Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat

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Vertebrate photoreceptor cells are the basic sensory apparatus of the retina, capable of converting the energy of absorbed photons into neuronal signals. The proximal portions of mammalian photoreceptor outer segments are synthesized daily by cell bodies, and outer segment tips are shed with a circadian rhythm, resulting in a complete turnover of outer segments about every 9 days. The shed outer segments are phagocytosed by adjacent retinal pigment epithelial (RPE) cells, and metabolites are recycled to photoreceptors. The Royal College of Surgeons (RCS) rat is a widely studied, classic model of recessively inherited retinal degeneration in which the RPE fails to phagocytose shed outer segments, and photoreceptor cells subsequently die. We have used a positional cloning approach to study the rdy (retinal dystrophy) locus of the RCS rat. Within a 0.3 cM genetic inclusion interval, we have discovered a small deletion of RCS DNA that disrupts the gene encoding the receptor tyrosine kinase Mertk. The deletion includes the splice acceptor site upstream of the second coding exon of Mertk and results in a shortened transcript that lacks this exon. The aberrant transcript joins the first and third coding exons, leading to a frameshift and a translation termination signal 20 codons after the AUG. The concordance of these and other data indicate that Mertk is probably the gene for rdy. Our results provide genetic evidence for an essential role of a receptor tyrosine kinase in a specialized form of phagocytosis and suggest a molecular model for ingestion of outer segments by RPE cells.

INTRODUCTION

Proper functioning of the basic sensory apparatus of the mammalian retina depends on the cooperation of two distinct cell types, the photoreceptor cell and the retinal pigment epithelial (RPE) cell, which are intimately associated in the outer retina. The continual synthesis and shedding of photoreceptor outer segments places a substantial biosynthetic load on the photoreceptor cell. Part of the function of the RPE cell is to lighten this burden, through phagocytosis of shed outer segments and recycling of retinoids and membrane lipids to the photoreceptor cell (1,2). The Royal College of Surgeons (RCS) rat is an animal model of recessively inherited retinal degeneration in which the process of cooperation between the photoreceptor cell and the RPE has gone awry, resulting in a progressive, postnatal loss of photoreceptor cells (3,4). Histological examination of the RCS retina reveals an abnormal build-up of outer segment debris between the photoreceptor cell outer segment layer and the RPE that occurs prior to and concomitant with photoreceptor cell death (5–8). Genetic chimera (9) and RPE cell transplantation (10) experiments have localized the defect in the RCS rat to the neuroectoderm-derived RPE cells, and RCS RPE cells fail to phagocytose isolated outer segments in culture (11).

One hypothesis regarding the nature of the RCS RPE phagocytosis defect is that ingestion may require binding of a specific receptor on the RPE to a ligand on the outer segment (12), and that RCS RPE cells lack functional receptors. Indeed, normal rat RPE cells show a degree of specificity for ingestion of outer segments in vitro when offered a variety of particles (13). The phagocytosis defect is not general because RCS RPE cells or explants can ingest latex beads and carbon particles (11,14) and RCS peritoneal phagocytes display normal function in culture (15). Candidate receptors that have been proposed include αvβ5 integrin (16), mannose receptor (17) and CD36 (18), but none of these proteins has been shown conclusively to be defective in the RCS rat. Recently, extensive genetic and genomic resources for the laboratory rat have become widely available (19–23), facilitating work on this important model organism. We therefore decided to take a positional cloning approach to identification of the retinal dystrophy locus (rdy) with the aim of gaining insight into the normal and abnormal interactions between the RPE and photoreceptor cells, and into the molecular mechanisms of outer segment ingestion by the RPE cells.

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RESULTS

Genetic mapping of rdy

Rudimentary genetic mapping previously had localized rdy ∼19 cM from nonagouti and 32 cM from Svp-1 on rat chromosome 3 (24). To confirm this localization, we scored 30 microsatellite markers from distal chromosome 3 (25) on DNA from RCS and a congenic strain RCS-**rdy**<sup>+</sup> (26) into which a wild-type rdy allele from the Fischer 344 strain had been introgressed. RCS alleles were observed for most markers, but markers D3Mit11 and D3Mit12 displayed an F344 allele in the congenic strain, indicating retention of a block of F344 DNA through selection for the wild-type rdy allele.

To locate rdy precisely, we constructed a high resolution genetic map of the region. DNA genotyping and retinal histology were performed for an initial group of progeny from an RCS × F344 backcross, and the locus was localized to an ∼15 cM interval between markers D3Mit4 and D3Rat104. Animals with recombination breakpoints within this interval were scored with additional markers, and rdy was confined to a 4–5 cM subinterval bounded by markers D3Mit12 and D3Rat126. The backcross was expanded to 110 N<sub>2</sub> progeny, and additional markers were mapped to create a high resolution genetic map of the region and confine rdy to an inclusion interval of ∼0.3 cM and bracketed by D3Rat161/D3Rat257 and D3Rat256 (Fig. 1a).

![Figure 1](http://waldo.wi.mit.edu/rat/public/) (a) A total of 1718 N<sub>2</sub> progeny from an RCS × BN backcross were genotyped with D3Mit12 and D3Rat126, which bracket rdy. Recombinant chromosomes were scored with additional markers within the interval and genotypes at rdy were inferred by retinal histology. Genetic distance in cM or the number of recombinants between adjacent markers is shown. (b) Physical map of the rdy genetic inclusion interval. Shown are selected BAC clones (horizontal black lines) and sequence-tagged sites (STSs; vertical dashed lines) within the inclusion interval between D3Rat161/257 and marker 3374, and which are part of a much larger BAC/STS content map. Most STSs derive from BAC insert ends. This, in combination with insert size information, provides an estimate of the extent of insert overlap and the distances between STSs (28). The **Mertk** gene is located to one side of the interval, and all of the coding exons fall within BAC 499P2, although the precise location of the first coding exon was not determined. No other genes were detected in the inclusion interval.

Construction of physical maps of the rdy region

Our efforts to construct a genetic map of the rdy region were aided by simultaneous construction of physical maps because the higher resolution of the physical maps and lack of a requirement for polymorphism allowed us to order markers that were unresolved on genetic maps. Yeast artificial chromosome (YAC) clones were isolated by screening a library with D3Mit12 and D3Rat126, and genotypes were determined for 110 N<sub>2</sub> progeny, and additional markers were mapped to create a high resolution genetic map of the region and confine rdy to an inclusion interval of ∼0.3 cM and bracketed by D3Rat161/D3Rat257 and D3Rat256 (Fig. 1a).
We began isolating bacterial artificial chromosome (BAC) clones across the interval covered by the YAC contig because BACs are largely free of the cloning artifacts found in YACs. As the genetic mapping progressed, our efforts focused on the interval between D3Rat161/Rat257 and D3Rat256. We constructed a highly redundant BAC contig spanning this interval and beyond by simultaneous walking from multiple entry points (28). A new polymorphic microsatellite marker (3374) was generated from a BAC end sequence and used to narrow further the genetic inclusion interval, which is more than covered by three minimally overlapping BAC clones (Fig. 1b).

Routine BLAST analysis of sequences near insert ends revealed two with a high degree of similarity to different parts of a murine Merk cDNA sequence. The order of the two end sequences indicated that the orientation of transcription of the gene is from centromere to telomere. Placement on the BAC contig of an STS developed with oligonucleotide primers from the 3′-untranslated region (3′-UTR) of murine Merk, and sequencing from BAC clone 499P2 with primers from the beginning of the open reading frame, demonstrated that the entire coding portion of the gene lies within the rd/y genetic inclusion interval (Fig. 1b).

Sequence and expression analysis of Merk in normal and dystrophic rats

Merk encodes a receptor tyrosine kinase expressed in monocytes, epithelia and reproductive tissues (29) that is a member of a family of at least three receptor tyrosine kinases, which also includes Axl and Tyro3, with ectodomains composed of two immunoglobulin and two fibronectin domains, similar to neural cell adhesion molecules (30). The prior association of Merk with adhesion, epithelia and phagocytic monocytes, along with location of the gene within the genetic inclusion interval, made it an attractive rd/y candidate.

We used oligonucleotide primers designed from partial rat sequence and murine Merk cDNA sequence to amplify overlapping segments covering the entire coding region of rat Merk by RT–PCR. Sequencing of RT–PCR products revealed that the rat gene encodes a 994 amino acid protein and shares 91.8% nucleotide identity and 91.5% amino acid identity with murine Merk, suggesting that the two genes are orthologous.

We assessed expression of Merk in normal and dystrophic animals by northern analysis and RT–PCR (Fig. 2). The expression profile in normal, outbred Sprague–Dawley rats (Fig. 2a) is similar to that in mice (31). Comparison of RPE/sclera, neural retina and kidney mRNA samples from RCS and RCS-rd/y strains revealed a slightly smaller transcript in dystrophic animals (Fig. 2b). RT–PCR analysis was consistent with the northern results. Overlapping segments spanning most of the coding region could be amplified from both RCS and RCS-rd/y samples, but primer pairs from the 5′ end of the coding region either amplified a smaller segment (Fig. 2c, primers a + b) or failed to amplify sequences (Fig. 2c, primers c + d) from RCS cDNA, indicating a deletion of 5′ sequences from the RCS Merk transcript.

Mutation of Merk in RCS rats

We determined the genomic structure of the 5′ coding region of rat Merk by sequence walking on DNA from clone 499P2 (Fig. 3). Comparison of the genomic structure with the sequence of the a + b RT–PCR product from RCS cDNA (Fig. 2c) revealed a precise deletion in the aberrant transcript of 409 bases,
corresponding to the second coding exon. Conceptual translation of the deleted mRNA shows a frameshift after codon 19, followed immediately by a translation termination codon (Fig. 3).

PCR analysis of RCS and RCS-\textit{rdy} genomic DNA demonstrated that, although the 3' half of exon 2 and adjoining intron sequences could be amplified from both strains, primers from the 5' half of exon 2 or upstream intron sequences failed to yield a product from RCS DNA (Fig. 3), indicating a deletion of sequences from the RCS genome. Southern analysis of RCS and RCS-\textit{rdy} genomic DNA digested with one of nine different restriction enzymes and probed with a segment from the second coding exon (the location of which is shown in Fig. 3) revealed that the restriction fragment detected in RCS DNA was usually 1.5–2 kb smaller than that detected in RCS-\textit{rdy} DNA, with the exception of fragments generated by \textit{Pst}I digestion, where the RCS fragment was \textasciitilde 8 kb larger than the RCS-\textit{rdy} fragment (data not shown). The 5' end of the smaller, wild-type \textit{Pst}I fragment is located within the second coding exon. A deletion of RCS DNA that includes the \textit{Pst}I site in the second coding exon and extends 1.5–2 kb into adjoining 5' intron sequences is consistent with the Southern hybridization and genomic PCR data.

Together, the structures of the RCS \textit{Mertk} transcript and genomic DNA indicate that a small deletion, which includes the splice acceptor site next to the second coding exon, has resulted in expression of an aberrant transcript that lacks this exon and that is incapable of encoding a functional protein.

**DISCUSSION**

The \textit{Mertk} mutation is non-recombinant with \textit{rdy}

We used the presence or absence of a c+d amplification product as a genetic marker to score spleen RNA samples from the five RCS \texttimes BN backcross progeny with recombination events closest to \textit{rdy} (Fig. 1a), and found that the \textit{Mertk} RT–PCR marker was non-recombinant with the \textit{rdy} mutation. We also used the presence or absence of a PCR product from the 5' portion of the second coding exon to score the genomic DNA of all recombinants within the \textit{D3Mit12} and \textit{D3Rat126} interval detected in both the RCS \texttimes BN and the RCS \texttimes F344 backcrosses. The deletion is non-recombinant with \textit{rdy} in both crosses, a total of 3173 meiotic events.

**Figure 3.** Structure of \textit{Mertk} 5' genomic and cDNA sequences in normal and dystrophic rats. The genomic structure of the first four coding exons and adjoining introns of wild-type \textit{Mertk} is depicted in the middle. Partial cDNA sequences from the 5' end of \textit{Mertk}, obtained from RCS and RCS-\textit{rdy}+ animals, are shown above, with the locations of splice junctions indicated by arrows. Sequence at the beginning of the third coding exon is underlined, and a premature translation termination codon in the RCS transcript is indicated by three asterisks. A depiction of PCR results obtained by amplifying segments in and around the second coding exon (GenBank accession no. AF208236) is shown below. Gray horizontal bars indicate products amplified from RCS-\textit{rdy}+ DNA that could not be amplified from RCS DNA. Black bars indicate products that were obtained from both DNAs. The lower portion of the figure is drawn to scale. The hatched bar at the bottom denotes the location of a hybridization probe used for Southern analysis of restriction digested RCS and RCS-\textit{rdy}+ DNA. P, \textit{Pst}I site.
Other, less likely scenarios are also possible, such as the existence of a second mutant gene in the inclusion interval that alone is responsible for the phenotype, or deleterious effects by the deletion on a second gene near to Mertk. Functional complementation of the RCS defect by wild-type Mertk would rule out these alternatives conclusively.

A mouse strain homozygous for a targeted mutation of Mertk that destroys tyrosine kinase activity by removing a single exon near the 3’ end of the coding region exhibits splenomegaly and an increased sensitivity to endotoxins (32). The retinas of the mutant mice apparently were not examined, so a retinal defect may have passed unnoticed. Alternatively, it would not be surprising if the mutant mouse and RCS rat expressed different retinal phenotypes because the mutant murine Mertk protein was intended to have residual function (32). Moreover, a similar mutant Axl receptor with a missense mutation that ablates tyrosine kinase activity can still promote cell adhesion in culture (33). Regardless of which explanation is correct, our results highlight the utility of a phenotype-driven approach to understanding gene function.

**A molecular model for phagocytosis of outer segments by RPE**

The identification of Mertk as the likely rdy gene provides insight into the RCS retinal dystrophic phenotype and the mechanism of phagocytosis of outer segments by the RPE cells. Cell culture studies implicate αβ integrin, a vitronectin receptor which is present at the RPE–outer segment interface (34), in the binding but not internalization of outer segments by RPE cells (16). It is plausible that Mertk cooperates with αβ to phagocytose outer segments and signal the event within RPE cells. Signaling through other receptor tyrosine kinases can promote cell growth (35) or cell migration (36) in a vitronectin receptor-dependent manner, and there are mechanistic similarities between cell migration and phagocytosis (37).

Mertk signaling of phagocytosis may also be important for trophic support of photoreceptors by the RPE. Transplantation of normal RPE cells to the retinas of RCS rats slows degeneration of photoreceptor cells over an area larger than the transplant site (38), and targeted mutation of Mertk, Tyro3 and Axl in mice apparently leads to decreased trophic support for developing sperm and other cells, and results in postnatal degeneration of rods and cones in the retina (39).

Gas6, a secreted vitamin K-dependent protein expressed in a variety of tissues, including the retina (D. Vollrath and M.M. LaVail, unpublished data), is a ligand for all three Mer-family receptors (40,41). Gas6 can bind to the outside of cells and promote heterotypic intercellular adhesion through the Axl receptor (33), supporting a role for Mertk and Gas6 in adhesion of the RPE to photoreceptor outer segments. Gas6 preferentially binds to phosphatidylserine (42), an abundant constituent of photoreceptor membranes (43). Phosphatidylserine is present predominantly in the inner membrane leaflet, but is exposed to the exterior of apoptotic cells and oxidized red blood cells (44). Binding of Gas6 to photoreceptor membranes that display phosphatidylserine may provide a mechanism for selective phagocytosis of older outer segments by the RPE.

**Relevance to human disease**

Mutations responsible for a number of forms of early-onset retinal degeneration in humans have been identified, including mutations associated with Leber congenital amaurosis and retinitis pigmentosa. A large number of other human retinal degeneration loci have been mapped, but mutations have not been described. Human MERTK has been mapped to chromosome 2q14.1 (45). To our knowledge, no retinal degeneration locus has yet been linked to this region, but the extensive genetic heterogeneity of the condition in humans and the simple loss-of-function nature of the mutation we have characterized leave open the possibility that mutations in MERTK are responsible for a fraction of human early-onset retinal degeneration. Although most genes linked to retinal degeneration are expressed in photoreceptors, a growing minority of genes are expressed in the RPE cells (46–49). The RPE cells are thought to be relevant to the pathogenesis of a common late-onset form of human retinal degeneration, age-related macular degeneration (AMD), because abnormal deposits of lipofuscin-like material known as drusen accumulate in and around the RPE cells in this condition. Given the RPE cell-autonomous nature of the RCS phagocytosis defect and the importance of outer segment renewal to the well being of the photoreceptor cell, it is possible that MERTK, or a molecule with which it interacts, is involved in the pathogenesis of AMD.

**MATERIALS AND METHODS**

**Genetic mapping**

DNA for genotyping was isolated from rat spleens by a modification of a previously described protocol (50). A total of 1718 N2 progeny from a BN × RCS backcross were scored with D3Mit12 and D3Rat126 by resolving amplification products on agarose gels stained with ethidium bromide, and recombinants subsequently were scored with additional markers, some of which were labeled with 32P and resolved on sequencing gels (Fig. 1a). A polymorphic CA repeat (3374) was amplified with oligonucleotide primers (CTGGCCCTCCATTGTTGTG; TTGAGCAGGGACAGA) and Taq Gold (PE Biosystems, Foster City, CA) by a touchdown PCR protocol. An ∼7–10 bp size difference between the BN allele and the smaller RCS allele was detected by agarose gel electrophoresis.

**Retinal histology**

To infer genotype at rdy, P21–P35 backcross progeny were euthanized with carbon dioxide, eyes were immediately enucleated, fixed in a mixture of formaldehyde and glutaraldehyde, bisected along the vertical meridian and embedded in a mixture of Epon–Araldite. Sections of 1 μm of the entire retina were cut and stained with toluidine blue as described elsewhere (8). All animal procedures adhered to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

**Physical mapping**

YAC clones from the SHR strain were isolated from a commercially available library (Research Genetics, Huntsville, AL) by PCR screening. STSs were generated from YAC-containing clones and primers m and n (T3 side) or o and p (T7 side) (Table 1). STSs derived from chimeric ends were detected by radiation hybrid mapping using a commercially available mapping panel (Research Genetics). BAC
Table 1. Oligonucleotide sequences

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<tr>
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<tr>
<td>f</td>
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<td>h</td>
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*Marine sequence.

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