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Evaluation of human diacylglycerol kinaseι, DGKI, a homolog of *Drosophila rdgA*, in inherited retinopathy mapping to 7q

Sara J. Bowne[1], **Lori S. Sullivan**[1], **Li Ding**[2], **Elie Traer**[2], **Stephen M. Prescott**[2], **David G. Birch**[3], **Avril Kennan**[4], **Peter Humphries**[4], and **Stephen P. Daiger**[1]

[1]*Human Genetics Center, School of Public Health, and Department of Ophthalmology and Visual Science, The University of Texas-Houston Health Science Center, Houston, TX* [2]*Huntsman Cancer Center Institute, The University of Utah, Salt Lake City, UT* [3]*Retina Foundation of the Southwest, Dallas, TX* [4]*Genetics Department, Trinity College, Dublin, Ireland*

Abstract

Purpose—To determine the genomic organization of diacylglycerol kinase$_{\iota}$ and to test whether defects in this gene are present in individuals affected with autosomal dominant retinitis pigmentosa (adRP). Diacylglycerol kinaseι has been mapped to the RP10 locus on 7q and shows 49% sequence similarity to the *Drosophila*DGK2 *rdgA* gene. Since mutations in the DGK2 *rdgA* gene cause photoreceptor degeneration in *Drosophila*, it is possible that mutations in diacylglycerol kinaseι could be responsible for human retinal degeneration.

Methods—DNA sequence from genomic clones containing diacylglycerol kinase$_{\iota}$ was compared with the cDNA sequence to identify intron/exon boundaries. Single-strand conformational analysis and PCR product sequencing were used to screen members of one family previously mapped to the RP10 locus and 47 small unmapped families with autosomal dominant retinitis pigmentosa.

Results—Diacylglycerol kinaseι is divided into 35 exons with the initiation codon being present in exon 2. Mutational analysis found a missense change (Lys153Phe) in three adRP families; however, it did not segregate with disease in one of the families. Silent substitutions were seen in codons 865 and 875. Intronic variation was detected in the amplifications of exons 3,5,18, 28, and 32; these do not affect splice site consensus sequences. Typing of a polymorphic variant detected in intron 31 in members of the RP10 family gave a LOD score of -4.2 at 0% recombination.

Conclusions—No evidence of disease-associated mutations was found in any of the samples tested. Based on the linkage data and mutation screening, diacylglycerol kinaseι is excluded as a candidate for the RP10 form of adRP and cannot be a frequent cause of other forms of adRP. Mutations in diacylglycerol kinaseι may yet be the cause of recessive forms of retinal degeneration in humans, either in homozygous or compound heterozygous forms. The data provided here will permit testing of this hypothesis.

Retinitis pigmentosa (RP) is a group of clinically and genetically heterogeneous hereditary disorders that affect the photoreceptors and retinal pigment epithelium. To date, thirteen autosomal recessive (arRP), eleven autosomal dominant (adRP) and six X-linked (XlRP) loci have been mapped. Of the eleven adRP loci, only five disease-associated genes have been identified; rhodopsin, peripherin/*RDS*, *CRX*, *NRL*, and *RP1* (RetNet, 1 October 1999). One of

Correspondence to: Stephen P. Daiger, PhD, P.O. Box 20334, Houston, TX, 77225-0334; Phone: (713) 500-9829; Fax: (713) 500-0900; email: sdaiger@utsph.sph.uth.tmc.edu.

the unidentified adRP loci, RP10, has been mapped to 7q31-q35 using linkage data from three independent families [1-4].

Recently a novel retinal-expressed diacylglycerol kinase (DGK), diacylglycerol kinaseι (*DGKI*), has been identified and mapped by fluorescence in situ hybridization to 7q32.2-q33 [5]. DGK controls diacylglycerol levels by phosphorylating diacylglycerol into phosphatidic acid. Through this interaction, DGK plays an important role in complex lipid biosynthesis and signal transduction [5-7]. Diacylglycerol kinaseι is a member of the DGK type IV subfamily, which is characterized by the presence of four ankyrin repeats located at the C-terminus and a MARCKs phosphorylation site [8]. *DGKI* has 49% sequence similarity with another type IV subfamily member, the *Drosophila* DGK2 *rdgA* gene [5].

The *Drosophila* DGK2 *rdgA* gene was first identified by Masai et al. in 1993 [9] as the mutant gene responsible for the retinal degeneration in the homozygous *rdgA* fruit fly. Studies have shown that the photoreceptor degeneration in the *rdgA* mutant is due to the disruption of the subrhabdomeric cisternae which are perforated extensions of the endoplasmic reticulum. Subrhabdomeric cisternae play an important role in the maintenance of fly photoreceptors by transporting phospholipids to the photoreceptor membrane. Recent studies have indicated that the *rdgA* protein is associated with subrhabdomeric cisternae, and it is the reduced levels of this diacylglycerol kinase in the *rdgA* mutant that cause a deficiency of phosphatidic acid and subsequent photoreceptor degeneration [9,10].

DGKI has the highest sequence similarity of any known human gene to the *Drosophila rdgA* gene, and is the most likely *rdgA* ortholog. Due to the high sequence similarity of human *DGKI* to the *Drosophila* DGK2 *rdgA* gene, and proximity of the diacylglycerol kinaseι locus to the RP10 disease region, it is possible that mutations in the *DGKI* gene could be responsible for human retinal degeneration. To test this hypothesis we determined the genomic organization of *DGKI* for subsequent mutation testing of affected individuals. Samples from one RP10 family, UTAD045, and 47 small adRP families were tested for mutations.

METHODS

Genomic Characterization

Genomic clones were isolated by Genome Systems (St. Louis, MS) using fragments of the *DGKI* cDNA and genomic primers. Positive genomic clones were analyzed by restriction mapping and Southern blot analysis. Southern blots of the positive genomic clones were probed with cDNA and positive bands were subcloned into conventional vectors and sequenced by automated dideoxy sequencing. Alternatively, some intron/exon boundaries were identified by direct sequencing of P1, BAC and PAC genomic clones. Comparison of the genomic sequence with the cDNA sequence identified intron/exon boundaries. Intron/exon boundaries for exons 3 through 35 were identified using the above method. The 3’ boundary of exon 2 was determined using Vectorette (Genosys, Woodlands, TX) modification of a CEPH YAC in the region and PCR cycle sequencing with the Amplicycle™ Sequencing Kit (Perkin Elmer, Branchburg, NJ). All boundaries were consistent with conserved splice site sequences. PCR amplification in genomic DNA was used to determine the location of the initiation codon. All unpublished sequences from this study have been deposited in GenBank (accession numbers AF219907-AF219939). Portions of the *DGKI* genomic sequence have been recently deposited in GenBankas part of the large-scale sequencing of 7q for the Human Genome Project. BLAST analysis of these sequences confirms the location of several intron/exon junctions.

Patients and Families

Members of the large American RP10 family, UTAD045, were identified and blood collected as previously described [2,3]. Members of 47 small adRP families were ascertained at either (1) the Anderson Vision Research Center, Retina Foundation of the Southwest, Dallas or (2) the Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles. Informed consent was obtained from all subjects. For each family member tested, a clinical examination revealed the presence of RP. Autosomal dominant inheritance was established through pedigree evaluation.

Mutational Analysis

DNA was isolated from peripheral blood using standard extraction procedures. Individual exons were amplified by PCR with AmpliTaq Gold polymerase (Perkin Elmer) using the primers in Table 1 and standard cycling parameters. PCR products were radiolabeled by incorporating 1μCi ^{32}P-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). The amplified products of exons 3, 21, and 29 were digested with *Sma* I, *Sau3* I, and *Pal* I, respectively, in order to reduce fragment sizes. PCR products were denatured and separated on 0.6% MDE gels (FMC Bioproducts, Rockland, ME) at room temperature and 4 °C. Gels were dried and subjected to autoradiography after electrophoresis.

The underlying DNA change for each SSCP variant was determined by PCR product sequencing. Genomic DNA from patients was amplified by PCR using the conditions described above. PCR product sequencing was performed using either the Sequenase™ PCR Product Sequencing Kit (Amersham) or the Amplicycle™ Sequencing Kit (Perkin Elmer). PCR product was treated with Exonuclease I and Shrimp Alkaline Phosphatase (United States Biochemical, Cleveland, OH) prior to sequencing with the Amplicycle™ Sequencing Kit. Samples were run on either 6% Acryl-a-Mix™ (Promega) or 6% LongRanger™ (FMC Bioproducts) denaturing gels.

To insure that sequence variants present in the RP10 family were not missed due to the limitations of SSCP analysis, automated dideoxy sequencing of PCR product was performed using DNA from two affected and one unaffected member of UTAD045. Sequencing was terminated once linkage analysis of the polymorphism in intron 31 (see below) excluded diacylglycerol kinaseι as the cause of disease in UTAD045. All coding sequences except exons 2 and 27 were tested prior to the termination of sequencing.

SSCP and sequencing analysis revealed the presence of a sequence polymorphism in intron 31 at 3130-39 (G->A). This polymorphism was typed in members of UTAD045 using *Eco*R I digestion of PCR product. After digestion, PCR products were visualized on 2% agarose gels (Promega).

RESULTS

In this study we determined that *DGKI* is encoded in 35 exons with the initiation codon beginning in exon 2. Comparison of genomic sequence from clones containing *DGKI* with the cDNA sequence was used to determine the intron/exon junctions for exons 3 through 35. The 3’ intron/exon junction of exon 2 was identified using alternative methods. Intron/exon junctions for exons 2 through 35 are described in Table 2. The exact location of exon 1 and the 5’ exon 2 intron/exon junction could not be determined due to the presence of several repetitive sequences in the 5’ region of the *DGKI* cDNA. No information regarding this region was present in the databases. The genomic structure and flanking intronic sequence of *DGKI* was used to design primers for mutational analysis. PCR primers for exon 2 were designed based on the cDNA sequence and contain all but the first 17 bp of the coding sequence.

Samples from the American RP10 family, UTAD045, and 47 small adRP families without mutations in known adRP genes were tested for mutations in *DGKI*. Mutational analysis found one missense substitution, two silent substitutions, and five intronic variants. None of these variants appear to be disease-causing, and linkage testing of one of the polymorphic intronic variants in UTAD045 excludes mutations in *DGKI*as the cause of RP10.

Members of three small adRP families had a Lys153Phe (CTT->TTT) missense substitution in exon 3. Further testing demonstrated that this substitution did not segregate with disease in one of the families tested. Two silent substitutions, Thr865Thr (ACA->ACG) and Glu875Glu (GAA->GAG) were also identified in the samples tested. The Glu875Glu substitution is polymorphic in the African-American population (data not shown).

Intronic variation was detected in amplifications for exons 3, 5, 18, 28, and 32. None of these variants affect the splice site consensus sequence. The G->A transition at 3130-39 in intron 31 occurred at polymorphic levels in the population. This two-allele marker was typed in members of UTAD045. Recombinant individuals were detected in UTAD045 and linkage analysis of this marker with disease gave a LOD score of -4.2 at 0% recombination. Table 3 summarizes the sequence variants detected in this study.

DISCUSSION

No apparent disease-causing mutations were identified in *DGKI*, in adRP families. It is unlikely that the missense sequence change (Lys153Phe) seen in several of the families is disease causing since it does not segregate with disease in at least one of the families tested. None of the intronic variants detected in this study affect consensus splice sequences so they are also unlikely causes of disease. Identification of the sequence polymorphism in intron 31 allowed linkage testing between the disease locus in the UTAD045 family and *DGKI*. A LOD score of -4.2 at 0% recombination excludes *DGKI* as the possible cause of disease in this family, although portions of the gene 3’ of intron 31 are not formally excluded.

DGKI is excluded as the cause of RP10 by mutation screening and linkage analysis. Further, it is unlikely that mutations in *DGKI* are a common cause of other forms of autosomal dominant RP. It is possible though, that mutations in *DGKI* may yet be the cause of retinal degeneration in humans, either in homozygous or compound heterozygous forms. In order for disease to manifest in the *rdgA Drosophila*, both alleles of the diacylglycerol kinase have to be defective, therefore it is possible that both alleles of *DGKI* must also be mutated in humans to cause retinal degeneration [9]. Data presented here will make it possible to determine if mutations in *DGKI* are the cause of disease in patients with autosomal recessive RP or other forms of retinopathy.

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Table 1

PCR Primers Designed to Amplify *DGKI*

Exon	5' Primer	3' Primer	Annealing Temp. (\deg C)	Product Size (bp)
2A	GCTGCCATTTGCTGCCCC	CTTCCGCTGCTGCTGCTG	56	202
2B	CGCCATGAACCCCAGCTC	CCTGTACGAGACCTGCTTCC	60	249
3	TTTTTCCCCACAGTGGTCCC	AGGTATCTGAGGACAGGTCAGCAG	55	229,248*
4	CCCTGGAAAAAAAAAATTG	TCATTCAGATCCACAGGC	50	190
5	GAACTCTTCAGAACAGACTG	GCAATGTAACAAAACTGC	55	172
6	GAATCCACTGAATCAATGAG	CAGTCTAGTCTGATGCTTATC	52	174
7	TCCAGTGTGGGTGACAGAG	CTCAGTTGCTTGCAGAGG	52	208
8	TCATTGCACTTCAGGAGG	GACAAAACATGAGCCCAC	52	195
9	TTACTCTTTGACCAGAATTG	TCATTTTTCTCTGCACTG	50	210
10	TGGTTCTGCATGGGAAAG	ACTATGGAAAGAAAGTGG	52	194
11	AGCCAAGGGTGTGTATCC	AGTGAGAGTTGTGTCGTCAAG	52	208
12	AATGAGCAGTTATTCTTGGG	AGCAGAAAATTCTCACTTGC	55	209
13	GCTCTAGCCAGTGAAATGTTC	GATAGAATTGGCAGAGAACTG	55	157
14	CTCTGTTTCTCAAACTGTTTTC	TGATGATATGGGCAGTCC	55	226
15	GCTTCACTGTTCTCCACTCTGG	ATGCTCCAGGGCTCCTTTC	55	233
16	GGTTTTATTCTTCTCTTTCTGCCTG	TGGGGAGATGGAAATGGGAC	55	175
17	TCGCTACCTTTAGTGAGG	GCAACTTTATGATACACAGTG	52	179
18	GCAAGGATTTGGTATCAC	TTGGGAGTTTTTTGTGGC	52	190
19	GTGACTACTTCAAAACCC	TCAGAGTTCACGTCAAAC	52	160
20	GGTCTAACTACAAACTAACAC	CCTGTGAATTGAATAAACTC	52	261
21	AACTCCTTGCTCCCCTTTG	CCTGGACTTTTTTCCTCTTCC	55	162,185*
22	CATGGCTACCAATTCCGC	CGCATGAAATCAATGAGGC	52	191
23	TCATTCCTGTCCATATTCTGATTC	CAGCCCACAAAGATGAGAAAG	55	165
24	TTTTAACCACGAGAAACAC	CAAAAATGGAAAGTTGAGG	52	177
25	AGTTGTGGGGCAGCTTGTTC	GAAAGATGGACTTCATTTGGC	55	174
26	GAATGCTGAGTGAGGGAAGC	GATGCCTTGCTCTTCAACTTC	55	200
28	CTTCATGCAACAGACTTC	ATGGATGTCACCCTAAGGC	52	210
29	GGGTAGTATCTGGTAAACACTTC	TTGAAAGAGAATCCTGACC	55	135,266*
30	CCATTTAATTCAATTTGAGGG	TGAACTGCAAAGACCAGGG	50	194
31	GGGTCTTCTACTAATGGG	AGGGGATGATAAATGATAG	52	153
32	AAACATTGTGGTCTGCTAC	TACTTACCGTGGTCAAGG	52	230
33	CCAAGGCATTTTAATTCTGTG	CATTCTTCTCCCAGATATGTTC	50	195
34	TGGTTTCCCATTTCATCTTTG	GGAGGATGTTTGCACTCACTC	52	158
35A	TTTGTATTTCACGACTAGCG	GTCAAACAGCAGTTTCCAG	52	189
35B	CCGTCAGAACTATAAGGTCATTGGC	AAAGCTAGTGGCATGGTCTCAGG	55	198

*Size of product after enzyme restriction.

Table 2

Position and Nucleotide Sequence of Intron/Exon Boundaries of Diacylglycerol Kinaseι

Exon	Position in cDNA*	Acceptor Splice Site	Donor Splice Site
2	….-707		…TACAG/gtaggtg
3	708-815	gctgcag/GAAAG…	…GGAGT/gtaggtg
4	816-912	cttgcag/GAGAA…	…TTGCA/gtgagtg
5	913-987	attgtag/AAATC…	…AAAAG/gtgagtg
6	988-1044	atgacag/ATTAA…	…GAGAA/gtgagta
7	1045-1110	cccacag/AATTT…	…GTAAG/gtaagag
8	1111-1182	atcccag/GGCTT…	…AGGCG/gtaagat
9	1183-1299	tcttcag/TTTCA…	…CTCAG/gtaactg
10	1300-1373	cctacag/AACTC…	…TGGAA/gtaagtg
11	1374-1473	ccttcaa/CAGGA…	…ACCAG/gtgagta
12	1474-1556	tttacag/GGAAC…	…GATGC/gtaagtc
13	1557-1617	tctacag/GCTTG…	…GAACG/gtaggtc
14	1618-1731	cctgtag/GTGGG…	…GAGGG/gtaagaa
15	1732-1869	tcctcag/GGCTA…	…GTAAG/gttagag
16	1870-1948	tttgcag/CTCCC…	…CAGAG/gtgagga
17	1949-2004	ttcacag/AAGCA…	…CAGGG/gtatata
18	2005-2067	ggtatag/GCAGC…	…TTGTT/gtaagta
19	2068-2141	gtttcag/TGTGA…	…CCCAG/gtaggct
20	2142-2253	cttgcag/ATATT…	…CTTTG/gtgagca
21	2254-2453	tttatag/GCAGC…	…AATGA/gtgagtc
22	2454-2554	tttgcag/TCCCC…	…AGCTT/gtaagtt
23	2555-2608	atttcag/CTATT…	…TGGAG/gtaagaa
24	2609-2688	cccttag/CGATA…	…AGGAG/gtgagtt
25	2689-2756	tcattag/GACCT…	…CCCAG/gtaacaa
26	2756-2802	tccccag/GGCTC…	…AGATG/gtgagtt
27	2803-2813	cgaatag/ACAGA…	…CTCAG/gtaagtt
28	2814-2942	tccttag/GAACA…	…TCGGG/gtgagtg
29	2943-3065	ctctcag/GATCT…	…CTACA/gtcacca
30	3066-3091	cttttag/GTCAC…	…TCATG/gtaagaa
31	3092-3129	ttcacag/CAATT…	…TGAAG/gtaagat
32	3130-3250	tttgcag/CTAAT…	…CCACG/gtaagta
33	3251-3287	tatttag/GACCT…	…GAAAC/gtgagtg
34	3288-3387	gatttag/GGGTG…	…CCAAG/gtaactc
35	3388-	cgtatag/GGTAA…	

*Positions in the cDNA are given according to the GenBank *DGKI* cDNA sequence accession AF061936

Table 3

Sequence Variants Identified in Diacylglycerol kinase ι

Location	cDNA variant	Protein change	Frequency
Exon 3	743 C-\gt T	Lys153Phe	0.03
Exon 28	3001 A-\gt G	Thr865Thr	0.05
Exon 28	3031 A-\gt G	Glu875Glu	0.14*
Intron 31	3130-39 G-\gt A	none	0.49

* Frequency in African-American population. This substitution was not seen in any Caucasians tested.