time when visual sensitivity drops markedly (Fig. 5 [inset]) (13, 14, 41). Retardation of rhodopsin regeneration because of IRBP depletion would account for the disappearance of rhodopsin in the light-reared animals well before the volume of rod–outer-segment membrane debris starts to diminish, and would also account for the observation that the amount of rhodopsin more closely parallels the amount of this debris in dark-reared or black-eyed dystrophic rats (14, 31).

IRBP may play a role in photoreceptor cell death. The depletion of IRBP could permit a damaging accumulation of retinol in the light and could account for the observation that photoreceptor cell death and the loss of rod–outer-segment membrane debris is retarded in darkness (14, 31). The two retinol-binding sites of IRBP also appear to be capable of binding α-tocopherol (21). Loss of IRBP could therefore accelerate the degradation of photoreceptor membranes by depriving them of a compound that protects them against oxidative damage.

In summary, our observations on the RCS rat, coupled with those from other laboratories, suggest that IRBP may be synthesized by the photoreceptors, that it plays a role in the transport of retinoids between the retina and RPE, and that its depletion as the disease progresses may accelerate photoreceptor degeneration.

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