MUTATION UPDATE

CRB1 Mutation Spectrum in Inherited Retinal Dystrophies

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Mutations in the Crumbs homologue 1 (*CRB1*) gene have been reported in patients with a variety of autosomal recessive retinal dystrophies, including retinitis pigmentosa (RP) with preserved paraarteriolar retinal pigment epithelium (PPRPE), RP with Coats-like exudative vasculopathy, early onset RP without PPRPE, and Leber congenital amaurosis (LCA). We extended our investigations of *CRB1* in these retinal dystrophies, and identified nine novel *CRB1* sequence variants. In addition, we screened patients with "classic" RP and classic Coats disease (without RP), but no pathologic sequence variants were found in the *CRB1* gene. In total, 71 different sequence variants have been identified on 184 *CRB1* alleles of patients with retinal dystrophies, including amino acid substitutions, frameshift, nonsense, and splice site mutations, in-frame deletions, and large insertions. Recent studies in two animal models, mouse and *Drosophila*, and in vivo high-resolution microscopy in patients with LCA, have shed light on the role of CRB1 in the pathogenesis of retinal dystrophies and its function in the photoreceptors. In this article, we provide an overview of the currently known *CRB1* sequence variants, predict their effect, and propose a genotype–phenotype correlation model for *CRB1* mutations. Hum Mutat 24:355–369, 2004. © **2004 Wiley-Liss, Inc.**

KEY WORDS: CRB1; Crumbs; Leber congenital amaurosis; LCA; retinitis pigmentosa; RP; Coats disease; genotype–phenotype

DATABASES:
CRB1 – OMIM: 604210, 600105 (RP12), 204000 (LCA); GenBank: AY043324.1 (*Homo sapiens*, isoform I), AY043325.1 (*Homo sapiens*, isoform II), NT_004671.15 (*Homo sapiens*, genomic sequence), AF406641.1 (*Mus musculus*), U42839.2 (*Caenorhabditis elegans*), M33753.1 (*Drosophilia melanogaster*)
www.sph.uth.tmc.edu/Retnet (RetNet Retinal Information Network)

INTRODUCTION

The Crumbs homologue 1 (*CRB1*) gene (MIM# 604210) maps to chromosome 1q31.3, and contains 12 exons, encompassing 210 kb of genomic DNA. The gene exhibits alternative splicing at its 3′ end [den Hollander et al., 2001b]. The alternative splice variants are predicted to encode either a 1,376-aa extracellular protein (AY043324.1), or a 1,406-aa transmembrane protein with a 37-amino acid cytoplasmic domain (AY043325.1). Both proteins contain a signal peptide, 19 EGF-like domains, and three laminin A G-like domains. Multiple *Crb1* splice variants and two novel alternative exons have been detected in mouse [Mehalow et al., 2003; Watanabe et al., 2004]. *CRB1* expression was found to be restricted to retina and brain [den Hollander et al., 1999, 2002], although some reports describe expression in other tissues, such as kidney, colon, stomach, lung, and testis [Roh et al., 2002; Watanabe et al., 2004]. An alternative splice variant of mouse *Crb1* encoding a C-terminally

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truncated secreted protein (*Crb1s*) is expressed in a wider range of tissues and during skin development [Watanabe et al., 2004].

Mutations in the *CRB1* gene lead to severe retinal dystrophies. *CRB1* mutations have been found in patients with retinitis pigmentosa (RP) type 12 [den Hollander et al., 1999; Bernal et al., 2003; Khaliq et al., 2003], a specific form of RP characterized by a preserved paraarteriolar retinal pigment epithelium (PPRPE), an early onset and progressive loss of the visual field, optic nerve head drusen, vascular sheathing, nystagmus, and hyperopia (MIM# 600105) [Heckenlively, 1982; van den Born et al., 1994]. *CRB1* mutations have also been detected in patients with early onset RP without PPRPE but with other RP12 characteristics [Lotery et al., 2001b; Bernal et al., 2003], and in RP patients who had developed Coats-like exudative vasculopathy, a relatively rare complication of RP characterized by vascular abnormalities (retinal telangiectasia and choroid to retina anastomoses), yellow extravascular lipid depositions, and in severe cases retinal detachment [den Hollander et al., 2001a]. In addition, mutations in the *CRB1* gene have been detected in 10 to 13% of patients with Leber congenital amaurosis (LCA), the most severe retinal dystrophy leading to blindness or severe visual impairment in the first year of life (MIM# 204000) [den Hollander et al., 2001a; Lotery et al., 2001a; Hanein et al., 2004].

Using in vivo high-resolution microscopy, it was shown that the retinas of patients with *CRB1* mutations, in contrast to other inherited retinal degenerations, are remarkably thick in cross-section and lack the distinct layers of normal adult retina. The abnormal retinal architecture resembles that of immature normal retina, and it has been suggested that disruption of CRB1 function disturbs the development of normal human retinal organization by interrupting naturally occurring apoptosis [Jacobson et al., 2003]. In contrast, the retinal architecture of the retinal degeneration 8 (*rd8*) mouse, carrying a homozygous 1-bp deletion in exon 9 of *Crb1*, is in general normal [Mehalow et al., 2003]. Unlike other models in which photoreceptor degeneration occurs throughout the retina, degeneration in *rd8* mice is focal. The inferior nasal quadrant of the fundus exhibits large, irregularly shaped spots, which correspond to regions with retinal folds and pseudorosettes that involve the photoreceptors. In these regions, the photoreceptors have shortened inner and outer segments shortly after birth, and the outer segments are lost during the first months of life [Mehalow et al., 2003]. In the normal mouse retina, the CRB1 protein localizes to the outer limiting membrane, which is composed of complexes of adherens junctions between photoreceptors and Müller cells [Pellikka et al., 2002; Mehalow et al., 2003]. In the *rd8* mouse, the outer limiting membrane is fragmented throughout the retina, even in areas that are not affected by folds and pseudorosettes. Consequently, the outer limiting membrane looses its barrier function, leading to a disorganization of the photoreceptor cells in some regions of the retina [Mehalow et al., 2003]. The phenotypic differences between the *rd8* mouse and patients with *CRB1* mutations may be caused by species differences, which has also been reported for other mouse models of inherited retinal dystrophies [Mehalow et al., 2003].

CRB1 is homologous to *Drosophila* Crumbs (Crb) protein, an important determinant of apicobasal polarity in epithelial cells and crucial for the assembly of the zonula adherens [Tepass et al., 1990; Tepass, 1996]. Recently, it was established that Crb is also essential for proper morphogenesis of the photoreceptor cells in *Drosophila* [Izaddoost et al., 2002; Pellikka et al., 2002]. The defects caused by *Crb* mutations in *Drosophila* photoreceptors are similar to those seen in the *rd8* mouse [Mehalow et al., 2003]. The rhabdomeres, equivalent to mammalian outer segments, are shortened and the zonula adherens is fragmented [Izaddoost et al., 2002; Pellikka et al., 2002]. Interestingly, massive photoreceptor degeneration is seen when flies with *Crb* mutations are subjected to constant light exposure [Johnson et al., 2002]. It has been speculated that RP patients with *CRB1* mutations may benefit from reduced amounts and/or intensities of daylight [Johnson et al., 2002].

In this work, we extended *CRB1* mutation analysis in patients with RP with PPRPE, RP with Coats-like exudative vasculopathy, and LCA, and in addition screened 93 patients with autosomal recessive or isolated "classic" RP and 18 patients with classic Coats disease (without RP). We provide an overview of the currently known *CRB1* sequence variants, predict their effect, and propose a genotype–phenotype correlation model for *CRB1* mutations.

CRB1 MUTATIONS IN RP WITH PPRPE AND/OR COATS-LIKE EXUDATIVE VASCULOPATHY

CRB1 mutations have been identified in 10 out of 15 unrelated patients (Table 1) [den Hollander et al., 1999] and in two families with RP and PPRPE [Khaliq et al., 2003]. In addition, *CRB1* mutations were found in 5 out of 9 patients with RP and Coats-like exudative vasculopathy (Table 1) [den Hollander et al., 2001a]. *CRB1* mutations were also identified in two families with RP but without PPRPE [Lotery et al., 2001b]; however the affected individuals did exhibit other features of RP12, such as early disease onset, optic nerve head drusen, yellow spots in the posterior pole, vascular sheathing, and nystagmus. Mutation screening of 92 autosomal recessive RP families from Spain revealed *CRB1* mutations in six families (Table 1) [Bernal et al., 2003]. PPRPE was seen in one of these families, and affected individuals in the remaining families exhibited other characteristics of RP12, such as early disease onset and hyperopia.

In this study, we screened 12 additional patients with RP and PPRPE and seven additional patients with RP and Coats-like exudative vasculopathy for mutations in the *CRB1* gene by sequence analysis. In the patients with RP and PPRPE, we identified sequence variants on both

TABLE 1. Results of Mutation Analysis of the *CRBl* Gene in Various Patient Groups

Patient group	Patients with 2 *CRBl* alleles	Patients with 1 *CRBl* allele	Patients with no *CRBl* mutations	Total	Reference
RP+PPRPE	10 (67%)	0 (0%)	5 (33%)	15	den Hollander et al. [1999]
RP+PPRPE	8 (67%)	2 (17%)	2 (17%)	12	This study
RP+Coats	4 (44%)	1 (11%)	4 (44%)	9	den Hollander et al. [2001a]
RP+Coats	1 (14%)	1 (14%)	5 (71%)	7	This study
RP	4 (4%)[a]	2 (2%)[a]	86 (93%)	92	Bernal et al. [2003]
RP	0 (0%)	0 (0%)	93 (100%)	93	This study
LCA	6 (11%)	1 (2%)	45 (87%)	52	Den Hollander et al. [2001a]
LCA	6 (3%)	15 (8%)	169 (89%)	190	Lotery et al. [2001a]
LCA	18 (10%)	0 (0%)	161 (90%)	179	Hanein et al. [2004]
LCA	0 (0%)	0 (0%)	44 (100%)	44	This study
Classic Coats disease	0 (0%)	0 (0%)	18 (100%)	18	This study

[a]Patients in one family exhibited PPRPE, and patients in the remaining families had other characteristics of RP with PPRPE, such as early onset and/or hyperopia.

CRB1 alleles in eight patients, a sequence variant on one *CRB1* allele in two patients, and no sequence variants in two patients (Tables 1 and 2). Segregation of *CRB1* sequence variants was confirmed in family members of three probands (Patients 12723, 17679, 17964; data not shown). Six novel *CRB1* sequence variants (c.584G>T (p.C195F), c.2506C>A (p.P836T), c.2548G>A (p.G850S), c.2957A>T (p.N986I), c.3427delT (p.C1143fsX66), and c.4148G>A (p.R1383H)) were not found in 372 chromosomes of ethnically matched control individuals (Table 3).

In the patients with RP and Coats-like exudative vasculopathy, we identified sequence variants on both *CRB1* alleles in one patient, a sequence variant on one *CRB1* allele in another patient, and no sequence variants in five patients (Tables 1 and 2). Three novel *CRB1* amino acid substitutions (c.1733T>A (p.V578E), c.1760G>A (p.C587Y), and c.2875G>A (p.G959S)) were not found in 372 control chromosomes. In two patients (Patients 18803 and 18858) we identified a novel sequence variant in intron 6 (c.2128+15A>C, Table 4).

In total, we identified *CRB1* sequence variants in 20 out of 27 RP patients with PPRPE and 7 out of 16 RP patients with Coats-like exudates (Table 1). RP with PPRPE and RP with Coats-like exudative vasculopathy are partly overlapping clinical entities, since patients who have RP with PPRPE have a higher-than-average incidence of Coats-like changes [van den Born et al., 1994]. In 5 out of 7 patients with RP and Coats-like exudative vasculopathy, RP12 characteristics were present, such as PPRPE and early onset of the disease. However, two patients were clearly distinct from RP12 (I. van den Born, A. den Hollender, F. Cremers, unpublished results) [den Hollander et al., 2001a].

In two RP patients with PPRPE, and two patients with RP and Coats-like exudates, a sequence variant on only one *CRB1* allele was identified (Tables 1 and 2). The second *CRB1* sequence variant in these patients may reside in intronic or regulatory sequences that were not analyzed, or may represent a heterozygous deletion of one or more exons, which is missed in PCR-based mutation analysis. Another possibility is that the disease in these patients is caused by digenic inheritance, which has been described for other retinal dystrophies [Kajiwara et al., 1994; Katsanis et al., 2001].

No *CRB1* sequence variants were identified in 7 out of 27 patients with RP and PPRPE, and 9 out of 16 patients with RP and Coats-like exudates (Table 1), suggesting that the underlying *CRB1* mutations were missed by PCR-based mutation analysis, or that these specific forms of RP are genetically heterogeneous.

CRB1 MUTATIONS IN AUTOSOMAL RECESSIVE AND ISOLATED "CLASSIC" RP

To determine the frequency of *CRB1* mutations in autosomal recessive and isolated "classic" RP, we screened the *CRB1* gene in 93 unrelated patients by single-strand conformation polymorphism (SSCP) analysis and subsequent sequencing of shifted bands. We identified sequence variants in two probands (Patients 9402 and 14155). Patient 14155 has a single nucleotide substitution (c.2307C>T) that does not change the amino acid (p.R769R) (Table 4).

Patient 9402 carries a heterozygous single nucleotide substitution (c.614T>C), leading to a nonconservative amino acid change (p.I205T). Sequence analysis of all protein coding exons, the flanking splice sites, and 800 bp of the putative promoter sequence did not reveal a second *CRB1* allele that carried a mutation. This sequence variant was not identified in 372 control chromosomes, but was also not identified in the affected sister of the patient, suggesting that it is a rare, nonpathogenic sequence variant (Table 4). The same sequence variant was recently detected in a Spanish RP family, in which it segregated with the disease phenotype, but no second *CRB1* sequence variant was identified in this family either [Bernal et al., 2003].

In conclusion, we did not detect any pathogenic sequence variants in the *CRB1* gene of 93 probands with autosomal recessive or isolated "classic" RP. In contrast, we identified *CRB1* sequence variants in 20 out of 27 RP patients with PPRPE and 7 out of 16 RP patients with Coats-like exudative vasculopathy (Table 1); other publications have described *CRB1* mutations in RP

TABLE 2. *CRBI* Sequence Variants in Patients With RP With PPRPE, Early Onset RP Without PPRPE, RP With Coats–Like Exudative Vasculopathy, and Leber Congenital Amaurosis

Disease and patient number[a]	Allele 1[b]		Allele 2[b]		Reference
RP with PPRPE					
25983	c.482C>T	p.A161V	c.482C>T	p.A161V	den Hollander et al. [1999]
17679	c.584G>T	p.C195F	c.2843G>A	p.C948Y	This study
RP112	c.750T>G	p.C250W	c.750T>G	p.C250W	den Hollander et al. [1999]
24228	**c.1208C>G**	**p.S403X**	c.2290C>T	p.R764C	den Hollander et al. [1999]
25977	**c.2185_2186ins*Alu*[c]**	**Unknown**	**c.2185_2186ins*Alu*[c]**	**Unknown**	den Hollander et al. [1999]
24868	c.2234C>T	p.T745M	c.2234C>T	p.T745M	den Hollander et al. [1999]
13080	c.2234C>T	p.T745M	c.2234C>T	p.T745M	This study
25540	c.2234C>T	p.T745M	c.2843G>A	p.C948Y	den Hollander et al. [1999]
12723	c.2234C>T	p.T745M	c.2843G>A	p.C948Y	This study
14489	c.2234C>T	p.T745M	c.2843G>A	p.C948Y	This study
M–641	c.2245_2247delTCA	p.S749del	c.2843G>A	p.C948Y	Bernal et al. [2003]
13066	c.2290C>T	p.R764C	**c.2401A>T**	**p.K801X**	This study
26023	c.2290C>T	p.R764C	**c.2983G>T**	**p.E995X**	den Hollander et al. [1999]
18803	c.2506C>A	p.P836T	–	–	This study
3330RP	c.2536G>A	p.G846R	c.2536G>A	p.G846R	Khaliq et al. [2003]
15278	c.2548G>A	p.G850S	c.2843G>A	p.C948Y	This study
25710	c.2842+5G>A	Splice defect	c.2843G>A	p.C948Y	den Hollander et al. [1999]
17964	c.2957A>T	p.N986I	**c.3427delT**	**p.C1143fsX66**	This study
22147	c.3122T>C	p.M1041T	c.3122T>C	p.M1041T	den Hollander et al. [1999]
RP0136	c.3212T>C	p.L1071P	c.3212T>C	p.L1071P	den Hollander et al. [1999]
111RP	c.3212T>C	p.L1071P	c.3212T>C	p.L1071P	Khaliq et al. [2003]
15850	c.3299T>C	p.I1100T	c.3299T>C	p.I1100T	This study
15849	c.4148G>A	p.R1383H	–	–	This study
Early onset RP without PPRPE					
M–717	**c.481dupG**	**p.A161fsX7**	**c.481dupG**	**p.A161fsX7**	Bernal et al. [2003]
B–102	c.2671T>G	p.C891G	c.3299T>C	p.I1100T	Bemal et al. [2003]
M–69 (2 patients)	c.2843G>A	p.C948Y	c.2843G>A	p.C948Y	Bernal et al. [2003]
M–69 (1 patient)	c.2843G>A	p.C948Y	c.3299T>C	p.I1100T	Bernal et al. [2003]
B–15	c.2884_2886delTTA	p.L962del	–	–	Bemal et al. [2003]
DRP–2	**c.3343_3352del**	**p.G1115fsX22**	**c.3343_3352del**	**p.G1115fsX22**	Lotery et al. [2001b]
DRP–1	c.3961T>A	p.C1321S	c.3961T>A	p.C1321S	Lotery et al. [2001b]
RP with coats					
9439	**c.1208C>G; c.1298A>G**	**p.S403X; p.Y433C**	c.2290C>T	p.R764C	den Hollander et al. [2001a]
17658	**c.2401A>T**	**p.K801X**	c.3541T>C	p.C1181R	den Hollander et al. [2001a]
16937	c.2509G>C; c.4060G>A	p.D837H; p.A1354T	c.2843G>A	p.C948Y	den Hollander et al. [2001a]
16894	c.2681A>G	p.N894S	–	–	den Hollander et al. [2001a]
16968	c.2842+5G>A	Splice defect	c.2843G>A	p.C948Y	den Hollander et al. [2001a]
17659	c.2875G>A	p.G959S	–	–	This study
18858	c.1733T>A	p.V578E	c.1760G>A	p.C587Y	This study
Leber congenital amaurosis					
–	**c.111delT**	**p.S38fsX32**	–	–	Lotery et al. [2001a]
7/F/29	**c.257_258dupTG**	**p.N87fsX0**	–	–	Jacobson et al. [2003]
1	**c.428_432delGATTC**	**p.R143fsXI**	–	–	Lotery et al. [2001a]
2	c.430T>G	p.F144V	–	–	Lotery et al. [2001a]
3	**c.613_619del**	**p.I205fsX12**	c.1438T>C	p.C480R	Lotery et al. [2001a]
16690	**c.613_619del**	**p.I205fsX12**	**c.2401A>T**	**p.K801X**	den Hollander et al. [2001a]

187 S	**c.613_619del**	**p.I205fsX12**	c.2843G>A	p.C948Y	Hanein et al. [2004]
6	**c.613_619del**	**p.I205fsX12**	–	–	Lotery et al. [2001a]
5	c.1148G>A	p.C383Y	**c.2611_2613insT**[d]	**Frameshift**	Lotery et al. [2001a]
–	c.1438T>G	p.C480G	c.1438T>G	p.C480G	Lotery et al. [2001a]
200 S	c.1750G>T	p.D584Y	c.1750G>T	p.D584Y	Hanein et al. [2004]
7	c.2042G>A	p.C681Y	–	–	Lotery et al. [2001a]
2 F	c.2128G>C	p.E710Q	c.2128G>C	p.E710Q	Hanein et al. [2004]
2.2 F	c.2128G>C	p.E710Q	c.3320T>G	p.L1107R	Hanein et al. [2004]
131 S	c.2222T>C	p.M741T	**c.3988delG**	**p.E1330fsX10**	Hanein et al. [2004]
161 S	c.2234C>T	p.T745M	c.3074G>T	p.S1025I	Hanein et al. [2004]
6/M/26	c.2245_2247delTCA	p.S749del	c.2245_2247delTCA	p.S749del	Jacobson et al. [2003]
206 S	c.2290C>T	p.R764C	**c.2479G>T**	**p.G827X**	Hanein et al. [2004]
9	c.2290C>T	p.R764C	c.2843G>A	p.C948Y	Lotery et al. [2001a]
8	c.2290C>T	p.R764C	–	–	Lotery et al. [2001a]
4/F/18[e]	c.2290C>T	p.R764C	–	–	Jacobson et al. [2003]
5/F/24	c.2290C>T	p.R764C	–	–	Jacobson et al. [2003]
12862	**c.2401A>T**	**p.K801X**	**c.2401A>T**	**p.K801X**	den Hollander et al. [2001a]
8/M/50	**c.2401A>T**	**p.K801X**	–	–	Jacobson et al. [2003]
–	**c.2438_2439ins>100A**[f]	**Unknown**	–	–	Lotery et al. [2001a]
10	**c.2548_2551delGGCT**	**p.G850fsX4**	–	–	Lotery et al. [2001a]
163 S	c.2555T>C	p.I852T	**c.4005+1G>A**	**Splice defect**	Hanein et al. [2004]
42 S	**c.2688T>A**	**p.C896X**	**c.2688T>A**	**p.C896X**	Hanein et al. [2004]
15	c.2843G>A	p.C948Y	**c.257_258dupTG**	**p.N87fsX0**	Lotery et al. [2001a]
12831	c.2843G>A	p.C948Y	c.2843G>A	p.C948Y	den Hollander et al. [2001a]
16507	c.2843G>A	p.C948Y	c.2843G>A	p.C948Y	den Hollander et al. [2001a]
16	c.2843G>A	p.C948Y	c.2843G>A	p.C948Y	Lotery et al. [2001a]
I/F/2	c.2843G>A	p.C948Y	c.2843G>A	p.C948Y	Jacobson et al. [2003]
41 S	c.2843G>A	p.C948Y	c.2843G>A	p.C948Y	Hanein et al. [2004]
3/F/14[e]	c.2843G>A	p.C948Y	c.3653G>T	p.C1218F	Jacobson et al. [2003]
54.1 S	c.2843G>A	p.C948Y	c.3961T>A	p.C1321S	Hanein et al. [2004]
11	c.2843G>A	p.C948Y	–	–	Lotery et al. [2001a]
12	c.2843G>A	p.C948Y	–	–	Lotery et al. [2001a]
13	c2843G>A	p.C948Y	–	–	Lotery et al. [2001a]
14	c.2843G>A	p.C948Y	–	–	Lotery et al. [2001a]
2/M/12	c.2843G>A	p.C948Y	–	–	Jacobson et al. [2003]
124 S	**c.2853dupT**	**p.A952fsX3**	**c.2853dupT**	**p.A952fsX3**	Hanein et al. [2004]
010LCA	c.2966T>C	p.I989T	c.2966T>C	p.I989T	Khaliq et al. [2003]
164 S	c.3074G>T	p.S1025I	c.3320T>C	p.L1107P	Hanein et al. [2004]
12859	c.3299T>G	p.I1100R	**c.3331G>T**	**p.E1333X**	den Hollander et al. [2001a]
154 S	c.3307G>A	p.G1103R	**c.3347delT**	**p.F1116fsX24**	Hanein et al. [2004]
2.1 F	c.3320T>G	p.L1107R	c.3320T>G	p.L1107R	Hanein et al. [2004]
14 S	c.3320T>G	p.L1107R	c.3320T>G	p.L1107R	Hanein et al. [2004]
13067	**c.3331G>T**	**p.E1111X**	**c.3878+1G>T**	**Splice defect**	den Hollander et al. [2001a]
17	c.3613G>A	p.G1205R	–	–	Lotery et al. [2001a]
202 S	**c.3879G>A**	**p.W1293X**	**c.3879G>A**	**p.W1293X**	Hanein et al. [2004]
18	c.3949A>C	p.N1317H	–	–	Lotery et al. [2001a]
19	**c.2996C>A**	**p.C1332X**	–	–	Lotery et al. [2001a]
–	**c.4121 4130del**	**p.A1374fsX19**	**c.4121_4130del**	**p.A1374fsX19**	Gerber et al. [2002]
80 F	**c.4121 4130del**	**p.A1374fsX19**	**c.4121_4130del**	**p.A1374fsX19**	Hanein et al. [2004]

[a]Patient M–489 [Bernal et al., 2003] excluded; p.I205T not pathogenic. Patient 12872 [den Hollander et al., 2001a] excluded; p.R1331H not pathogenic. Patient 4 [Lotery et al., 2001a] excluded, p.T289M not pathogenic.

[b]Nucleotide position in AY043325.1; A of ATG is 1. Intron sequences can be found in NT_004671.15. Null mutations are indicated in bold. Nomenclature as suggested by den Dunnen and Antonarakis [2000].

[c]This *Alu* insertion is oriented in the antisense direction, contains a >70–nt poly(A) tail, and is flanked by a 12–bp direct repeat consisting of nucleotides 2174 to 2185.The exact size of the insertion in could not be determined for technical reasons.

[d]This sequence variant was originally described as a 1–bp insertion in codon 871.TheT is either inserted between nucleotide 2611 and 2612 or between 2612 and 2613.

[e]LCA or early–onset severe RP.

[f]This mutation was originally described as a >100 poly(A) insertion, the exact size of the insertion is unknown.

patients with PPRPE and in RP patients without PPRPE, but with other characteristics of this type of RP [Lotery et al., 2001b; Bernal et al., 2003; Khaliq et al., 2003]. Bernal et al. [2003] identified *CRB1* mutations in 6 out of 92 autosomal recessive Spanish RP families (Table 1). Patients in one family presented with PPRPE. However, patients in all families had other characteristics of RP with PPRPE, such as early onset and/or hyperopia [Bernal et al., 2003]. Therefore, we can conclude that *CRB1* mutations are not a frequent cause of "classic" RP, but are an important cause of RP with PPRPE and/or Coats-like exudates and early onset RP without PPRPE but with other characteristics seen in patients with PPRPE.

CRB1 MUTATIONS IN LEBER CONGENITAL AMAUROSIS

Previously, *CRB1* mutations have been detected in 10 to 13% of LCA patients [den Hollander et al., 2001a; Lotery et al., 2001a; Hanein et al., 2004]. We screened another cohort of 44 LCA patients (24 from Quebec, Canada and 20 from other countries worldwide) for mutations in the *CRB1* gene by SSCP analysis and subsequent sequencing of shifted bands. We identified a sequence variant in only one patient (Patient 18240). This patient carries a heterozygous single nucleotide substitution (c.2714G>A), leading to an amino acid change (p.R905Q) (Table 4). Sequence analysis of the entire protein coding region, splice junctions, and an 800-bp segment of the putative promoter did not reveal a second mutated allele. The sequence variant was not present in 372 control chromosomes. Segregation analysis with polymorphic markers for the RP12/*CRB1* locus in family members of Patient 18240 showed that one unaffected sibling has the same *CRB1* alleles as the patient. Although this excludes autosomal recessive inheritance of *CRB1* mutations, we cannot exclude digenic inheritance.

The absence of pathogenic *CRB1* sequence variants in this group of LCA patients is remarkable, since relatively high frequencies of mutations were previously reported in a group of LCA patients mainly of German origin (13%) [den Hollander et al., 2001a], in a group of LCA patients mainly from the United States (11%) [Lotery et al., 2001a], and in a group of LCA patients mainly from France and North Africa (10%) [Hanein et al., 2004] (Table 1). This indicates that the frequency of *CRB1* mutations can vary considerably between populations.

ANALYSIS OF THE ROLE OF *CRB1* MUTATIONS IN CLASSIC COATS DISEASE

Since we previously showed that *CRB1* mutations are an important risk factor for the development of Coats-like exudative vasculopathy in RP [den Hollander et al., 2001a], we hypothesized that heterozygous *CRB1* mutations may form a risk factor for the development of classic Coats disease, a separate entity that develops in patients without RP [Shields et al., 2001]. Mutation analysis of the *CRB1* gene in 18 unrelated patients with classic Coats disease revealed no sequence variants in the *CRB1* gene (Table 1).

DISTRIBUTION OF *CRB1* MUTATIONS

Figure 1 depicts all *CRB1* mutations identified to date, classified according to the type of mutations and grouped per phenotype, from this study and others [den Hollander et al., 1999, 2001a; Lotery et al., 2001a, 2001b; Gerber et al., 2002; Khaliq et al., 2003; Bernal et al., 2003; Jacobson et al., 2003; Hanein et al., 2004]. In total, 71 different sequence variants have been identified on 184 *CRB1* alleles, including 42 amino acid substitutions, 13 frameshift mutations, nine nonsense mutations, three splice site mutations, two in-frame deletions, and two large insertions (Table 3). The most frequently encountered mutations are c.2843G>A (p.C948Y) (32/184 alleles), c.2234C>T (p.T745M) (8/184 alleles), c.2290C>T (p.R764C) (8/184 alleles), and c.2401A>T (p.K801X) (6/123 alleles). Most sequence variants (43/71) have been found on only one allele (Fig. 1; Table 3).

A clustering of sequence variants is found in exons 7 and 9, which encode the second and third laminin A G-like domains (Fig. 1; Table 3). Out of the 42 different amino acid substitutions, 23 (55%) are located in the laminin A G-like domains; nine were found in the second laminin A G-like domain (G2), 11 in the third laminin A G-like domain (G3), but only three in the first laminin A G-like domain (G1). A total of 17 amino acid substitutions (40%) reside in the EGF-like domains, and are distributed evenly through these domains (Fig. 1; Table 3).

EFFECT OF *CRB1* MUTATIONS

All *CRB1* nonsense mutations and all frameshift mutations, except for the frameshift mutation in exon 12 (c.4121_4130del (p.A1374fsX19)), are predicted to result in the truncation of variable parts of the extracellular domain and removal of the transmembrane and 37–amino acid cytoplasmic domains. Alternatively, *CRB1* nonsense mutations and frameshift mutations leading to premature stop codons may result in low levels of protein expression due to nonsense-mediated decay of the mutant mRNA [Frischmeyer and Dietz, 1999]. The frameshift mutation in exon 12 is less likely to induce nonsense-mediated decay, since it is located near the 3′-end of the gene, and is not followed by a downstream intron [Frischmeyer and Dietz, 1999]. The frameshift removes the last 33 amino acids of the cytoplasmic domain and introduces 19 erroneous amino acids followed by a premature stop codon [Gerber et al., 2002].

The cytoplasmic domain of CRB1 is highly conserved and functionally related to the cytoplasmic domain of *Drosophila* Crb [den Hollander et al., 2001b]. Eight residues are completely conserved between several Crb homologues from different species (Fig. 2) [Klebes and

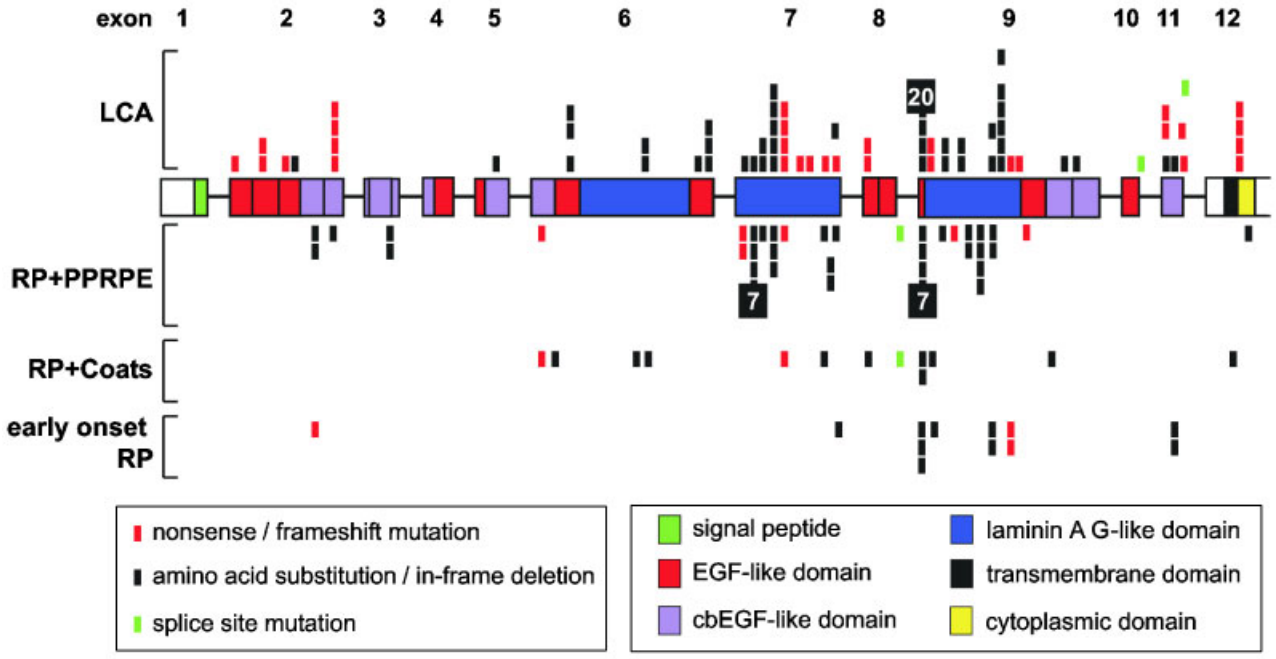

FIGURE 1. **Schematic representation of the intron–exon structure of the *CRB1* gene and sequence variants that are likely to be pathogenic, classified according to the types of variants and grouped per phenotype. Sequence variants observed in one to five alleles are depicted as vertical bars; the number of alleles in excess of six is depicted in boxes. Untranslated cDNA sequences are indicated by open boxes. Exons are drawn to scale, introns are not. Protein domains are shown with colored boxes. References for sequence variants: this study, den Hollander *et al.* [1999, 2001a], Lotery *et al.* [2001a, b], Gerber *et al.* [2002], Bernal *et al.* [2003], Jacobson *et al.* [2003], Khaliq *et al.* [2003], and Hanein *et al.* [2004].**

Knust, 2000; Bossinger et al., 2001; den Hollander et al., 2001b; Izaddoost et al., 2002; Roh et al., 2003]. The cytoplasmic domain is of crucial importance since it has been shown to link Crb homologues to several cytoplasmic proteins [Bhat et al., 1999; Klebes and Knust, 2000; Bachmann et al., 2001; Hong et al., 2001; den Hollander et al., 2002; Lemmers et al., 2002; Medina et al., 2002; Roh et al., 2002, 2003]. The conserved C-terminal ERLI motif binds to PDZ domains, and the other four conserved residues (p.G1377, p.Y1379, p.P1381, and p.E1385) are proposed to be part of a FERM-domain binding site (Fig. 2) [Izaddoost et al., 2002]. The frameshift mutation in exon 12 (c.4121_4130del (p.A1374fsX19)) removes both binding domains and abolishes interaction of CRB1 with cytoplasmic proteins. The c.4148G>A (p.R1383H) amino acid substitution affects a residue that is not conserved in other Crb homologues, but is located in the FERM-domain binding site and therefore may affect its binding properties (Fig. 2). However, since no second *CRB1* sequence variant was identified in Patient 15849 (Table 2), it is possible that this amino acid substitution is not pathogenic (see Distinction Between Pathogenic and Nonpathogenic *CRB1* Sequence Variants).

Laminin A G-like or ALPS (agrin, laminin, perlecan, slit) domains were originally identified as a five-fold repetition of 158–180 amino acid residues in the C-terminal globular domain of the laminin α1 chain [Sasaki et al., 1988]. Laminin A G-like domains have been identified in a variety of proteins, and can serve as protein interaction modules. These domains exhibit low overall homology, but have some residues that are highly conserved [Beckmann et al., 1998]. Figure 3 depicts an alignment of the laminin A G-like domains of human CRB1, mouse Crb1 and *Drosophila* Crb, and the amino acid substitutions in the *CRB1* gene that are located in these domains. Two amino acid substitutions (c.2234C>T (p.T745M) and c.2548G>A (p.G850S)) affect residues that are completely conserved between the nine laminin A G-like domains of hCRB1, mCRB1 and *Drosophila* Crb. A total of 11 amino acid substitutions (c.2222T>C (p.M741T), c.2506C>A (p.P836T), c.2509G>C (p.D837H), c.2555T>C (p.I852T), c.2966T>C (p.I989T), c.3122T>C (p.M1041T), c.3212T>C (p.L1071P), c.3299T>C (p.I1100T), c.3299T>G (p.I1100R), c.3320T>G (p.L1107R), and c.3320T>C (p.L1107P)) affect residues that are identical or similar in at least four of these domains. Laminin A G-like domains contain cysteine residues, which are proposed to form disulfide bridges [Beckmann et al., 1998]. The first laminin A G-like domain of the CRB1 protein contains six cysteine residues, which may form three disulfide bridges, and the second and third laminin A G-like domains of CRB1 each contain two cysteine residues. One amino acid substitution (c.1760G>A (p.C587Y)) affects the second cysteine residue of the first laminin A G-like domain, which may disrupt the secondary structure of this domain. Another amino acid substitution (c.2290C>T (p.R764C)) introduces a cysteine residue in the second laminin A G-like domain,

TABLE 3. Overview of *CRB1* Sequence Variants That Are Likely to be Pathogenic

Nucleotide change[a]	Effect	Exon	Protein domain	Proof of pathogenicity	Disease	No. of alleles	Reference
c.111delT	**p.S38fsX32**	2		Protein truncation	LCA	1	Lotery et al. [2001a]
c.257_258dupTG	**p.N87fsX0**	2		Protein truncation	LCA	2	Lotery et al. [2001a]; Jacobson et al. [2003]
c.428_432delGATTC	**p.R143fsX1**	2		Protein truncation	LCA	1	Lotery et al. [2001a]
c.430T>G	p.F144V	2	EGF3	–	LCA	1	Lotery et al. [2003]
c.481dupG	**p.A161fsX7**	2		Protein truncation, segregation	Early onset RP	1	Bernal et al. [2003]
c.482C>T	p.A161V	2	EGF4	Conserved residue	RP+PPRPE	2	den Hollander et al. [1999]
c.584G>T	p.C195F	2	EGF5	Conserved residue	RP+PPRPE	1	This study
c.613_619del	**p.I205fsX12**	2		Protein truncation	LCA	4	den Hollander et al. [2001a]; Lotery et al. [2001a]; Hanein et al. [2004]
c.750T>G	p.C250W	3	EGF6	Conserved residue, segregation	RP+PPRPE	2	den Hollander et al. [1999]
c.1148G>A	p.C383Y	5	EGF9	Conserved residue	LCA	1	Lotery et al. [2001a]
c.1208C>G	**p.S403X**	6		Protein truncation, segregation	RP+PPRPE, RP+Coats	1,1	den Hollander et al. [2001a]
c.1298A>G	p.Y433C	6	EGF10	May disrupt secondary structure	RP+Coats	1	den Hollander et al. [2001a]
c.1438T>G	p.C480G	6	EGF11	Conserved residue	LCA	2	Lotery et al. [2001a]
c.1438T>C	p.C480R	6	EGF11	Conserved residue	LCA	1	Lotery et al. [2001]
c.1733T>A	p.V578E	6	G1	BLOSUM62 score –2	RP+Coats	1	This study
c.1750G>T	p.D584Y	6	G1	BLOSUM62 score –3	LCA	2	Hanein et al. [2004]
c.1760G>A	p.C587Y	6	G1	May disrupt secondary structure	RP+Coats	1	This study
c.2042G>A	p.C681Y	6	EGF12	Conserved residue	LCA	1	Lotery et al. [2001a]
c.2128G>C	p.E710Q	6	G2	Segregation	LCA	3	Hanein et al. [2004]
c.2185_2186ins*Alu*[b]	**Unknown**	7		Severe disruption, Segregation	RP+PPRPE	2	den Hollander et al. [1999]
c.2222T>C	p.M741T	7	G2	Conserved residue	LCA	1	Hanein et al. [2004]
c.2234C>T	p.T745M	7	G2	Conserved residue	LCA, RP+PPRPE	1,7	den Hollander et al. [1999]; Hanein et al. [2004]
c.2245_2247delTCA	p.S749del	7	G2	Segregation	LCA, RP+PPRPE	2,1	Bernal et al. [2003]; Jacobson et al. [2003]
c.2290C>T	p.R764C	7	G2	May disrupt secondary structure, segregation	LCA, RP+PPRPE	5,3	den Hollander et al. [1999]; Lotery et al. [2001a]; Jacobson et al. [2003]; Hanein et al. [2004]; This study
c.2401A>T	**p.K801X**	7		Protein truncation, segregation	LCA, RP+PPRPE, RP+Coats	4,1,1	den Hollander et al. [2001a]; Jacobson et al. [2003]; This study
c.2438_2439ins>100A[c]	**Unknown**	7		Severe disruption	LCA	1	Lotery et al. [2001a]
c.2479G>T	p.G827X	7		Protein truncation	LCA	1	Hanein et al. [2004]
c.2506C>A	p.P836T	7	G2	Conserved residue	RP+PPRPE	1	This study
c.2509G>C	p.D837H	7	G2	Conserved residue	RP+Coats	1	den Hollander et al. [2001a]
c.2536G>A	p.G846R	7	G2	Segregation	RP+PPRPE	2	Khaliq et al. [2003]
c.2548_2551delGGCT	**p.G850fsX4**	7		Protein truncation	LCA	1	Lotery et al. [2001a]
c.2548G>A	p.G850S	7	G2	Conserved residue	RP+PPRPE	1	This study
c.2555T>C	p.I852T	7	G2	Conserved residue	LCA	1	Hanein et al. [2004]
c.2611_2613insT[d]	**Frameshift**	7		Protein truncation	LCA	1	Lotery et al. [2001a]
c.2671T>G	p.C891G	7	EGF13	Conserved residue, segregation	Early onset RP	1	Bernal et al. [2003]
c.2681A>G	p.N894S	8	EGF13	Segregation	RP+Coats	1	den Hollander et al. [2001a]
c.2688T>A	**p.C896X**	8		Protein truncation		2	Hanein et al. [2004]
c.2842+5G>A	Splice defect	Intron 8		Segregation	RP+PPRPE, RP+Coats	1,1	den Hollander et al. [1999]; den Hollander et al. [2001a]

c.2843G>A	p.C948Y	9	EGF14	Conserved residue, segregation	LCA, RP+PPRPE, RP+Coats, early onset RP	20, 7, 2, 3	den Hollander et al. [1999]; den Hollander et al. [2001a]; Lotery et al. [2001a]; Bernal et al. [2003]; Jacobson et al. [2003]; Hanein et al. [2004];This study
c.2853dupT	**p.A952fsX3**	9		Protein truncation	LCA	2	Hanein et al. [2004]
c.2875G>A	p.G959S	9	G3		RP+Coats	1	This study
c.2884_2886delTTA	p.L962del	9	G3	Segregation	Early onset RP	1	Bernal et al. [2003]
c.2957A>T	p.N986I	9	G3	Segregation	RP+PPRPE	1	This study
c.2966T>C	p.I989T	9	G3	Conserved residue, segregation	LCA	2	Khaliq et al. [2003]
c.2983G>T	**p.E995X**	9		Protein truncation	RP+PPRPE	1	den Hollander et al. [1999]
c.3074G>T	p.S10251	9	G3	BLOSUM62 score−2	LCA	2	Hanein et al. [2004]
c.3122T>C	p.M1041T	9	G3	Conserved residue, segregation	RP+PPRPE	2	den Hollander et al. [1999]
c.3212T>C	p.L1071P	9	G3	Conserved residue, segregation	RP+PPRPE	4	den Hollander et al. [1999]; Khaliq et al. [2003]
c.3299T>C	p.I1100T	9	G3	Conserved residue, segregation	RP+PPRPE, early onset RP	2,2	Bernal et al. 2003;This study
c.3299T>G	p.I1100R	9	G3	Conserved residue, segregation	LCA	1	den Hollander et al. [2001a]
c.3307G>A	p.G1103R	9	G3	BLOSUM62 score −2	LCA	1	Hanein et al. [2004]
c.3320T>G	p.L1107R	9	G3	Conserved residue, segregation	LCA	5	Hanein et al. [2004]
c.3320T>C	p.L1107P	9	G3	Conserved residue	LCA	1	Hanein et al. [2004]
c.3331G>T	**p.E1111X**	9		Protein truncation	LCA	1	den Hollander et al. [2001a]
c.3343_3352del	**p.G1115fsX22**	9		Protein truncation, segregation	Early onset RP	2	Lotery et al. [2001b]
c.3347delT	**p.F1116fsX24**	9		Protein truncation	LCA	1	Hanein et al. [2004]
c.3427delT	**p.C1143fsX66**	9		Protein truncation	RP+PPRPE	1	This study
c.3541T>C	p.C1181R	9	EGF16	Conserved residue, segregation	RP+Coats	1	den Hollander et al. [2001a]
c.3613G>A	p.G1205R	9	EGF16	Conserved residue	LCA	1	Lotery et al. [2001a]
c.3653G>T	p.C1218F	9	EGF17	Conserved residue	LCA	1	Jacobson et al. [2003]
c.3878+1G>T	**Splice defect**	Intron 10		Conserved nucleotide of splice site	LCA	1	den Hollander et al. [2001a]
c.3879G>A	**p.W1293X**	11		Protein truncation	LCA	2	Hanein et al. [2004]
c.3949A>C	p.N1317H	11	EGF19		LCA	1	Lotery et al. [2001a]
c.3961T>A	p.C1321S	11	EGF19	Conserved residue, segregation	LCA, early onset RP	1,2	Lotery et al. [2001b]; Hanein et al. [2004]
c.3988delG	**p.E1330fsX10**	11		Protein truncation	LCA	1	Hanein et al. [2004]
c.3996C>A	**p.C1332X**	11		Protein truncation	LCA	1	Lotery et al. [2001a]
c.3997G>T	**p.E1333X**	11		Protein truncation, segregation	LCA	1	den Hollander et al. [2001a]
c.4005+1G>A	**Splice defect**	Intron 11		Conserved nucleotide of splice site	LCA	1	Hanein et al. [2004]
c.4060G>A	p.A1354T	12	TM	–	RP+Coats	1	den Hollander et al. [2001a]
c.4121_4130del	**p.A1374fsX19**	12		Protein truncation, segregation	LCA	4	Gerber et al. [2002]; Hanein et al. [2004]
c.4148G>A	p.R1383H	12	Cyto	–	RP+PPRPE	1	This study

[a]Nucleotide position in AY043325.1; A of ATG is 1. Intron sequences can be found in NT_004671.15. Null mutations are indicated in bold. Nomenclature as suggested by den Dunnen and Antonarakis [2000].
[b]This *Alu* insertion is oriented in the antisense direction, contains a >70–nt poly(A) tail, and is flanked by a 12–bp direct repeat consisting of nucleotides 2174 to 2185.The exact size of the insertion in could not be determined for technical reasons.
[c]This mutation was originally described as a >100 poly(A) insertion, the exact size of the insertion is unknown.
[d]This sequence variant was originally described as a 1–bp insertion in codon 871. TheT is either inserted between nucleotide 2611 and 2612 or between 2612 and 2613.

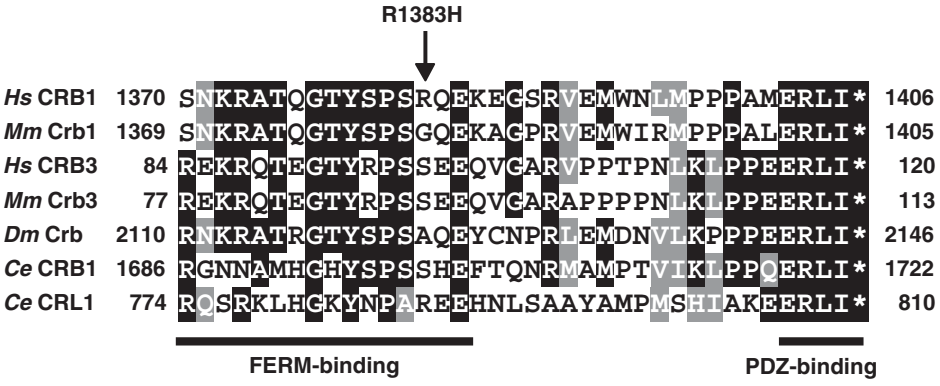

FIGURE 2. **Alignment of cytoplasmic domains of human (*Hs*) CRB1 (AY043325.1), mouse (*Mm*) Crb1 (AF406641.1), human CRB3 (AY103469.1), mouse Crb3 (BC024462.1), *Drosophila* (*Dm*) Crb (M33753.1), *C. elegans* (*Ce*) CRB1 (U42839.2), and CRL1 (AL008869.1). Identical amino acids are indicated in black boxes and conserved residues in gray boxes. Sequences were aligned with ClustalW and boxed with BoxShade 3.21 (www.ch.embnet.org/software). Amino acid positions are indicated.**

which may disrupt folding and/or form a disulfide bond inappropriately with another protein.

EGF-like domains typically consist of six cysteine residues that interact with each other by the formation of disulfide bridges. These stabilize the native fold, which comprises a major and minor β-sheet. Disulfide bridges are formed between the first and third cysteine residues, the second and fourth residues, and the fifth and sixth residues [Cooke et al., 1987]. The CRB1 protein has 19 EGF-like domains, however the 14th EGF-like domain (EGF14) is truncated, since it contains only four cysteine residues. A distinct subgroup of EGF-like domains has been identified that contains a consensus sequence associated with calcium binding (cb) (Fig. 4) [Handford et al., 1991]. In other proteins that contain tandemly repeated cbEGF domains, such as fibrillin-1, Ca^{2+} is predicted to rigidify the interdomain region, resulting in a rod-like structure [Downing et al., 1996]. In CRB1, EGF-like domains 4–7, 9–10, 16–17, and 19 contain a calcium binding sequence (Fig. 1). Of the 17 amino acid substitutions that localize to EGF-domains, the majority affects highly conserved residues known to be important for EGF-like domain structure and is likely to cause disruption of the native fold (Fig. 4). A total of 11 amino acid substitutions (c.584G>T (p.C195F), c.750T>G (p.C250W), c.1148G>A (p.C383Y), c.1438T>G (p.C480G), c.1438T>C (p.C480R), c.2042G>A (p.C681Y), c.2671T>G (p.C891G), c.2843G>A (p.C948Y), c.3541T>C (p.C1181R), c.3653G>T (p.C1218F), and c.3961T>A (p.C1321S)) affect the conserved cysteine residues involved in disulfide bond formation. One amino acid substitution, c.1298A>G (p.Y433C), changes a highly conserved aromatic residue to a cysteine. This amino acid change may disrupt folding of CRB1, and/or result in an inappropriate disulfide bond with another protein. The amino acid substitution c.482C>T (p.A161V) alters a highly conserved alanine residue located in a turn at one end of the major two stranded antiparallel β-sheet. A glycine to serine change at this position in human fibrillin-1 has been shown to cause a mild folding change that results in a connective tissue disease [Whiteman et al., 2001]. The amino acid substitution c.3613G>A (p.G1205R) may also result in a similar disruption of protein folding, since a highly conserved glycine residue is located at this position in a turn at one end of the minor β-sheet. Of particular interest are the three amino acid substitutions c.3949A>C (p.N1317H), c.2681A>G (p.N894S), and c.430T>G (p.F144V), which occur at nonconserved residues and whose effects are not easily explained in terms of structure. One can hypothesize that these residues may disrupt intra- or intermolecular interactions. However, since no second *CRB1* sequence variation was identified in the patients that carry these amino acid substitutions (Table 2), it is possible that they are not pathogenic (see Distinction Between Pathogenic and Nonpathogenic *CRB1* Sequence Variants).

Next to intradomain effects, long-range structural consequences may result from the effects of amino acid substitutions when EGF-like domains are tightly linked to neighboring modules. Structural effects of calcium binding mutations in fibrillin-1 domain pairs have been shown to be highly dependent upon domain context. [McGettrick et al., 2000]. Determination of the long range consequences of CRB1 sequence variants will require further structural information about the linkage of EGF-like domains, since there is a difference in the number of linker residues between tandemly repeated cbEGF domains in CRB1 compared to fibrillin-1, which may result in alternative pairwise interactions [Downing et al., 1996].

DISTINCTION BETWEEN PATHOGENIC AND NONPATHOGENIC *CRB1* SEQUENCE VARIANTS

Most *CRB1* sequence variants lead to a severe disruption of the protein, affect a conserved residue, are likely to disrupt the secondary structure of the protein or lead to splice defects (Table 3). For some sequence variants the effect is not clear, but their segregation has been demonstrated in family members (Table 3). Particularly for amino acid substitutions that do not affect a conserved residue or disrupt the secondary structure, it can be difficult to determine

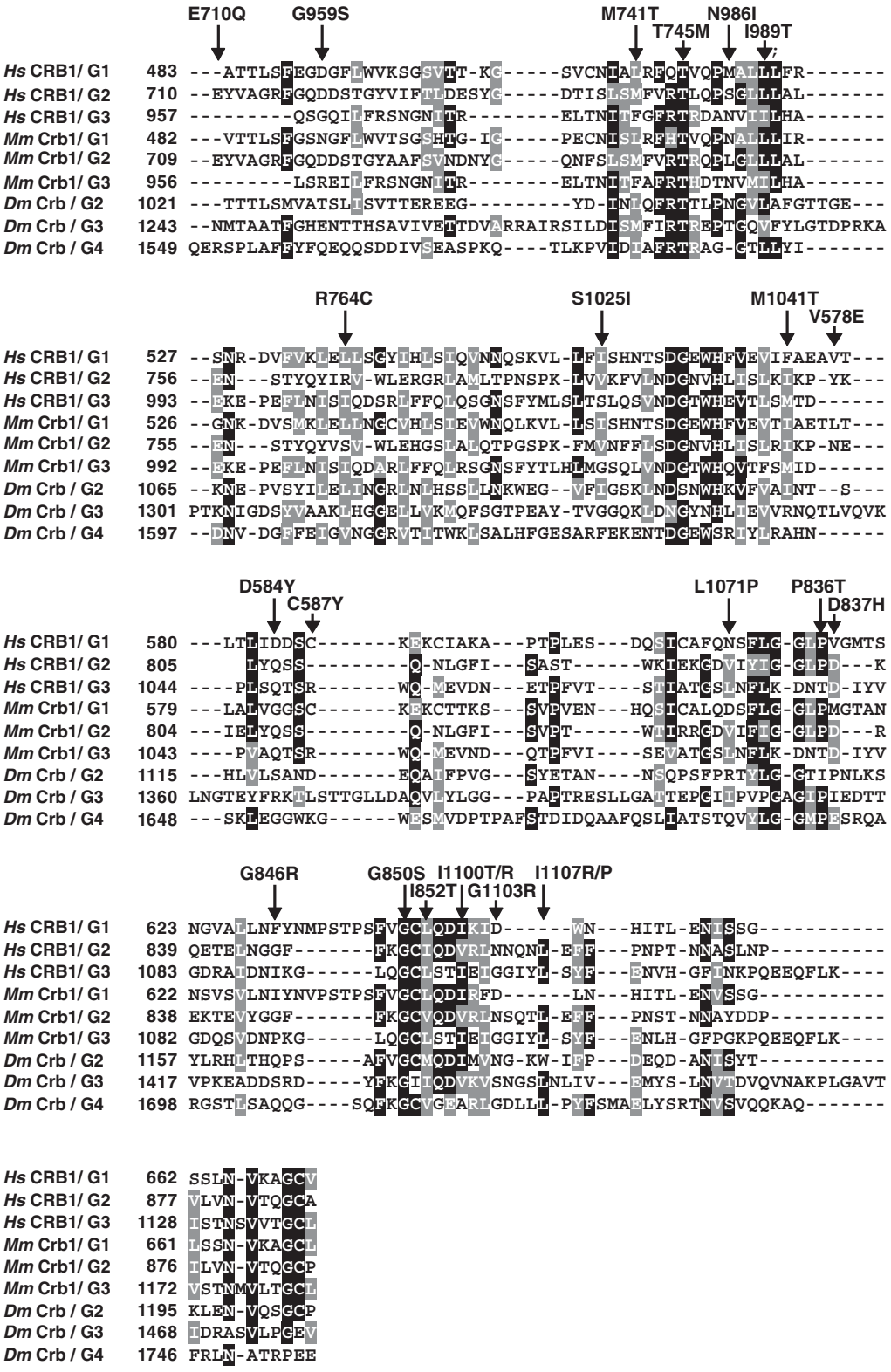

FIGURE 3. **Alignment of laminin A G-like domains of human (*Hs*) CRB1 (AY043325.1), mouse (*Mm*) Crb1 (AF406641.1), and *Drosophila* (*Dm*) Crb (M33753.1), and *CRB1* amino acid substitutions identified in these domains. Identical amino acids are indicated in black boxes and conserved residues in gray boxes. Sequences were aligned with ClustalW and boxed with BoxShade 3.21. Amino acid positions are indicated.**

whether they are pathogenic or not. Several amino acid substitutions (c.614T>C (p.I205T), c.866C>T (p.T289M), c.2035C>G (p.Q679E), c.2306_2307GC>AG (p.R769Q), c.2306G>A (p.R769H), c.2714G>A (p.R905Q), and c.3992G>A (p.R1331H)) are not pathogenic since they do not segregate with the

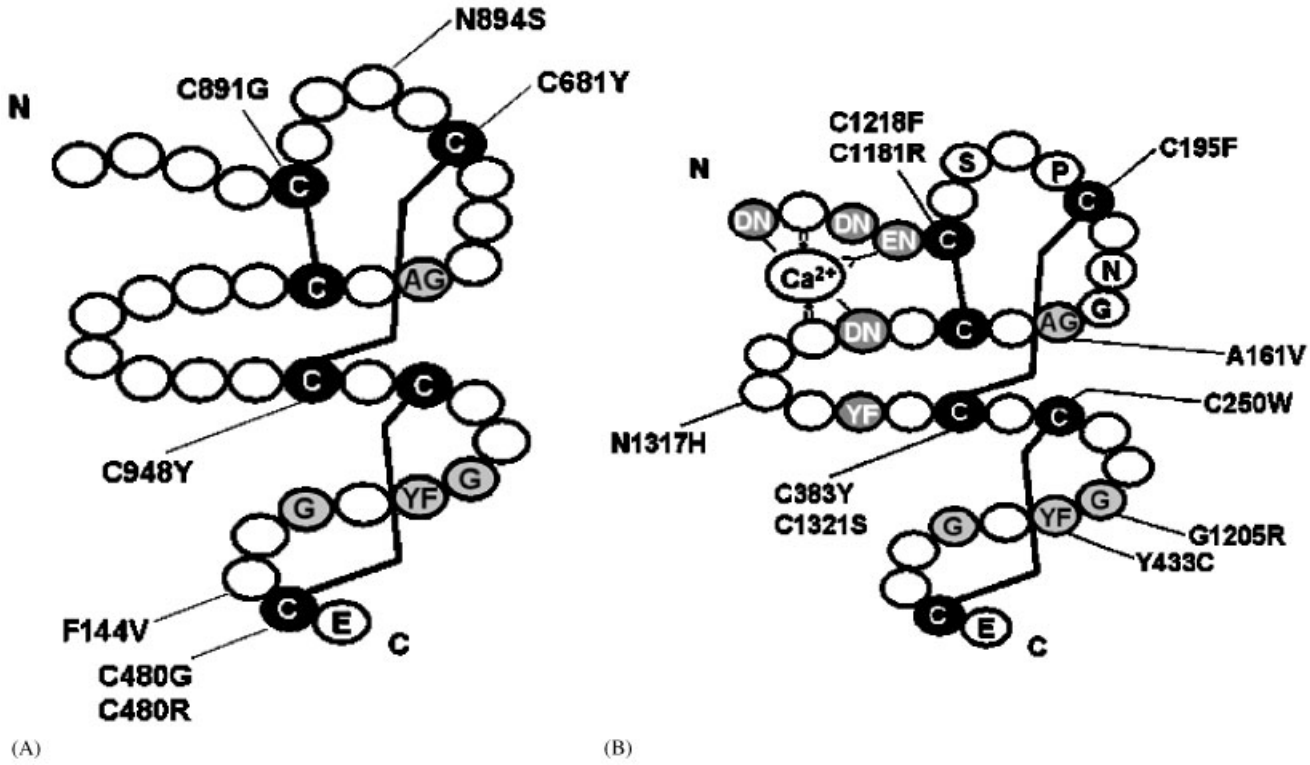

FIGURE 4. **Consensus sequence of non-cbEGF-like domains (A) and cbEGF-like domains (B), and *CRB1* amino acid substitutions identified in these domains. Conserved cysteine residues are indicated in black, residues conserved in both types of EGF-like domains in gray (black letters), and conserved residues in cbEGF-like domains in gray (white letters).**

phenotype in family members, or are present in control alleles (Table 4). Four amino acid substitutions (c.1733T>A (p.V578E), c.1750G>T (p.D584Y), c.3074G>T (p.S1025I), and c.3307G>A (p.G1103R)) are likely to be pathogenic since they lead to substitutions of amino acids that are evolutionary not related, and therefore have negative BLOSUM62 matrix scores (Table 3) [Henikoff and Henikoff, 1992]. Four amino acid substitutions (c.2875G>A (p.G959S), c.3949A>C (p.N1317H), c.4060G>A (p.A1354T), and c.4148G>A (p.R1383H)) have positive BLOSUM62 scores, and are therefore less likely to be pathogenic. One of these substitutions (c.4060G>A (p.A1354T)) is located on the same allele as c.2509G>C (p.D837H) (Table 2), which is more likely to be pathogenic since it affects a conserved residue in the second laminin A G-like domain. In the patients carrying the amino acid substitutions c.430T>G (p.F144V), c.2875G>A (p.G959S), c.3949A>C (p.N1317H), and c.4148G>A (p.R1383H), no second *CRB1* sequence variant was identified (Table 2). This suggests that either the second sequence variant was missed by PCR-based mutation analysis, or that these amino acid substitutions are not pathogenic.

GENOTYPE–PHENOTYPE CORRELATION FOR *CRB1* MUTATIONS

We previously hypothesized that LCA may be associated with complete loss of function of CRB1, while RP patients (early onset RP with or without PPRPE, and RP with Coats-like exudative vasculopathy) may have residual CRB1 function [den Hollander et al., 2001a]. For our calculations below, null mutations were defined as nonsense and frameshift mutations, and mutations affecting the invariable AG or GT dinucleotides of splice sites. Some *CRB1* amino acid substitutions may also represent null mutations, however, functional evidence is lacking to support this.

Our hypothesis is supported by the observation that LCA patients carry *CRB1* null mutations more frequently than RP patients (Tables 2 and 3). Out of 90 mutated *CRB1* alleles identified in LCA patients, 33 (37%) are null mutations, compared to 13 out of 69 (19%) mutated *CRB1* alleles identified in RP patients ($P = 0.01$; Fisher's exact test). However, if our hypothesis is correct, one would expect more LCA patients than RP patients to carry null mutations on both alleles. Sequence variants were detected on both *CRB1* alleles in 32 out of 37 RP patients, and 3 out of 32 (9%) carry null mutations on both alleles (Table 2). Sequence variants were detected on both *CRB1* alleles in 35 out of 55 LCA patients, and 8 out of 35 (23%) LCA patients carry null mutations on both alleles (Table 2). The percentage of LCA patients that carry null mutations on both alleles is therefore not significantly higher than the percentage of RP patients that carry null mutations on both alleles ($P = 0.1$; Fisher's exact test). However, sequence variants were identified more frequently on both *CRB1* alleles in RP patients than in LCA patients.

TABLE 4. Overview of Nonpathogenic *CRB1* Sequence Variants

Nucleotide change[a]	Effect	Exon	Protein domain	Proof of nontaathogenicity	Reference
c.–268G>A	–	5'UTR		–	Bernal et al. [2003]
c.71–12A>T	–	Intron 1		Present in control alleles, no segregation	Lotery et al. [2003]; Bernal et al. [2003]
c.614T>C	p.I205T	2	EGF5	No segregation	Bernal et al. [2003]; This study
c.652+42T>A	–	Intron2		No segregation	Bernal et al. [2003]
c.653–44_653–41delTGCT	–	Intron 2		–	Lotery et al. [2001a]
c.866C>T	p.T289M	4	EGF7	No segregation	den Hollander et al. [2001a]; Lotery et al. [2001a]; Bernal et al. [2003]
c.989–53T>G	–	Intron 4		No segregation	Bernal et al. [2003]
c.1171+35C>T	–	Intron 5		Present in control alleles	Lotery et al. [2001a]
c.1172–64T>G	–	Intron 5		No segregation	Bernal et al. [2003]
c.1172–54G>T[b]	–	Intron 5		–	Lotery et al. [2001a]
c.1410G>A	(p.L470)	6	EGF11	Synonymous codon change, no segregation	Lotery et al. [2001a]; Bernal et al. [2003]
c.1428C>T	(p.T476)	6	EGF11	Synonymous codon change	Lotery et al. [2001a]
c.1647T>C	(p.N549)	6	G1	Synonymous codon change, no segregation	Lotery et al. [2001a]; Bernal et al. [2003]
c.2035C>G	p.Q679E	6	EGF12	No segregation	Bernal et al. [2003]
c.2128+15A>C	–	Intron 6		–	This study
c.2306_2307GC>AG	p.R769Q	7	G2	Present in control alleles	Lotery et al. [2001a]
c.2306G>A	p.R769H	7	G2	No segregation	Bernal et al. [2003]
c.2307C>T	(p.R769)	7	G2	Synonymous codon change	This study
c.2714G>A	p.R905Q	8	EGF13	No segregation	This study
c.2823G>A	(p.P941)	8	EGF14	Synonymous codon change, present in control alleles	Lotery et al. [2001a]
c.3171C>T	(p.N1057)	9	G3	Synonymous codon change, no segregation	Lotery et al. [2001a]; Bernal et al. [2003]
c.3992G>A	p.R1331H	11	EGF19	Present in control alleles, no segregation	den Hollander et al. [2001a]; Lotery et al. [2001a]; Bernal et al. [2003]

[a]Nucleotide position in AY043325.1; A of ATG is 1. Intron sequences can be found in NT_004671.15. Nomenclature as suggested by den Dunnen and Antonarakis [2000].
[b]This sequence variant was originally described as a G>T substitution 54 bp 5'to exon 6. However, the nucleotide at this position is not a G.

This may reflect the higher number of sequence variants that were missed by PCR-based mutation analysis in LCA patients, for example heterozygous deletions spanning one or more exons. Nevertheless, the existence of at least some RP patients with two null mutations indicates that complete loss of function of CRB1 is not sufficient for causing LCA vs. RP. A possible explanation is that environmental factors or genetic modifiers may influence the severity of the disease.

Interestingly, the amino acid substitution c.2843G>A (p.C948Y) is found homozygously in 5 out of 35 (14%) LCA patients (Table 2) and only in 1 out of 32 RP probands. In Family M-69 [Bernal et al., 2003], two affected family members were homozygous for c.2843G>A (p.C948Y), and one was compound heterozygous for c.2843G>A (p.C948Y) and c.3299T>C (p.I1100T). The patients that are homozygous for c.2843G>A (p.C948Y) have a more severe phenotype, resembling LCA, compared to the patient who is compound heterozygous for the mutation. This suggests that this amino acid substitution is a severe mutation, or may even represent a null allele.

FUTURE DIRECTIONS

In vivo high-resolution microscopy in patients with *CRB1* mutations and studies in model organisms have shed light on the function of *CRB1* in retinal development and pathogenesis. Additional studies are required to determine whether or not the mechanisms underlying light-induced photoreceptor degeneration observed in *Drosophila* can be extrapolated to humans, and if patients with *CRB1* mutations may benefit from reduced amounts and/or intensities of daylight [Johnson et al., 2002].

Routine DNA diagnostics for patients with LCA and autosomal recessive RP would enable clinicians to establish more accurate diagnoses and prognoses, would allow genetic counseling in the family of the patient, and may be important to select patients for gene-specific therapies in the future [Cremers et al., 2002]. Genetic heterogeneity of LCA and RP has hampered the development of a routine DNA diagnostic test. Identification of a substantial number of sequence variants in *CRB1* and other LCA genes has allowed the development of a genotyping chip for LCA, which will be available as a diagnostic test in the near future (R. Allikmets, personal communication).

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