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Homozygous Deletion in the Coding Sequence of the *c-mer* Gene in RCS Rats Unravels General Mechanisms of Physiological Cell Adhesion and Apoptosis

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The RCS rat presents an autosomal recessive retinal pigment epithelium dystrophy characterized by the outer segments of photoreceptors being phagocytosis-deficient. A systematic genetic study allowed us to restrict the interval containing the *rdy* locus to that between the markers D3Mit13 and D3Rat256. We report the chromosomal localization of the rat *c-mer* gene in the cytogenetic bands 3q35-36, based on genetic analysis and radiation hybrid mapping. Using a systematic biocomputing analysis, we identified two strong related candidate genes encoding protein tyrosine kinase receptors of the *AXL* subfamily. The comparison of their expression patterns in human and mice tissues suggested that the *c-mer* gene was the best gene to screen for mutations. RCS *rdy*⁻ and RCS *rdy*⁺ cDNAs were sequenced. The RCS *rdy*⁻ cDNAs carried a significant deletion in the 5' part of the coding sequence of the *c-mer* gene resulting in a shortened aberrant transcript encoding a 20 amino acid peptide. The *c-mer* gene contains characteristic motifs of neural cell adhesion. A ligand of the *c-mer* receptor, *Gas6*, exhibits antiapoptotic properties. © 2000 Academic Press

Key Words: RCS rats; retinal pigment epithelium; macrophages; phagocytosis; *c-mer*; apoptosis.

INTRODUCTION

One of the major functions of the retinal pigment epithelium (RPE) adjacent to the photoreceptor outer segments (POS) is the ingestion and degradation of the oldest discs discarded daily by the photoreceptor cells (PR). In the rat, an intense circadian phagocytic activity degrades 25,000–30,000 discs every day (La

Vail, 1976). The inherited retinal degeneration of the Royal College of Surgeons (RCS) rat was the first spontaneous animal model of inherited retinal pigment epithelium defect to be recognized (Bourne *et al.*, 1938; Mullen & La Vail, 1976), and is one of the major animal models used for the study of human retinal dystrophies. In the RCS rat retina, the RPE fails to phagocytose shedding discs (Mullen & La Vail, 1976); the accumulation of debris between the RPE cell layer and the POS results in photoreceptor cell death (Dowling & Sidman, 1962). The PR degeneration *stricto sensu*

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can first be detected histologically and electroretinographically at 18 days and is completed by 3 months of age (Dowling & Sidman, 1962). The RCS retinal degeneration is transmitted as a monogenic autosomal recessive trait. The mutated gene called *rdy* for *retinal dystrophy* causing the loss of vision of RCS rats had not previously been identified (Strauss *et al.*, 1998). Obviously, the identification of the protein encoded by the *rdy* gene and the understanding of its function is of outstanding importance for elucidating the phagocytosis mechanisms involved in the ingestion of POS by the RPE. The identification of the *rdy* gene will provide fundamental insights into physiological cell adhesion and apoptotic mechanisms occurring during development and contributing to maintenance of diverse types of cells, tissues and organs during adulthood. The *rdy* gene was assigned to linkage group IV, i.e., to rat chromosome 3 between the *agouti* and *Svp-1* loci on the basis of co-segregation of the RCS retinal phenotype with morphological and polymorphic protein markers (La Vail, 1981a). The recent availability of numerous highly polymorphic (CA)_n repeats spanning the whole rat genome allowed us to refine the *rdy* gene localization. From an extensive biocomputing analysis of gene homology databases and a careful study of the literature, the *c-mer* tyrosine kinase proto-oncogene emerged as the best candidate gene mutations of which could cause the RCS retinal degeneration. The sequencing of RCS *rdy*⁻ and RCS *rdy*⁺ cDNAs led us to identify a significant deletion in the 5' part of the coding sequence of the RCS *rdy-c-mer* tyrosine kinase gene. This deletion results in a shortened aberrant transcript encoding a 20 amino acid peptide. Normal *c-mer* transcripts are ubiquitous in rat tissues. This is consistent with the general cell adhesion properties of the *c-mer*-encoded tyrosine kinase receptor and a widespread involvement of the protein in the phagocytic ingestion phase of apoptotic bodies.

MATERIAL AND METHODS

All animals were handled in strict accordance with the Helsinki Declaration and with the Association for Research in Vision Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research. The animals were kept at 21°C with a 12-h light:12-h dark cycle and fed *ad libitum*.

Intraspecific Backcross Breeding

Rat pedigrees were generated for linkage analysis by intraspecific breeding. Affected RCS *rdy*⁻ p⁺ and RCS *rdy*⁻ p⁻ males (obtained from M.M. La Vail) were crossed with Brown Norway females (Charles River, France). F1 heterozygous females were backcrossed with RCS parental males to obtain F2 recombined backcross animals.

Fundus Photography and Fluorescein Angiography

These studies were performed on both pigmented RCS *rdy*⁺ and *rdy*⁻ rats at 21 days, 1 month and a half, 3 months and 1 year of age (kindly provided by M.M. La Vail, University of California, San Francisco, CA). A Canon CF-60ZA human retinal camera modified as previously described was used for fundus photography and fluorescein angiography (DiLoreto *et al.*, 1994). Rats were intraperitoneally injected with 0.15 g/kg of 10% sodium fluorescein (CIBAVision, France) (Shen *et al.*, 1998) and serial photographs were taken the 10 min following injection.

Electroretinogram and Evoked Visual Potential Measurement

Electroretinograms (ERGs) are used to measure the electric activity of rod and cone photoreceptors which are the first cells to receive light signals and transduce them into electric membrane potential variations which are, in turn, transmitted to the brain. Visual-Evoked Potentials (VEPs) can be used to check whether the visual cortex of the animal still receives meaningful signals allowing visual perception. ERGs and VEPs were recorded in separate sessions. Three rats were analyzed for each age chosen, i.e., 3 weeks and 2 months. Rats were dark-adapted over-night for each recording. ERGs were recorded as previously described (Peachey *et al.*, 1997). VEPs were recorded using a Grass needle electrode placed under the scalp overlaying the visual cortex. The ground lead was placed as for the ERG recordings, and the reference lead behind the ear at the same level on the skull as the recording electrode to avoid a nonspecific heart signal.

Tissue Sampling

Rats were sacrificed by asphyxia in CO₂ at 2 months of age for the backcross animals, and at 2 years for congenic parental and control rats. Both eyecups were

removed and a part of the tail was cut off. Tissues were frozen in dry ice and stored at -80°C until further use.

Histological Phenotyping

The two eyecups taken from each F2 rat were frozen, fixed and embedded in OCT Compound (Sakura). Fourteen-micrometer-thick cryosections of each eye were collected on five slides, previously coated with 2% 3-aminopropyltriethoxysilane solution in acetone. Each slide contained eight sections from the same eye. Sections were air dried, stained for 1–3 min in a 5% alun carmin solution and mounted in an aqueous mounting media for microscopy (Aquatex, Merck). The phenotype of the backcross rats was determined under the photonic microscopy by counting the number of rows of photoreceptor nuclei in the outer nuclear layer of each rat retina using 14- μm -thick frozen retinal tissue sections. At 2 months of age, the normal rat retina has 10–12 rows of photoreceptor nuclei whereas only 0–2 rows of photoreceptor nuclei can be observed in a degenerated RCS retina. This anatomical difference is significant and sufficient to discriminate unambiguously between F2 animals affected by the retinal degeneration and F2 rats which are normal.

Rat Genotyping

Genomic DNA samples were extracted from sectioned rat tails according to a standard technique. Based on the assignment of the *rdy* gene (La Vail, 1981a) and on our preliminary results, 90 (CA)_n repeat type polymorphic markers located on chromosome 3 were selected from the genetic maps available for the rat genome (Bihoreau *et al.*, 1997; Jacob *et al.*, 1995; Brown *et al.*, 1998; <http://waldo.wi.mit.edu/rat/public/>). For each primer pair, PCR conditions were optimized according to standard methods. Genomic DNA was genotyped as described elsewhere (Gauguier *et al.*, 1996).

Radiation Hybrid Mapping

The flanking markers of the gene region were mapped with a Rat Radiation Hybrid panel from Research Genetics (Huntsville, AL). Twenty-five nanograms of DNA from each of the 106 cell lines was used as PCR template for the primers D3Mit13 and D3Rat256 and each marker was screened at least

twice. Using standard PCR conditions and protocols, the products were analyzed on a 3% agarose gel. The samples were scored for the presence (score = 1) or absence (score = 0) of a PCR product of the expected size. Samples which gave conflicting results were scored 2. The data were submitted to the Laboratory for Genetic Research at the Medical College of Wisconsin (http://ares.ifrc.mcw.edu/maps-bin/rh_placement.cgi) and the Otsuka GEN Research Institute (<http://e4000.otsuka.gr.jp:8012/cgi-bin/RH/rhNgv.pl>) where they were tested against the framework maps.

Biocomputing Analysis of Gene Databases

Using the rat CA repeats markers and radiation hybrid mapping, we were able to map the *rdy* locus to the genetic interval bracketed by D3Mit13 and D3Rat256 and corresponding to the rat chromosomal localization 3q35-q36. We systematically detected in the OXFORD grid (<http://www.jax.org/>) all the identified genes shared by the rat chromosome 3 and the various human chromosomes containing homologous genes, to pinpoint and ultimately identify potential candidate rat genes. A similar strategy has been suggested in a recent report (Gauguier *et al.*, 1999).

RT-PCR Assays

Total RPE RNA was extracted from RCS *rdy*⁻ and RCS *rdy*⁺ rats by a standard protocol. One microgram of total RPE RNA from RCS *rdy*⁻ or RCS *rdy*⁺ rats was used to produce the first strand of cDNA with Superscript II (Gibco BRL), according to manufacturer's instructions. From the mouse cDNA sequence (NM_008587), 25 mer oligonucleotides were designed for heterologous PCR on rat cDNAs. Primers spanning the whole cDNA were used:

- oligo1-5' 5'-CATCTGTCCGAGAGAACTGC-CAGA-3',
- oligo1-3' 5'-TTGTGGCCTCACAGCTGAAGACTG-3',
- oligo2-5' 5'-CAGTCTTCAGCTGTGAGGCCCA-CAA-3',
- oligo2-3' 5'-TGGTCTTCACTGCCACCTTCTGAGA-3',
- oligo3-5' 5'-TCTCAGAAGGTGGCAGTGAA-GACCA-3',
- oligo3-3' 5'-GGGATCAGCACTCCAGCAAGAG-TAC-3',

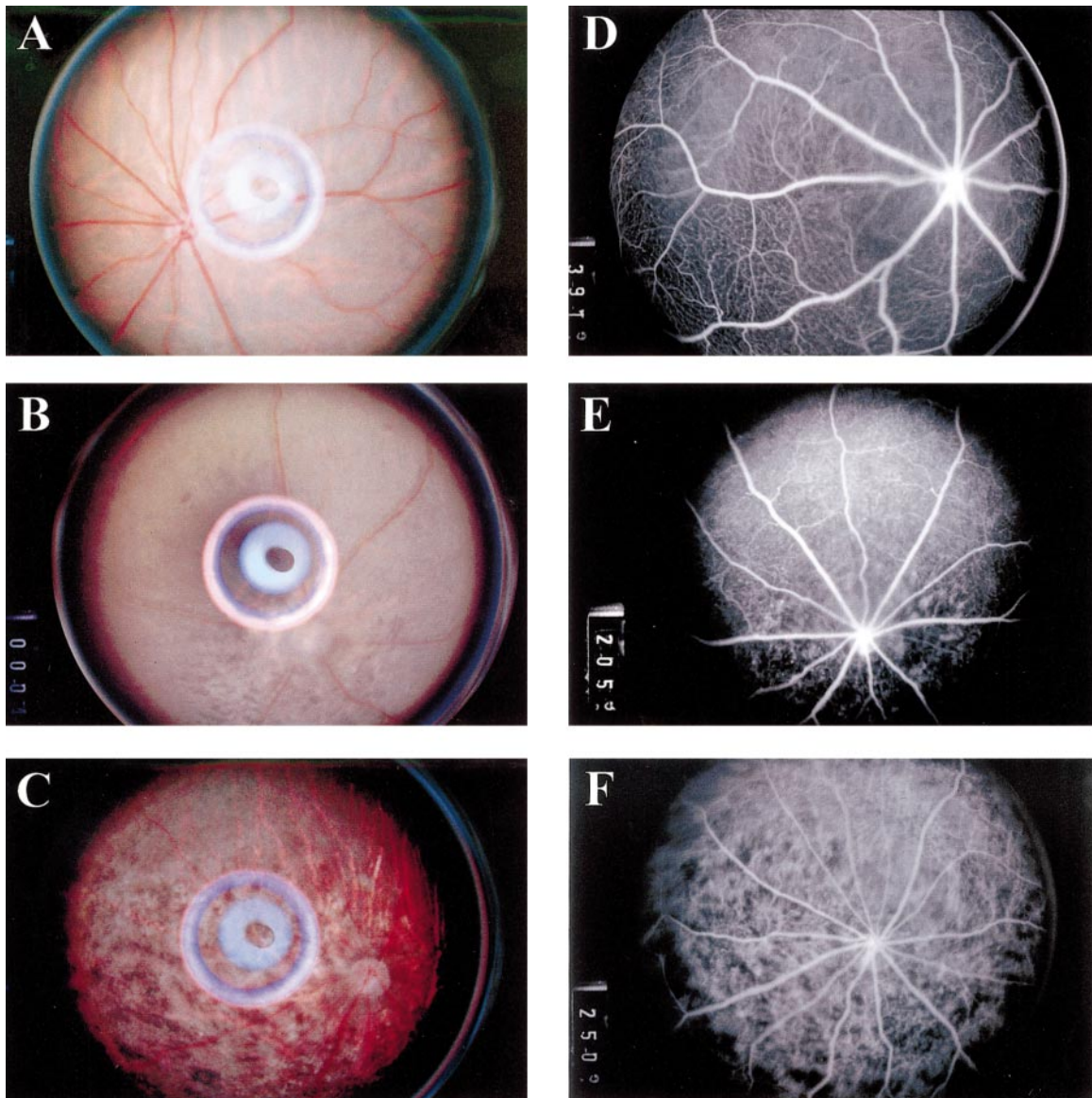


FIG. 1. (A) One-year-old RCS *rdy*⁺ rat. Venues are twice the diameter of the arterioles. The choroid and RPE are pigmented, larger choroïdal vessels can be seen through RPE and retina. Two concentric circles are the result of adaptation of the angiograph to small animals, and due to the reflection of the flash on the additional lens. (B) Color fundus photography of 3-month-old RCS *rdy*⁻ rat. RPE hypopigmentation and hyperpigmentation can be seen around optic nerve. Spots of RPE hyperpigmented predominate inferiorly. (C) One-year-old RCS *rdy*⁻ rat. Vessels and particularly arterioles show substantial narrowing; the optic nerve head is very pale. (D) RCS *rdy*⁻. Fluorescein angiogram at one month and a half. No abnormalities can be seen, all retinal arterioles and venules are filled with dye, details of the retinal capillary bed are easily visualized. (E) RCS *rdy*⁻. Angiogram at three months. Hypofluorescence around optic nerve is due to plaques of hyperpigmented RPE. (F) RCS *rdy*⁻. Angiogram at 1 year. Vascular narrowing is predominant on arterioles, hypofluorescent plaques due to multiple focal RPE hyperpigmentation predominate in the inferior part.

oligo4-5' 5'-GTACTCTTGCTGGAGTGCTGATCCC-3',
 oligo4-3' 5'-TCACATCAGAACTTCAGAGTCTTCC-3'.

One microliter of a 20 μ l RT reaction was used in a 20 μ l PCR. The PCR conditions used were: 5 min at 94°C, 35 cycles of 40 s at 94°C, 1 min at 55°C, 1 min 40 at 72°C, and an additional phase of 7 min at 72°C. The

amplification products were analyzed on a 1.5% agarose gel.

Sequencing Analysis

The *c-mer* RT-PCR products were sequenced on ABIprism A310 and ABI A377 automated sequencing machines (PE Biosystems, U.S.A.).

Northern Blot Analysis

A 1089-base cDNA probe, corresponding to one of the *c-mer* RCS *rdy*⁻ RT-PCR products, was radiolabeled with ³²P-ATP by random priming. A multiple rat tissue Northern blot (Clontech, Palo Alto, CA) was hybridized following manufacturer's instructions. Fifteen micrograms of macrophage total RNA from mice was transferred to a nylon membrane (Hybond-XL, Amersham Pharmacia Biotech). The membranes were probed for hybridization with a 786-base radiolabeled RT-PCR *c-mer* product according to a standard protocol.

RESULTS

To identify the RCS gene, we crossed homozygous wild-type rats from the most appropriate strain, likely to provide the optimal informativity of polymorphic markers, with RCS rats to obtain F1 heterozygous rats. These rats were backcrossed with parental homozygous RCS rats as described above. The phenotype and genotype of all the F2 rats were established and genotype/phenotype correlations used to identify the genetically recombinant animals. The minimal genetic and cytogenetic intervals containing the RCS gene were determined using the recombinant rats. The last step was to build a physical map of the critical region with PACs and BACs. A bio-computing analysis was performed in parallel to identify the gene, the alterations of which are responsible for the RCS retinal degeneration.

Choice of Rat Strains and Results of the Backcrosses

To maximize the informativity of polymorphic markers (Bihoreau *et al.*, 1997, <http://www.well.ox.ac.uk/~bihoreau/>), we genotyped three rat strains: Brown Norway (BN), Fischer and Lewis (Charles River, France). The BN strain displayed 76% polymorphism with the RCS strain and thus was chosen for the

backcrosses. We used both pigmented and pink-eyed rats for the crosses. Four hundred and forty-two F2 rats were obtained, phenotyped when 2 months old and genotyped.

Morphological and Physiological Studies of the RCS Rat Strain

To refine the clinical characterization of the RCS retinal degeneration and to determine confidently the best post natal stage at which unambiguous genotype/phenotype correlations could be established, normal control and RCS affected rats were phenotypically assessed by fluorescein angiography, electroretinography, visual evoked potentials analysis and histological analysis of retinas. In the RCS *rdy*⁻ rats, retinal abnormalities were first detected by color and black-and-white fundus photography, at 3 months of age (Figs. 1A–1C). Retinal angiograms of one month and a half, three months and 1-year-old RCS rats are shown in Figs. 1D–1F: changes in choroidal vascularization of RCS rats have been described previously (May *et al.*, 1996). We observed several other neuroretinal, epithelial, and vascular changes. A narrowing of retinal vessels, an abnormal remodeling of RPE causing hypo and hyperfluorescence, and pallor of the optic disks are clearly visible on fluorescent angiographs during the evolution of the RCS retinal degeneration. We performed electrophysiological studies on 3-week as well as on 2- and 3-month-old animals (Figs. 2A–2F). Despite the immaturity of ROS in rats until 35 days of age (Philp *et al.*, 1981), the RCS *rdy*⁻ rod activity was abnormally low at age 3 weeks and was absent from 2-month-old animals. Almost no rod photoreceptors were observed at this age (Dowling & Sidman, 1962). The cone activity in RCS *rdy*⁻ rats was also low, although measurable at 2 months: cones degenerate later during the RCS retinal dystrophy (Cotter *et al.*, 1984). No VEPs were measurable in 3-week-old RCS *rdy*⁺ control rats, but a VEP signal was observed at 2 months of age. In 2-month-old RCS *rdy*⁻ rats no VEP was detectable (data not shown). This is consistent with the destruction of the photoreceptor cell layer such that it cannot transmit a signal to secondary retinal neurons.

Histological Phenotyping of the Control and Backcross Animals

All the retinal sections, at least 40 per rat, were studied by two different researchers blind to the ge-

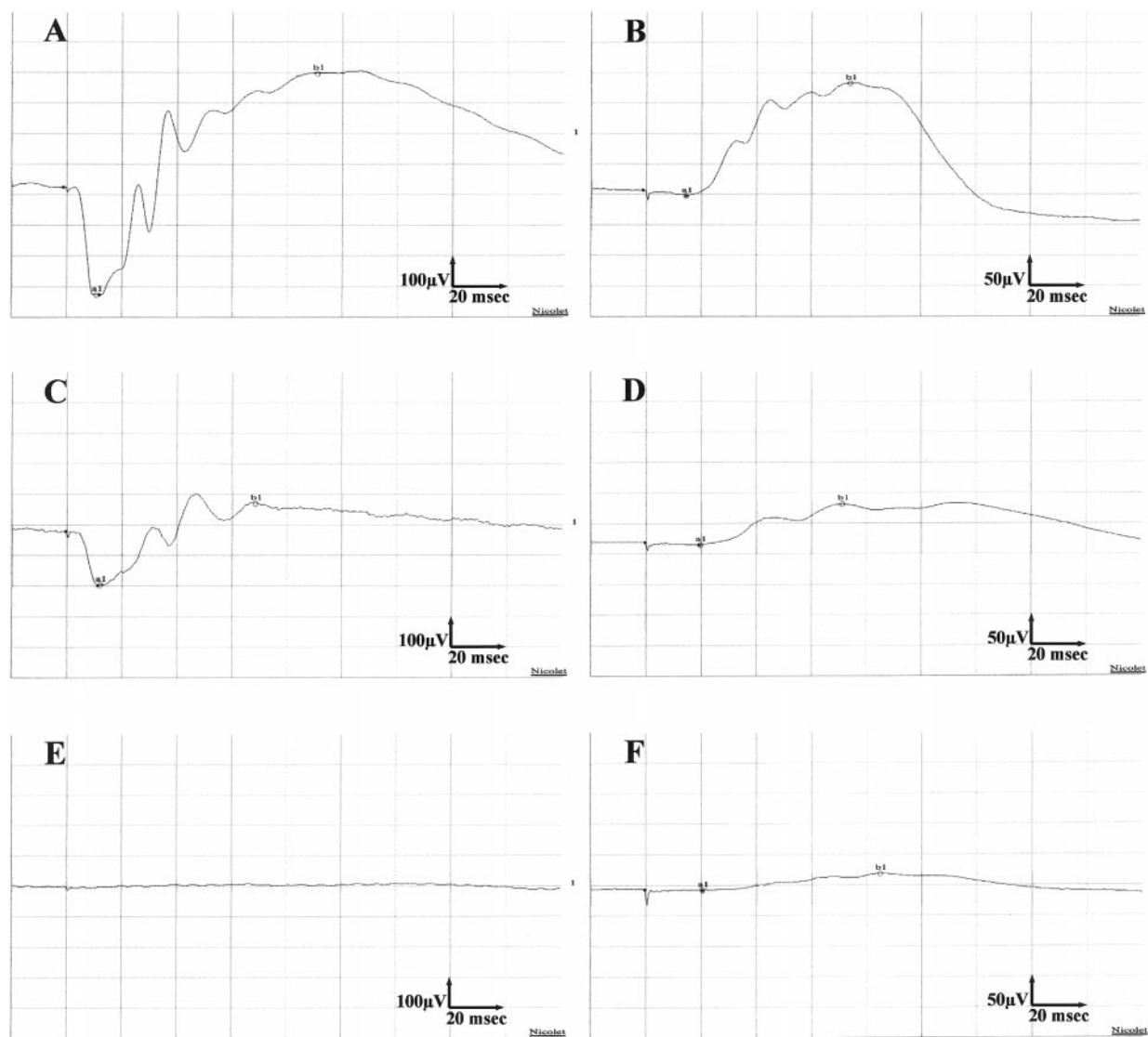


FIG. 2. Rod and cone electroretinogram measurements. (A, C, E) Dark-adapted ERGs recorded for the higher luminance strobe flash stimulus ($+0.5 \log \text{cd s/m}^2$) in RCS *rdy*⁻ rats. (B, D, F) Cone-mediated ERGs recorded for the higher luminance strobe flash stimulus ($+0.5 \log \text{cd s/m}^2$) in RCS *rdy*⁻ rats. Recordings were made 3 weeks (A, B), 2 (C, D), and 3 months (E, F) after birth.

nototype of the rats (Figs. 3A and 3B). Of the 442 F2 animals, 268 had abnormally few photoreceptor nuclei rows in both eyes, and the other 174 animals showed normal retinas.

Sequential Genotyping of the Rats and *rdy* Gene Localization

We compared allele sizes for (CA)_n repeat markers in RCS *rdy*⁻ affected with those in RCS *rdy*⁺ healthy rats: there were differences in alleles sizes for D3Mgh11, D3Mgh15, D3Mit12, DRFLP (La Vail,

1981b). We therefore presumed the *rdy* gene to map near one of these four markers. We constructed a refined map of the 12.7 cM region between D3Mgh12 and D3Mit3; the genetic distances are based on the ARB database site at <http://www.nih.gov/niams/scientific/ratgbase/> (Fig. 4). Five backcross F2 rats allowed us to determine the location of the *rdy* gene between D3Mit13 and D3Rat256, these 2 markers being excluded (Fig. 5). The remaining interval was estimated to cover less than one centimorgan. The last published map constructed with the Radiation Hybrid Panel from Cambridge University (UK) by the Otsuka

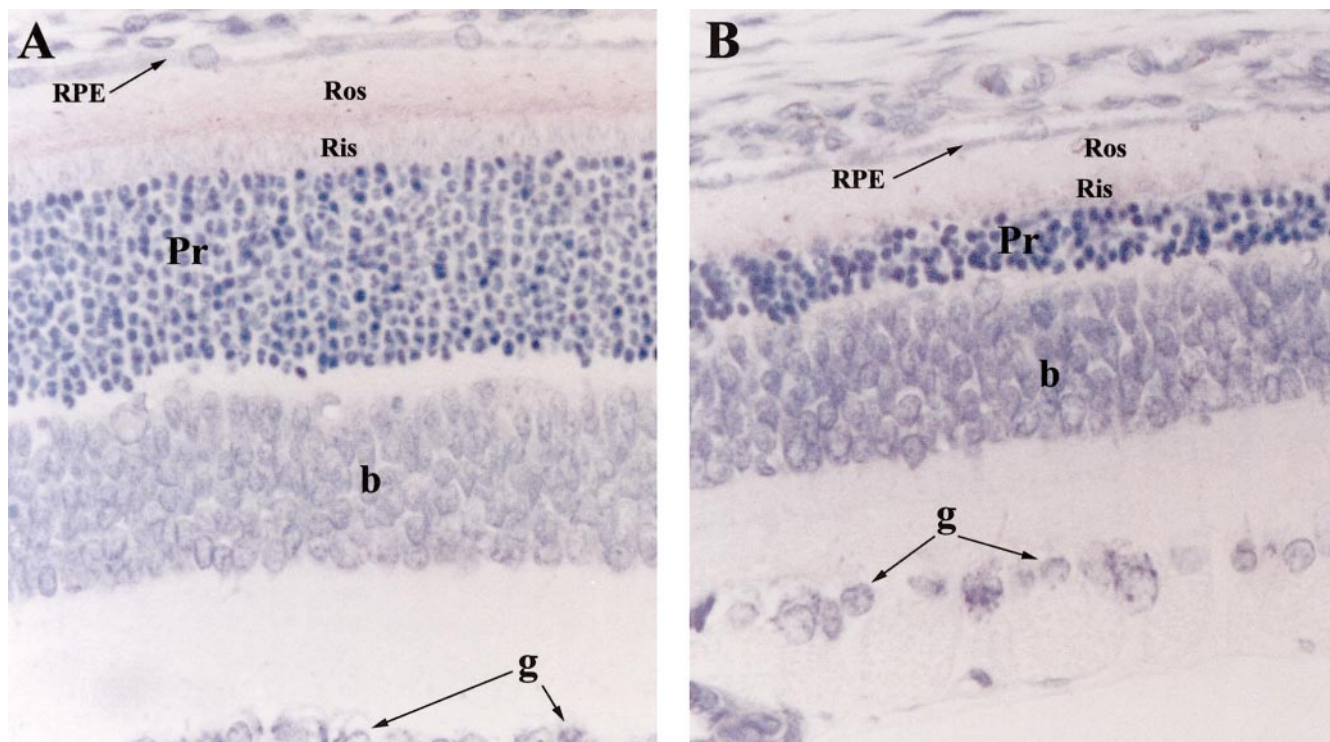


FIG. 3. Cryosections of RCS *rdy*⁺ normal control (A) and RCS *rdy*⁻ dystrophic rats (B). Magnification, $\times 400$. RPE, retinal pigment epithelium; Ros, rod outer segments; Ris, rod inner segments; Pr, photoreceptor cells; b, bipolar cells; and g, ganglion cells.

GEN Research Institute and the Wellcome Trust Centre for Human Genetics was not informative in our crosses (Watanabe *et al.*, 1999). To confirm physically that the two markers are indeed close to and flanking the *rdy* gene, we used PCR with the corresponding primers on the rat radiation hybrid mapping panel provided by Research Genetics Laboratories: the markers were linked to D3Rat21 with the Medical College of Wisconsin server and to D3Rat20 with the Otsuka GEN Research Institute server. They are also on the same radiation hybrid: the RH distance between them is 0.21 cR.

Determination of the Best Candidate Genes by Biocomputing Analysis

We genotyped our backcross rats with the first large set of CA repeats available. The *rdy* locus was located between *Adra2b* and *PLCb1* (D3Wox22). *Adra2b* has been assigned to mouse and human chromosomes 2 and *PLCb1* to mouse chromosome 2 and human chromosome 20. In view of the strong synteny between mouse chromosome 2 and rat chromosome 3, we checked all the human genes homologous to all the

genes of the rat chromosome 3. The rat critical chromosomal region is located on 3q35-q36 and corresponds to human chromosomal bands 20pcen-p13, 15q15-q21.1, 11p13, 9q34, and 2q11-q14.

As the *Tyro3* gene maps to the rat critical chromosomal region 3q35-q36, it is a candidate for the RCS retinal degeneration. *TYRO3* belongs to a subfamily of protein tyrosine kinase receptors comprising *AXL* and *C-MER*, all of which have the same ligand: GAS6. They are also involved in common processes such as the control of photoreceptor survival, and spermatogenesis (Lu *et al.*, 1999). Indeed, any of these proteins may be involved in the abrogation of phagocytosis of POS that characterizes the RCS retinal degeneration. The *AXL* gene has been mapped to 19q13.1 (Linz *et al.*, 1993) and was therefore excluded. The *C-MER* gene has just been assigned to human chromosome cytogenetic band 2q14.1 (Weier *et al.*, 1999). Its rat counterpart, *c-mer* is located within the genetic interval we identified. Similarly the human homolog of the rat *Tyro3* gene, *TYRO3*, maps to 20p13. Thus, both *c-mer* and *Tyro3* are candidate genes for the *rdy* locus. *Tyro3* is mostly expressed in the developing and differentiated central nervous system (CNS) (Polvi *et al.*, 1993)

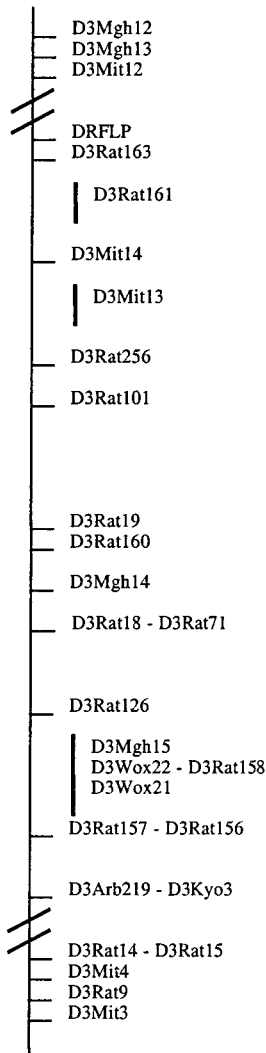


FIG. 4. Refined integrated map of the *rdy* gene region on chromosome 3q35-36. Anchored markers are known framework loci. Thick bars indicate the relative likelihoods for the location of other markers. The 2 markers flanking the *rdy* gene are D3Mit13 and D3Rat256.

and plays a role in hematopoiesis leading primarily to erythrocytopoietic and/or megacaryocytopoietic lineages (Mark *et al.*, 1994; Dai *et al.*, 1994; Lai *et al.*, 1994; Ohashi *et al.*, 1995b; Schulz *et al.*, 1995; Crosier *et al.*, 1995, 1996). *c-mer* is mostly expressed in monocytes/macrophages and epithelia, and is moderately expressed in the central nervous system (Jia & Hanafusa, 1994; Graham *et al.*, 1994, 1995). In view of the expression patterns of *tyro-3* and *c-mer*, and the epithelial and phagocytic properties of RPE cells, we considered *c-mer* to be the best candidate gene.

Analysis of the Rat *c-mer* cDNA Structure and Gene Expression Pattern

To determine the full length rat *c-mer* cDNA sequence, we used 4 pairs of oligonucleotides designed from the known mouse (NM_008587) and human (U08023) cDNA sequences. With these 25mer oligonucleotides, we performed RT-PCR starting from total RNA purified from normal control RCS *rdy*⁺ and affected microdissected rat RPE. With RCS *rdy*⁻ total RNA, we consistently obtained a deleted *c-mer* cDNA lacking 419 nucleotides present in the 5' part of the sequence of the controls (Fig. 6A). This deletion was present in all RCS *rdy*⁻ rats tested and absent from all normal control RCS *rdy*⁺ rats examined (Fig. 6B). The normal full-length *c-mer* transcripts were readily detectable in retinal pigment epithelium, neural retina, liver, testis (Fig. 6B), bone marrow, thymus, lung alveolar macrophages and peritoneal macrophages (Fig. 6C). Northern blot analysis (Fig. 7A) demonstrated that the *c-mer* gene is ubiquitously expressed, albeit most strongly in heart, liver, kidney, and testis. Moderate but significant *c-mer* gene transcription was detected in brain. In all RCS *rdy*⁻ rat tissues tested, we detected one species of 4.4 kb *c-mer* transcripts. Strong *c-mer* gene expression was also detected in two mouse macrophagic cell lines (Fig. 7B), namely the dendritic cell line D2SC and the macrophagic cell line J774, and in primary cultures of mouse bone marrow macrophages (BMMAC) and primary cultures of mouse

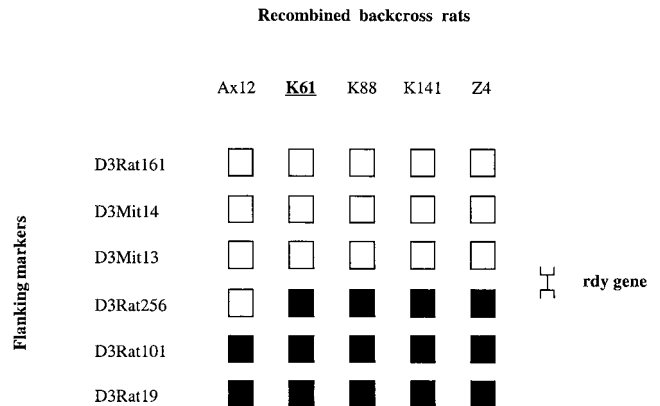


FIG. 5. Haplotypes of the 5 backcross animals delimiting the gene region to between markers D3Mit13 and D3Rat256, which are both excluded. Solid squares represent the homozygous RCS/RCS parental alleles responsible for the retinal dystrophy. Hollow squares represent the heterozygous RCS/BN parental alleles. Animals with a degenerated retina are in bold underlined characters. Correlation between the haplotypes and the histological diagnosis gives evidence for the genetic assignment of the gene.

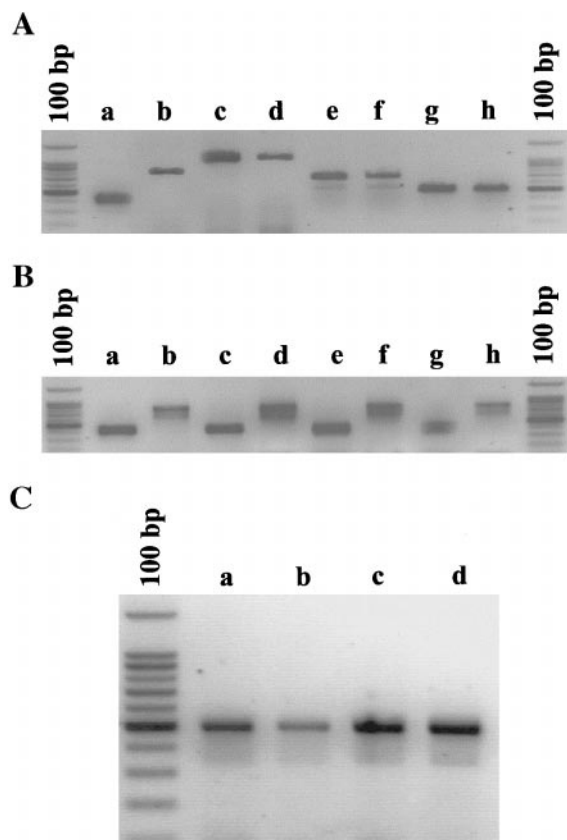


FIG. 6. (A) RT-PCR analysis of the *c-mer* mRNA in RPE cells from RCS *rdy*⁻ and RCS *rdy*⁺ rats. Primers spanning the whole cDNA sequence were used. The 419-bp deletion can be seen in the 5' part of the cDNA. Lanes a, c, e, g, RCS *rdy*⁻. Lanes b, d, f, h, RCS *rdy*⁺. Lanes a, b, primer pair 1; c, d, primer pair 2; e, f, primer pair 3; g, h, primer pair 4. PCR product sizes: primer pair 1, 411 bp for RCS *rdy*⁻ rats and 830 bp for RCS *rdy*⁺ rats; primer pair 2, 1089 bp; primer pair 3, 687 bp and primer pair 4, 512 bp. (B) RT-PCR analysis of the *c-mer* mRNA in various tissues in RCS *rdy*⁻ (lanes a, c, e, and g) and RCS *rdy*⁺ rats (lanes b, d, f, and h) using primers for the 5' part of the cDNA (primer pair 1). Lanes a, b, retinal pigment epithelium; lanes c, d, neural retina; lanes e, f, liver; lanes g, h, testis. The 411-bp product resulting from the deletion in RCS *rdy*⁻ rats is observed in all tissues tested. (C) RT-PCR analysis of the *c-mer* mRNA in various macrophagic cells from RCS *rdy*⁻ rats (primer pair 4). a, bone marrow; b, thymus; c, alveolar macrophage cells; d, peritoneal macrophage cells. The mRNA was detected in all macrophagic tissues tested.

dendritic cells (BMDC) (all these RNAs were generously provided by INSERM research unit 520 of the Curie Institute). The sequencing of RT-PCR products obtained from normal control and RCS rat RPE with the primer pair No. 1 provided a sequence of 830 bp in normal rat RPE. The 419-bp deletion was confirmed in affected RCS rat RPE, and caused a frameshift leading to a shortened *c-mer* peptide of 20 amino acids (Fig. 8).

DISCUSSION

Evidence for the Rat c-mer Gene Being the Gene Causing the RCS Phenotype

A biocomputing analysis combined with RT-PCR allowed us to identify a homozygous deletion in RCS *rdy*⁻ *c-mer* mRNA encoding a 20 amino acid truncated peptide, presumably devoid of any signaling capability. We sequenced the rat *c-mer* full-length cDNA both in normal RCS *rdy*⁺ and sick RCS *rdy*⁻ rats, and identified a 419-bp homozygous deletion in RCS *rdy*⁻ rat cDNA. Interestingly, the triple knock-out mice for the *Axl*, *Sky/Tyro3*, *c-mer* genes are blind owing to the postnatal degeneration of rods and cones in the retina (Lu et al., 1999). The histological and electrophysiological features of each single knock-out type of mice have not been reported. Their retinal functions during aging have not been described. While we had identified the accurate homozygous deletion in the RCS *rdy*⁻ rat cDNA, similar results, obtained by a classical positional cloning, were reported (D'Cruz et al., 2000). All these findings, taken together, are consistent with *c-mer* being the mutated gene causing the RCS phenotype. *Axl* and *Sky/Tyro3* are also potential candidate genes and their mutations can cause retinal degeneration. Although the rat *c-mer* gene is expressed in numerous tissue types, it is significantly expressed in retinal pigment epithelial cells and strongly in macrophages. These two classes of cells share a common permanent feature, that of phagocytosis. Phagocytosis involves the vitronectin receptors both in RPE and

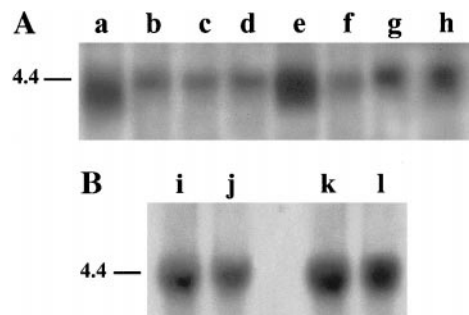


FIG. 7. (A) Hybridization of a 1089-base cDNA probe to a rat multiple tissue Northern blot (Clontech, Palo Alto, CA) containing mRNA from: a, heart; b, brain; c, spleen; d, lung; e, liver; f, skeletal muscle; g, kidney; h, testis. (B) Mouse *c-mer* mRNA in macrophagic cells. 15 µg of total RNA was loaded for each sample and hybridized with a 687-base mouse cDNA probe. The Northern contains the following samples: i, D2SC cell line; j, J774 cell line; k, bone marrow macrophage cells primary culture; l, bone marrow dendritic cells primary culture.

M V L A P L L

CATCTGTCCGGAGAGAACTGCCAGATCCGCGGCCCGCGATGGTTCGGCCCCACTACTG

L L G L L L L P A L W S

CTGGGGCTGCTGCTGCTATCCGCACCTCTGGAATGGAGCACTGCTGAAAAGGAGGAAGAA

ATCAAGCCAGATCAGCCATTTCAGGGCCCTTACCAGGGAGCCTACCAGCTGACCACAGG

CCATCTTCGCCCTCACTCCAGTGGGGACCAGCTGTCCACCATCCAGACTGGAAGATCA

CATCCAGCACACAGCCACTCCCGAGATGACCTCTGCAGCATCAAACCTCTGCCTCCT

GTTGCATTTAAAACCACAATGGACGCATAGTACTTTCGGAAACATAAAAAGTGTCAAATTT

AATTGCTCGATCAACATTCCTAACGTGTATCAAGAAACAGCTGGCATTTCGTGGTGAA

GATGAAAAGGAAGTCTTGGGGCACATTCATCAACACACAGTTTATCTCTGATGAGGAA

A -

GGGGTATCAATAATTGCATTGTTTCAGCATAAACAGTGTGCAGCGCTCAGACAATGGGTCC

TACATCTGTAAGATGAAGTGAACGATAGAGAGGTTGTGTCTGATCCCATATACGTGGAA

GTCCAAGGACTCCCTTACTTACTAAGCAGCCTGAGAGCGTGAATGTCAACCAGAAACACA

GCCTTCAACCTCACCTGCCAGCGTGTGGGACCCCTGAGCCGTCAACATCTTCTGGGTT

CAAAATAGCAGCCGTGTTAATGAAAATCCAGAAAGGTCCTCCCTGTCTCTAAGTGTCTGCT

GGTCTGACAGAGACCGCAGTCTTCAGCTGTGAGGCCACAA

FIG. 8. Sequence of a 830-bp normal RT-PCR product obtained with the primer pair 1 from mRNA originating from RCS rdy+ normal RPE. The sequence absent from RCS rdy- mRNA is indicated in underlined characters. The mutant amino acid sequence is indicated above the nucleotide sequence: the RCS rdy- truncated protein contains only 20 amino acids.

macrophages. Monocytes, macrophages and RPE cells can express $\alpha V\beta 5$ and $\alpha V\beta 3$ integrins.

However, RPE cells use $\alpha V\beta 5$ integrin due to the specific cellular context of PKC activation, whereas macrophages use $\alpha V\beta 3$ integrin to bind both POS and apoptotic cells (Finnemann & Rodriguez-Boulan, 1999). Thus, a simple molecular model of POS phagocytosis by RPE emerges where the POS are bound by the $\alpha V\beta 5$ vitronectin receptor and internalized by the *c-mer* receptor. However, this model does not take into account the role of phosphatidylserine (PS) moieties exposed on the outer leaflet of cellular membranes in most types of phagocytosis (Krahling *et al.*, 1999; Fadok *et al.*, 2000). Several data (Lu *et al.*, 1999; D'Cruz *et al.*, 2000) and our results support the participation of the *c-mer* receptor to the ingestion phase of phagocytosis in the normal rat retina under physiological conditions and a direct causative pathogenic effect of the complete deficiency of this receptor in the RPE of RCS rats. The final demonstration that the deletion of the *c-mer* gene in RCS rats is the cause of the inherited retinal degeneration requires restoration of the specific phagocytic function of RCS RPE cells *in vitro* and *in vivo*.

The Refined Phenotyping of the RCS Retinal Degeneration Suggests That the RCS Strain of Rats is a Specific Animal Model for Retinitis Pigmentosa

The histological method was the fastest method for phenotyping all the rats and was thus the most useful. However, the other methods, used for phenotyping RCS retinal degeneration, although slower, confirmed that accurate genotype/phenotype correlations were possible in all rats at 2 months of age. These methods also gave further insight into the pathophysiology of the RCS retinal degeneration. Our results suggest strongly that the RCS rat is a model of some forms of retinitis pigmentosa (RP). Indeed, the narrowing of retinal vessels, pallor of the optic disks and abnormal retinal pigmented deposits reminiscent of osteoblasts, detected in affected RCS rats by both fundus photography and angiography, were similar to pathognomonic ophthalmoscopic signs of retinitis pigmentosa (RP) in human patients. ERG and VEP studies strengthened the hypothesis of the RCS rat model being a specific model of RP affecting human patients. Although no human inherited retinal degeneration has yet been mapped to chromosome 2q14.1, it appears logical to analyze the *C-MER* gene in patients affected by inherited retinal dystrophies, and especially some forms of retinitis pigmentosa or AMD.

The Value of Biocomputing Research and Analysis for Identifying New Genes or New Functions for Characterized Genes

The availability of new genetic resources and the growing number of DNA and protein sequences available on the web have implications for the strategies to be used for identifying genes and genetic elements of interest. Several recent examples illustrate the power of the biocomputing approach (Laberge-le Couteulx *et al.*, 1999; Vanet *et al.*, 2000; Netchine *et al.*, 2000). Our biocomputing approach allowed us not only to identify the rat *c-mer* cDNA but also helped extract invaluable informations from several databases about the encoded protein. It indicated the structural and physiological properties common to the tyrosine kinase receptors of the *AXL* subfamily. The known gene expression patterns of *Axl*, *Tyro3/Sky*, and *c-mer* in different species (mouse and human) led us to focus our efforts on the rat *c-mer*, rather than on *Tyro3*, as the best candidate gene. However, all the receptors of the *AXL* family may participate directly or indirectly not only in the phagocytosis of POS but also in the phago-

cytosis of apoptotic bodies and senescent cells in general, since they share a common ligand GAS6 (Varnum *et al.*, 1995; Ohashi *et al.*, 1995a; Godowski *et al.*, 1995; Nagata *et al.*, 1996; Mark *et al.*, 1996). This ligand is encoded by the growth arrest-specific gene 6. The GAS6 protein binds specifically to PS moieties. The outer leaflet of normal eukaryotic cell membranes contains mostly cholinephospholipids, whereas the aminophospholipids, such as PS, are mainly present in the inner leaflet. Asymmetry seems to be the rule for normal cells (Verkleij *et al.*, 1973), and loss of membrane lipid asymmetry, in particular the emergence of PS at the cell surface, seems to be involved in the recognition and clearing of apoptotic (Fadok *et al.*, 1992, 1998; Krahling *et al.*, 1999) and senescent cells (Connor *et al.*, 1994; Ishimoto *et al.*, 2000).

A General Role for the c-mer Tyrosine Kinase Receptor and Related Proteins in Cell Adhesion

The widespread tissue distribution of *Axl*, *Tyros3/Sky*, and *c-mer* mRNAs and their common ligand implies a general role for these tyrosine kinase receptors in the development and physiology of mammals. The genes encoding *Axl* (O'Bryan *et al.*, 1991; Janssen *et al.*, 1991; Schulz *et al.*, 1993), *Sky/Dtk/Tyros3* (Ohashi *et al.*, 1994; Mark *et al.*, 1994; Fujimoto *et al.*, 1994; Lai *et al.*, 1994; Dai *et al.*, 1994; Crosier *et al.*, 1996), and *c-Eyk/c-mer* or *Mer* (Jia & Hanafusa, 1994; Graham *et al.*, 1994, 1995) are proto-oncogenes and are all involved in several types of malignancies. The loss of normal cell contact inhibition is a hallmark of cancerous cells, and reflects abnormal regulation of cell adhesion. The receptors encoded by the members of the *AXL* gene subfamily are typified by characteristic extracellular domains. They are composed of two immunoglobulin-like motifs in the NH₂ terminus followed by two fibronectin type III motifs, similar to those found in cell adhesion molecules such as neural cell adhesion molecules and receptor tyrosine phosphatases. The *GAS6* gene, which encodes the only known ligand of these receptors (Manfioletti *et al.*, 1993; Chen *et al.*, 1997), is widely expressed in the central nervous system (Prieto *et al.*, 1999), including the retina (D'Cruz *et al.*, 2000). Our data support *c-mer* gene expression in the adult rat brain. The spatial and temporal profiles of Gas6 protein and mRNA distribution suggest that the protein is the physiologically relevant ligand for *Axl*, *Tyros3*, and *c-mer* in the rat post-natal CNS. The *Axl/Tyros3/c-mer-GAS6* system may play a role in neuronal cell adhesion processes or in neuroglial cell interactions (Mark *et al.*, 1994; Ohashi *et al.*, 1994, 1995b;

Schulz *et al.*, 1995). Gas6, is also an adhesion molecule: Gas6 receptor-expressing cells bind to surfaces on which PS are exposed (Nakano *et al.*, 1997). GAS6 seems to bridge phagocytic cell membranes and PS-expressing membranes. One major biological outcome of the *Gas6/Axl-Tyros3-c-mer* interactions is to bring cells into close apposition. Complementary *Gas6/Axl*, *Gas6/Tyros3*, and *Gas6/c-mer* complexes may contribute to phenomena of organized aggregation of cells both during organogenesis and adulthood. These molecular interactions may be the molecular basis of the attachment of photoreceptors to RPE cells.

A General Role of the c-mer and Related Proteins in Phagocytosis and Apoptosis

PS residues are key markers exposed on the surface of apoptotic and senescent cells. All forms of phagocytosis engaging different types of receptors require at least one receptor binding to exposed PS residues. Rat Sertoli cells phagocytize apoptotic spermatogenic cells in primary culture, which consist mostly of spermatocytes, by recognizing PS exposed on the surface of degenerating spermatogenic cells (Shiratsuchi *et al.*, 1999). Triple knock out male mice for the *Axl*, *Tyros3*, and *c-mer* genes produce no mature sperm, owing to the progressive death of differentiating germ cells (Lu *et al.*, 1999). *Axl*, *Tyros3*, and *c-mer* receptors are all expressed by Sertoli cells during normal post-natal development, whereas their ligand Gas6 is produced by Leydig cells before sexual maturity and by both Leydig and Sertoli cells thereafter. Gas6 functions as an adhesion molecule that binds Gas6 receptor-expressing cells specifically to cells exposing PS moieties on their surface (Nakano *et al.*, 1997). This suggests that the *GAS6-Axl/Tyros3/c-mer* system is involved in the regulation of apoptosis. *In vitro* phagocytosis studies have shown that Gas6 enhanced the uptake of PS liposomes by macrophages and that the interaction of Gas6 with the surface of macrophages is essential to this enhancement. Gas6 also enhanced the uptake of PS-expressing apoptotic thymocytes by macrophages (Ishimoto *et al.*, 2000). Apoptotic bodies and POS compete for binding the vitronectin receptors $\alpha V\beta 3$ and $\alpha V\beta 5$ of RPE cells and macrophages *in vitro* (Finne-*mann* & Rodriguez-Boulan, 1999). Thus, Gas6 may help phagocytic cells—such as RPE cells—to recognize the tips of senescent POS, with PS exposed on their surface, already bound to the vitronectin receptor $\alpha V\beta 5$.

The major conclusion of this study is that members of the *Axl/Sky/Mer* tyrosine kinase family may play a

major physiological role in the regulation of cell adhesion and apoptotic pathways both during embryogenesis and during adulthood maintenance for diverse cell types, tissues and organs, including the retina.

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