Different \textit{RPGR} exon ORF15 mutations in \textit{Canids} provide insights into photoreceptor cell degeneration

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The canine disease, X-linked progressive retinal atrophy (XLPRA), is similar to human RP3, an X-linked form of retinitis pigmentosa, and maps to the same region in the X chromosome. Analysis of the physical map of the XLPRA and RP3 intervals shows a high degree of conservation in terms of genes and their order. We have found different mutations in exon ORF15 of the \textit{RPGR} gene in two distinct mutant dog strains (XLPRA1, XLPRA2). Microdeletions resulting in a premature stop or a frameshift mutation result in very different retinal phenotypes, which are allele-specific and consistent for each mutation. The phenotype associated with the frameshift mutation in XLPRA2 is very severe and manifests during retinal development; the phenotype resulting from the XLPRA1 nonsense mutation is expressed only after normal photoreceptor morphogenesis. Splicing of \textit{RPGR} mRNA transcripts in retina is complex, and either exon ORF15 or exon 19 can be a terminal exon. The retina-predominant transcript contains ORF15 as a terminal exon, and is expressed in normal and mutant retinas. The frameshift mutation dramatically alters the deduced amino acid sequence, and the protein aggregates in the endoplasmic reticulum of transfected cells. The cellular and molecular results in the two canine \textit{RPGR} exon ORF15 mutations have implications for understanding the phenotypic variability found in human RP3 families that carry similar mutations.

INTRODUCTION

Vision begins in the highly specialized photoreceptor cells of the retina. These cells and the adjacent retinal pigment epithelium (RPE) interact in synergy to maintain retinal function and viability. A multiplicity of mutations have been identified that cause naturally occurring or experimental degeneration (1). The human diseases predominantly associated with early rod photoreceptor degeneration are collectively termed retinitis pigmentosa (RP). Some of the responsible genes are involved in phototransduction, in maintaining photoreceptor structure, or in RPE retinoid metabolism. However, there remains a large number of diseases caused by genes whose function is poorly understood, including the RP3 form of X-linked RP (XLRP) caused by mutations in the RP GTPase regulator (RPGR) gene (2).

XLRP has a population prevalence of 1 in 25,000, and represents the most severe class of RP, with early onset of night blindness, visual field loss and eventual loss of central acuity (3,4). Of the five XLRP loci identified, causal mutations have only been established for RP2 (5) and RP3 (2). The RP3 region spans 500–600 kb in Xp21.1, and includes TCTE1L, SRPX, RPGR and OTC (2). This locus accounts for approximately 60–90% of affected XLRP pedigrees, although previous studies found RPGR mutations in only 20% of families (2,6,7). More recently, novel protein coding sequences have been identified in regions formerly regarded as introns of \textit{RPGR}. One of these, exon ORF15, is a mutational hot spot, and mutations in this exon now account for the majority of RP3 cases (8).

The role of RPGR in retinal function is unknown. The first 10 exons encode a domain that is highly conserved between

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\textsuperscript{2}AF385629, AF148800 and AF148798
species, consisting of six tandem repeats of 52–54 amino acids, which are homologous to the repeat structure of the RCC1 protein (2). Rpgr knockout mice in which exons 4–6 were replaced by a selectable marker that results in lack of Rpgr expression develop a slowly progressive post-developmental photoreceptor degeneration (9). The RCC1 domain has been used as a bait in yeast two-hybrid screens, resulting in the identification of two interacting proteins: the delta subunit of rod-specific cyclic GMP phosphodiesterase (10) and the Rpgr interacting protein (RPGRIP) (11,12). RPGRIP co-localizes with Rpgr in the photoreceptor outer segment (12) or connecting cilium (13), and may be involved in structural or transport functions, respectively.

There is little known of the function of the C-terminal half of Rpgr, particularly exon ORF15, in which most of the mutations are located (8). An alternative approach to address the question of the functional role of this domain is to identify other species with naturally occurring mutations in this region, and examine the resultant phenotypes. Such a comparative genomic approach is now possible in dogs, as there are sufficient resources to map and positionally clone disease genes (14).

Canine progressive retinal atrophy (PRA) comprises several naturally occurring retinal diseases with a similar phenotype to RP. Six distinct autosomal and one X-linked loci have been identified (15). XLPRA in the Siberian husky is the only spontaneous animal model for XLRP (16,17); the disease has been mapped to the short arm of the X chromosome, and showed no recombinants with Rpgr. Prior to recognition of the novel ORF15 exon of human RPGR, we characterized the RPGR splice variants, but found no mutations (18).

The original XLPRA disorder has now been renamed XLPRA1 to distinguish it from a second disease (XLPRA2) mapping to the same locus. Recombination events located the disease interval between microsatellite markers associated with TCE1L and OTC (19); these markers define a less than 1 cm zero recombination region, homologous to the RP3 interval, and containing the TCE1L, SRPX, RPGR and OTC orthologs. In the present study, we have completed the cloning and characterization of canine RPGR, including the 10 kb intron 15 containing exon ORF15. We have found three distinct microdeletions in exon ORF15 in canids, one benign and two disease-associated, which provide insights into the structure and function of the RPGR C-terminal domain and the human disease.

RESULTS

Comparative mapping of XLPRA1/XLPRA2 and RP3

To construct a physical map, we used published human gene sequence data for the RP3 interval to retrieve canine BACs, and

![Diagram](image_url)
made a contig extending from XK to OTC. This established that
gene order in the XLPRA and RP3 regions is the same (Fig. 1).
We found recombinations (3/64 and 2/107) with flanking
markers (19), effectively reducing the interval to a 550 kb
region extending from TCTE1L to OTC and including SRPX
and RPGR. Among these four genes, we excluded TCTE1L by
direct cDNA sequencing, and focused our efforts on the canine
ortholog of exon ORF15.

Cloning RPGR exon ORF15 and identification of three
microdeletions in canids

Primers from intron 14 and exon 16 were used to amplify the
10 kb intron 15 from a BAC clone positive for RPGR. The
product was cloned and directly sequenced using vector
primers followed by gene-specific primer walking. Exon
ORF15 (GenBank accession no. AF385629) is transcribed
continuously from exon 15, and extends about 2.8 kb into
introns 15; it is unusually rich in purines (about 70%) and
repetitive sequences. The stop codon (TAA) and poly(A) signal
(AAUAAA) are at positions 1717–1719 and 2805–2810,
respectively. Two unstable signals (AUUUA), recognition sites
for RNase E activity (20), were found in the 3′ UTR. As in
humans, the dog exon encodes the same set of charged amino
acids (Arg, Lys, Asp and Glu) alternated with glycines; the
encoded proteins have an identical number of glutamic and
aspartic acid residues and similar isoelectric point (4.01 versus
4.05). However, instead of the repetitive EEEE EEEE sequence of humans (8), the dog sequence contains GEEE-
EEEE and EEEEEEEE repeats. The C-terminal 35 amino acids are highly conserved between human
and dog. Because of the premature stop codons, the mutant
exon ORF15 sequences in XLPRA1 and 2 encode proteins
lacking this C-terminal tail.

Three microdeletions were found in canids in a 100 bp region
between nucleotides 1001 and 1100 of exon ORF15. In red
wolves, a three-nucleotide deletion eliminates a glutamic acid,
but does not alter the remainder of the protein and does not
cause disease (see below). A five-nucleotide deletion (delGA-
GAA) between 1028 and 1032 was identified in XLPRA1. In
XLPRA2, a two-nucleotide deletion (delGA) in 1084–1085
was identified, 51 nucleotides 3′ to the site of the XLPRA1
deletion (Figs 2 and 3).

The XLPRA1 mutation causes a frameshift and immediate
premature stop; the truncated protein is missing 230 C-terminal
amino acids, causing a slight decrease in the isoelectric point
(3.89 versus 4.01 in normal). By mutation scanning, we also
found the same five-nucleotide deletion in the Samoyed breed
with a clinically similar X-linked retinal degeneration (data not
shown). The XLPRA2 mutation results in a frameshift that
significantly changes the deduced peptide sequence, causing an
increased isoelectric point (4.30 versus 4.01), and leads to the
inclusion of 34 additional basic residues before prematurely
terminating translation 71 amino acids downstream. We
produced several compound heterozygotes that showed a more
severe clinical phenotype than either XLPRA1 or XLPRA2
hemizygotes or heterozygotes (see below). Figure 3C illustrates
the independent segregation of two mutant alleles in male
progeny of a compound heterozygote.

Exon ORF15 deletions determine retinal phenotype

Severe abnormalities in retinal structure and function were
found in XLPRA, and these were allele-specific. As previously
reported, photoreceptors in the XLPRA1 retina developed and
functioned normally (17). Retinal function remained normal
until 6 months of age or later, after which there was a decrease
in the amplitude of the dark-adapted rod and cone responses
(Fig. 4IA1–3). Morphologically, XLPRA1 photoreceptors
remained normal until young adulthood; thereafter, rods
showed distinct irregularities of the outer segments, followed
by degeneration. Cone abnormalities appeared less severe, and

Figure 2. Mutation analyses of exon ORF15 in canids. (A) Wild-type nucleotide sequence from the mutation region of canine exon ORF15 encompassing nucleo-
tides 1001–1100. The three deletions are illustrated in the top lines. The electropherograms show the deletions for affected and carrier dogs with XLPRA1 (B) and
XLPRA2 (C).
a proportionally larger number of cones remained in the visual cell layer in late disease (Fig. 4I–D). All affected males showed end-stage retinal disease by 4 years of age. In contrast, retinal development is aberrant in XLPRA2. ERG abnormalities were evident by 5–6 weeks of age; the responses were low in amplitude, abnormal in waveform, and deteriorated with age (Fig. 4IB1–3). There was relative preservation of cone flicker responses when rod function is severely compromised, but the cone signals are abnormally small and, in older animals, increased in timing. In parallel, photoreceptor morphogenesis is abnormal. Outer segments are highly disorganized and disoriented as they develop (Fig. 4IIE), and visual cell degeneration is present by 4 months and progresses (Fig. 4IIF, G). By 2 years, end-stage degeneration is present in all affected animals (Fig. 4IIH). Cone abnormalities were less severe, and a proportionally larger number of cones remained late in the disease.

In heterozygotes, the retinal functional abnormalities reflected the underlying photoreceptor pathology. Low-amplitude responses were present in young adult carriers of either genotype (Fig. 4IC–E), but progressive loss of function was found only in XLPRA2 (data not shown). Other than foci of patchy retinal degeneration, the remaining photoreceptors in XLPRA1 heterozygotes were normal (Fig. 4III). This was not the case for XLPRA2, since the carrier retina failed to develop normally, and surviving photoreceptors showed severe structural abnormalities (compare Fig. 4III with J and K). Such severe pathology in XLPRA2 carriers indicates that the mutation kills mutant photoreceptors early in development, and produces collateral damage in surviving normal ones. When this allele is expressed in the already compromised retina of a compound heterozygote, the disease is very severe (Fig. 4III).

Expression profile of RPGR splice variants and protein in normal and mutant retinas

To understand the disease mechanism resulting from exon ORF15 mutations, we have characterized RPGR alternative splicing in more detail, and examined its expression in normal and mutant retina using probes from different regions of the RPGR cDNA (Fig. 5).

Exon ORF 15 is transcribed with the RCC1-like domain and exon 14a. RPGR expression was examined using a normal retinal RNA sample loaded in separate lanes on the same gel, and hybridized with probes corresponding to different exons (Fig. 6A). Probes from exons of 3–10, 14a and ORF15 detected two novel common bands of 8.9 and 5.0 kb, suggesting that these exons are part of the same transcript, and that exon ORF15 is transcribed. We also demonstrate exon 14a expression in retina, and its transcription in frame with exon ORF15 (Fig. 6A).

Tissue expression profile reveals ORF15 as a retina-predominant terminal exon. Using probes for exons 3–10 or 16–19, we found, respectively, 3.2 and 2.8 kb bands in the northern blots (Fig. 6B). The 2.8 kb band is ubiquitously expressed in most tissues, but the 3.2 kb band appears to represent non-specific binding since it is not consistently found in repeat blotting of the same tissues. The 8.9 kb transcript, expressed predominantly in retina,
matches the expected sequence length (5284 bp) if exon ORF15 is used as a terminal exon.

XLPRA mutant retinas express RPGR transcripts. We examined expression in pre-degenerate (4–6 weeks of age) XLPRA retinas using probes for exons 3–10 and ORF15. At this age, the retinas show no cellular degeneration, although abnormalities of outer segment structure are present in XLPRA2. In addition to normals, we also used as controls retinas from animals of the same age that had non-allelic autosomal retinal diseases having comparable phenotypes (XLPRA1 and prcd, XLPRA2 and rcd1) (15). In affected and controls, the probes detected bands of 8.9 and 5 kb, and in XLPRA1 transcript abundance was the same as normal. Although a distinct message was found in XLPRA2, both probes found slightly lower levels of expression (Fig. 6C). These results indicate that the RPGR exon ORF15 is transcribed in mutant retinas.

We used in situ hybridization to examine the expression of the exon ORF15 retina predominant transcript. Comparable intense accumulation of silver grains was found in the normal and XLPRA1 affected retinas; tissues were not available from

Figure 4. ERG (I), pathology (II) and in situ hybridization (III) of normal and XLPRA. (I) Each panel shows the responses to scotopically balanced red and blue stimuli, white light, 5 Hz rod and 30 Hz cone stimuli. In XLPRA1, ERG develops normally (A1, 70 days) and remains normal until early adulthood. Amplitudes then decrease with age (A2, 1.3 years; A3, 2.5 years). XLPRA2 shows rod and cone responses that are reduced in amplitude or abnormal in waveform early (B1, 70 days; B2, 4 months). By 1 year (B3) the rod responses are nearly absent, but distinct cone signals with prolonged timing remain. XLPRA1 (D, 2.5 years) and XLPRA2 (E = 2 years) carriers retain normal ERG waveforms but lower amplitudes, especially in XLPRA2. C, normal control. Vertical lines or arrows, light stimulus onset; calibration marker, 100 µV and 50 ms except for rod flicker (100 ms). (II) In XLPRA1, retina and RPE are normal (A, 9.4 months) before developing outer segment (OS) abnormalities (B, 13 months). Degeneration occurs subsequently, but the rate and severity varies between affected animals (C, 15 months; D, 3 years). Remaining cones are prominent (oblique arrows). XLPRA2 retina develops abnormally; outer segments are disorganized and disoriented early (E, 1 month), and photoreceptors degenerate rapidly (F, 4 months; G, 7 months; H, 1.9 years). XLPRA1 carrier outside areas of patchy retinal degeneration shows a lower number of normal surviving photoreceptors (I, 5 years). XLPRA2 carrier (J, K, 1 year) shows patchy degeneration (J, horizontal bracket) and abnormally developed photoreceptors. The compound heterozygote (L, 9.7 months) shows advanced degeneration. Calibration bar, 50 µm; horizontal arrowhead, external limiting membrane. (III) Paired brightfield (A1, B1, C1, D1) and combined brightfield/epipolarization (A2, B2, C2, D2) images of normal (A, B, 10 months) and XLPRA1 retinas at 3.3 (C) and 11 (D) months hybridized with antisense (A, C, D) and sense (B) exon ORF15 probes (nucleotides 800–1451). Normal and mutant show the same pattern and intensity of hybridization. In peripheral retina of older mutant (D), label is present, but photoreceptor number is decreased. The wavy lines in panels C1,2 represent the photographic apposition of the RPE and retina. Calibration bar in D2, 26 µm; arrows, cone IS.
XLPRA2 animals. Label was found in the RPE, photoreceptor inner segments and the nuclear layers; cone inner segments showed dense clusters of label. No difference in label intensity or distribution was found between normal and XLPRA1-affected animals (compare Fig. 4IIIA1/2 with C1/2 and D1/2). In older affected animals, label intensity was reduced secondary to loss of visual cells, but the remaining cells showed normal label distribution (Fig. 4IIID1/2). The control sense probe showed very low and diffuse labeling in both the normal (Fig. 4IIIB1/2) and mutant (data not shown).

Western analysis. Antibody DR-39 raised against peptides of human RPGR exons 13 and 14 detected a 100 kDa band in the cytosol, but not in membrane fractions (Fig. 6D, top panel). We also found a 95 kDa major band and a smaller 90 kDa band in the membrane fraction with RPGR-254 antibody raised against the C-terminal 254 residues of RPGR (Fig. 6D, lower panel), but we could not identify any specific bands in the cytosolic fraction using the same antibody. However, no differences were found between control and mutant samples, an indication that the antibodies failed to detect protein(s) containing exon ORF15.

RPGR and other photoreceptor-specific proteins are normally expressed in mutant retina. We used different RPGR antibodies, and obtained the same results in normal and mutant retinas (Fig. 7). The polyclonal RPGR-33606 antibody intensely labeled the outer segments (Fig. 7A1,2) - results that are the same as previously reported (12). In contrast, antibody DR-39 intensely labeled the inner segments and the inner nuclear and ganglion cells (Fig. 7A3,4). The intensity and distribution of the labeling was the same in normal and mutant retina, even after photoreceptor degeneration had started (Fig. 7C3,4, D3,4). The three other antibodies (opsin, PDEγ and rod cGMP channel protein α subunit) produced the same labeling in normal and mutant (Fig. 7, columns 5–7), even in eyes that showed more disease-associated pathology. The normal labeling of the photoreceptor-specific proteins exam-

Figure 5. Schematic representation of the genomic organization of canine RPGR (A) not drawn to scale, and the location of the probes used to identify retinal expressed transcripts [B (18) and C (new transcript with ORF15 as a terminal exon)]. Note that brackets in (C) identify exons ORF 14 (exons 14 and 14a) and ORF15 (exon 15 and transcribed portion of intron 15) (B). An asterisk identifies the approximate position of the microdeletions associated with XLPRA1 and XLPRA2.

Figure 6. RPGR expression. (A) Antisense RNA probes from different exons of RPGR were hybridized to total retinal RNA (20 µg/lane). Probe locations are as follows: exons 3–10, nucleotides 258–1330; exons 16–19, nucleotides 1681–2374; exon14a, nucleotides 1842–2428; exon ORF15, nucleotides 803–1603. (B) RPGR expression in different tissues. A antisense probes of exons 3–10 (top) and exons 16–19 (middle) were hybridized to a dog tissue blot containing 20 µg total RNA/lane. Bottom panel shows the same blot hybridized with an 18S cDNA probe. (C) Expression of RPGR in normal (7.7 months), XLPRA1 and 2 and other non-allelic autosomal retinal diseases; affected samples were from pre-degenerate stages (4–6 weeks of age). Antisense RNA probes were hybridized to total retinal RNA (20 µg/lane) from normal and pre-degenerate retinas of XLPRA1, prcd, XLPRA2, and rcd1. The rcd1 and prcd samples serve as disease controls, and are from animals of the same age affected with non-allelic autosomal retinal diseases having comparable phenotype to XLPRA1 (prcd) and XLPRA2 (rcd1) (15). The bottom panel shows the hybridization of an 18S cDNA probe to the same blot. (D) Western blot of RPGR in retinal tissues of normal and affected dogs. Proteins (50 µg total protein/lane) from cytosol (top) and membrane (bottom) fractions were labeled with DR-39 and RPGR-254 antibodies, respectively. Sample source and age are listed for each lane.
Figure 7. Expression of photoreceptor proteins in normal (A), XL-PRA1 (B, before disease stage; C, early disease), and XL-PRA2 (D) dogs of the following ages: A, 9.6 months; B, 3.3 months; C, 11.3 months; D, 7 weeks (columns 1, 2, 5–7); 16 weeks (columns 3, 4). (A–C) are from 2μm DGD sections, (D) from 10μm cryosections. Columns 1, 2 and 3, 4 are paired Nomarski (1,3) and immunofluorescence (2,4) images using RPGR-33606 and DR-39 antibodies. Other columns represent single images of sections labeled with antibodies against opsin, PDEγ and rod cGMP channel protein α subunit. The same pattern and intensity of labeling is found in normal and mutant retinas. Calibration bar, 50μm.

Figure 8. Transient expression of different GFP–exon ORF15 constructs in COS7 cells. There is similar distribution in cells expressing vector (A), wild-type (B) or XL-PRA1 GFP–exon ORF15 (C) constructs. However, the XL-PRA2 construct results in GFP fluorescence that aggregates around the nucleus (D). The XL-PRA2 GFP–exon ORF15 protein aggregates (E1) and ER (E2) co-localize (E3).
ined indicates that their polarized distribution in the mutant visual cells is not altered.

**XLPRA2 mutant proteins aggregate in the ER**

We examined the subcellular localization of normal and mutant exon ORF15/GFP–fusion proteins using transfected COS7 cells. Signal from normal protein was mainly cytoplasmic, with some diffuse, faint nuclear presence (Fig. 8B); similar results were obtained following transfection with GFP vector alone (Fig. 8A) and with the exon ORF15 nonsense mutation (XLPRA1; Fig. 8C). In contrast, the frameshift mutation (XLPRA2) showed intense accumulation of protein aggregates in a distinct perinuclear subcellular compartment (Fig. 8D and E1). There was some faint, diffuse signal in the rest of the cytoplasm, but minimal signal in the nucleus. Staining with an endoplasmic reticular (ER)-specific marker confirms the co-localization of the aggregates and ER (Fig. 8E1–3).

**DISCUSSION**

Physical mapping of the XLPRA interval shows strong homology of this region of Xp between humans and dogs in terms of genes and their order. We mapped two distinct X-linked retinal disorders to this region, and found disease-associated mutations in RPGR exon ORF15. The specificity of the mutations is confirmed by finding no other sequence abnormalities in the characterized RPGR transcripts (18 and present study). Additionally, we scanned for the presence of the mutations in 137 dogs (211 chromosomes) of 20 different breeds, and found deletions only in XLPRA1 (Siberian husky and Samoyed) or XLPRA2 (mongrel-derived) affected or carrier dogs, thus confirming that the mutations are not polymorphisms. We also scanned 20 wolves (29 chromosomes; 19 red breeds, and found deletions only in XLPRA1 (Siberian husky and black wolf), and only found the deletion in two aged heterozygous red wolves that were asymptomatic. Based on the lack of a retinal disease phenotype in these females, the deletion is likely to be a rare, benign polymorphism (8).

A ‘hotspot’ for RPGR mutations in humans occurs in an approximately 1 kb purine-rich region of exon ORF15, in which out-of-frame mutations and premature stop are associated with disease. Of the 34 novel RP3 mutations reported recently, 23 were one-, two-, four- and five-nucleotide deletions that resulted in a frameshift with premature termination of translation (8). It was proposed that the purine-rich region may adopt unusual non-B-DNA conformations and show sequence motifs similar to DNA polymerase-α arrest sites found near other deletion hotspots; this can lead to microdeletions following slipped-strand mispairing events (21,22). We have found similar microdeletions in the present study, suggesting that RPGR exon ORF15 appears to be a mutation hotspot in canids.

Our studies demonstrate that the nature of the RPGR exon ORF15 deletions determine the retinal phenotype. Both disorders are distinct, and have remained so even though the diseases now are present in mixed-breed dogs of the same genetic background. This indicates that the phenotype results from differences between the two mutant proteins in vivo. The normal retinal development in XLPRA1 argues that the C-terminal 230 amino acids encoded by exon ORF15 are not vital for functional and structural differentiation of rods and cones. The same argument has been proposed for the role of the RCC1-like domain since photoreceptor development is normal in the Rprg knockout mouse (9). However, the severe disease that develops in young adult animals indicates that RPGR plays an essential role in photoreceptor viability. In contrast, the abnormal retinal development that occurs in XLPRA2, and the rapid degeneration that ensues, suggests that the mutation disrupts the critical differentiation of rods and cones, and causes their early degeneration.

Analysis of the heterozygous retinas provides insights into the role of the mutant alleles in retinal development and maintenance. Through random X-inactivation (23), approximately 50% of the retinal cells express the mutant or normal RPGR, and the expression of the mutant gene can be examined in the milieu where half of the photoreceptors are genetically normal. Carriers of either disease show ophthalmoscopically visible patchy retinal degeneration – an indication of mosaicism, the hallmark of retinal random X-inactivation (24). These changes, which are variable in extent and severity, are evident in most carriers by the time they are young adults, and in all by the time they are advanced in age. These abnormalities are stationary in XLPRA1, but some XLPRA2 carriers often show progressive disease that results in advanced retinal degeneration by 5–6 years of age or older. In contrast, lack of any disease in aged heterozygous red wolves suggests that the three-nucleotide deletion is a benign polymorphism.

The severe retinal disease phenotype in canine XLPRA is comparable to what has been reported for human patients with RPGR mutations, given the caveat that, at least in humans, only a limited number of phenotype-genotype correlation studies have been reported. With the exception of two reports that, retrospectively, represent mutations in exon ORF15 (25,26), all others have been limited primarily to exons 1–10 (e.g. 4,27). These studies show comparably severe clinical phenotypes in XLRP patients to those found in the two canine models. Also, the majority of carriers show clinical disease varying from mild to severe [impairment of night vision or visual field loss (3,24)]. In some families, the disease was sufficiently severe in females to be considered semidominant (28). Examination of exon ORF15 in these has identified null alleles in four of nine families (J.-M. Rozet, personal communication, 26 July 2001).

Expression studies of RPGR have shown that alternative splicing is complex, and species-specific variants may exist. The splice variants reported for RPGR are mainly after exon 13, especially in the brain and testes (18,25). Additionally, alternative exon usage of introns has been found frequently for RPGR transcripts; for example exon 14a (intron 14) in mouse (25) and dog (18), exons 15a, 15b1, 15b2 and ORF15 (intron 15) in human, mouse, cow and dog (8 and present study), and exon 19a in mouse (intron 18 (25)). The alternative splicing and exon usage suggests that different RPGR protein isoforms are produced for specific functions in vivo. The exon 16–19 probe mainly detected an 8.9 kb band, but failed to detect the 5 kb band that was recognized with probes for exons 3–10, exon 14a and ORF15. This result suggests that exon ORF15 can be used as a terminal exon (5 kb band), or alternatively spliced to exons 16–19 (8.9 kb band). We have found a putative consensus splice donor site, conserved between human and dog, located before the stop codon in the
that are of importance to humans and other species. Analysis, to aid in understanding gene function and diseases model, and the tools available for comparative genomic human RP3 families. Finally, we show the value of the canine mutations in this novel exon, a situation that is similar in indicate that very different retinal phenotypes can result from exon ORF15 in photoreceptor function and viability. They during development, but is essential later for their sustained viabiliy. In contrast, the XLPRA2 mutation affects photo-

The results of our studies highlight the importance of RPGR exon ORF15 in photoreceptor function and viability. They indicate that very different retinal phenotypes can result from mutations in this novel exon, a situation that is similar in human RP3 families. Finally, we show the value of the canine model, and the tools available for comparative genomic analysis, to aid in understanding gene function and diseases that are of importance to humans and other species.

METHODS

Animals, pedigrees and tissue collection

Two different outcrossed pedigrees were used to develop a meiotic map of canine Xp, fine map XLPRA1 and XLPRA2, and identify disease-causing mutations in RPGR. All procedures involving tissue collection were done under anesthesia or immediately following euthanasia with a barbiturate overdose. Blood samples from red wolves were collected under sedation after ophthalmic examination; these wolves were maintained for display or breeding in a zoo or nature center. We also examined and tested members of a pedigree of Samoyed dogs that had an X-linked retinal degeneration clinically identical to XLPRA1.

Canine BAC library screening, meiotic, radiation hybrid (RH) and physical mapping

The RPCI81 Canine BAC library was used (http://www.chori.org/bacpac/mcanine81.htm). Canine-specific probes of TCTE1L, SRPX, RPGR and OTC were prepared by PCR using degenerate primers, and the purified BAC DNA digested to identify microsatellite markers by hybridization. The experimental details for meiotic and RH mapping have been described (19). The contig was built with BAC clones positive for TCTE1L, SRPX, RPGR, and OTC and extended by end sequencing. The size of BAC clones was determined using NotI digestion, fingerprinting with EcoRV and separation by pulsed-field gel electrophoresis. The BLAST search of end and internal sequences for exons and conserved regions between human and dog was also used for contig assembly.

Cloning of canine RPGR intron 15, exon ORF15 and mutation identification

The intron 15 sequence was amplified by long-range PCR (forward primer RGF9 from intron 14, 5'-GAAAGTAAG-CATGGTCTCTTTACC-3', reverse primer RGR1 from exon 16, 5'-CACAGTTCTCTCATCTCTCTCTG-3'). The 10 kb PCR product was cloned into a TOPO-XL vector (Invitrogen, Carlsbad, CA). For mutation analysis, flanking primers were designed from normal sequence, and used to scan the mutation by PCR under the conditions of 98°C for 2 min and then 34 cycles of 98°C for 30 s, 62°C for 30 s and 72°C for 1 min with the primer pairs listed below, and the PCR products were separated on 8% polyacrylamide gel:

\[
\text{XLPRA1:} \quad \text{forward primer RGF14, 5'-AAGGGAGGAAAGGG-GAGGCT-3'} \\
\text{reverse primer RGR13, 5'-TCCCTCTTCTTCTCTCCCCTT-CTCA-3'}
\]

\[
\text{XLPRA2:} \quad \text{forward primer RGF14, 5'-TCCCTACTTCTCTTCTCCCCT-CTCA-3'}
\]

Sequencing was done by Taq cycle sequencing using DyeDeoxy terminators in an Applied Biosystems ABI 377 automated DNA sequencer.

Electroretinography (ERG) and morphology studies

ERGs were recorded from isoflurane-anesthetized dogs to stimuli and under conditions designed to enable separate evaluation of rod and cone-mediated responses (30); the preparation of eyecups for morphological examination utilized a triple fixation protocol (30). One-micrometer sections of plastic-embedded retina extending from the optic disc to the superior and inferior periphery were used for analysis.
Immunocytochemistry and in situ hybridization

Immunocytochemistry was done using normal and affected retinas collected in the light, fixed in 4% paraformaldehyde, and processed further in DGD (control and XLPR1 cut at 2 μm) (31) or OCT (control and XLPR2 cut at 10 μm) using standard methods. Different antibodies to RPGR and other retinal proteins were used, and are identified by target, source or reference. For RPGR: (a) polyclonal RPGR-254; C-terminal 254 amino acids of RPGR (partial exons 14, exons 15–19 (9)); (b) polyclonal RPGR-33606; human RGC1-like domain [residues 96–116 coded by partial exon 4 and 5 (12)]; (c) polyclonal DR-39, which targets 13 amino acids coded by human RPGR exons 13 and 14 (F. Manson). For Opsi: monoclonal R2-12N (32). For PDEγ: polyclonal anti-mouse (31). For rod cGMP channel protein α subunit: monoclonal anti-bovine (33). With the exception of the polyclonal RPGR-33606 antibody, all RPGR antibodies required an antigen retrieval step (heating the slides in 0.01 M citric acid for 10 min in a microwave at 70% power). Antibodies directed against exon ORF15 [polyclonal antibodies against synthetic peptides from human (nos 1877 and 1878) or mouse (nos 1875 and 1876) (F. Manson)] were not used because of lack of specificity (data not shown).

For in situ hybridization, paraformaldehyde-fixed/DGD-embedded sections (2 μm) were hybridized with antisense or sense RNA probes prepared from linearized exon ORF15 plasmid DNA. The slides were coated in Kodak NTB2 emulsion, exposed for 1–4 weeks at 4°C, developed, and stained with toluidine blue. Probes were synthesized using the MaxiScript kit (Ambion, Austin, TX) and labeled with [35S]UTP (1250 Ci/mmol; NEN Life Science).

Western and northern blots

Western analysis of RPGR in cytosolic and membrane fractions was done using antibodies RPGR-254 and DR-39. The procedure has been described previously (34,35). Retinal tissues were homogenized and centrifuged to separate cytosolic and membrane fractions; the crude pellets containing cell nuclei and membranes were sonicated and centrifuged. The proteins (50 μg/lane) were separated by 10% SDS-PAGE, and the bands in a nylon membrane (Imobilon) were visualized using an enhanced chemiluminescence western blotting detection kit (Amersham). For rod cGMP channel protein α subunit: monoclonal antibodies against synthetic peptides from human (nos 1877 and 1878) or mouse (nos 1875 and 1876) (F. Manson) were not used because of lack of specificity (data not shown).

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