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Journal

Biochimica et Biophysica Acta, 1260(3)

ISSN

0006-3002

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Publication Date

1995-02-01

DOI

10.1016/0167-4781(94)00231-q

Peer reviewed

Short Sequence-Paper

Sequence analysis and exclusion of phosducin as the gene for the recessive retinal degeneration of the abyssinian cat [☆]

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Received 18 August 1994; accepted 18 November 1994

Abstract

Phosducin was evaluated as a candidate gene for the recessive retinal degeneration in the Abyssinian cat, rdAc, using reverse transcription and polymerase chain amplification. The nucleotide sequence of the cat phosducin coding region was determined except for 23 bp at the 5' end. Single-strand conformation analysis of a silent polymorphism within the coding region established the nonlinkage of the phosducin gene with the rdAc locus.

Keywords: Retinal degeneration; Phosducin; Candidate gene; (Cat)

In cats homozygous for the mutation responsible for the recessive retinal degeneration, rdAc, ultrastructural alterations can be seen in photoreceptors even prior to retinal maturation [1]. Ophthalmoscopic abnormalities are not evident until 1.5 years of age, and visual loss progresses to complete blindness in approx. 3 years of age. Initially, visual function and electrophysiology appear relatively intact, but electroretinogram (ERG) changes are evident between the ages of 8–12 weeks. The loss of the rod B-wave amplitude correlates with the loss of rhodopsin, with no evidence that overall retinal sensitivity is reduced by other mechanisms. In this respect, the ERG changes in rdAc/rdAc animals are comparable to those observed for the diffuse form of human dominantly-inherited RP [2]. The onset and progression of retinal disease is considerably slower than that associated with the murine *rd* and *rd*s mutations or the rod-cone dysplasias of the Irish setter or collies but is comparable to the progressive rod-cone degeneration in miniature poodles [3]. Despite the early ultrastructural abnormalities, visual function is largely pre-

served until 2–3 years of age. The combination of electrophysiology, visual pigment reflectometry, light and ultrastructural histology all indicate that this recessive retinal disorder is primarily localized to a defect in the photoreceptors, with the initial disturbances being localized to the rods followed by secondary degeneration of the cones.

Phosducin is a highly conserved 33 kDa phosphoprotein that was initially found in the rod and cone photoreceptors, as well as in the pineal gland [4], but is now recognized to be present in other tissues [5,6]. This soluble protein is distributed within the cytoplasm of the photoreceptors. While the function of this protein is unknown, its phosphorylation state is tightly coupled to the light-adapted state of the eye. Specifically, phosphorylation is maximal in the dark-adapted condition and appears to be mediated by protein kinase A, which is sensitive to intracellular concentrations of cAMP [7]. The protein is dephosphorylated when the retina is illuminated. Both the phosphorylated and unphosphorylated forms of phosducin form tight complexes with the β and γ subunits of transducin [8]. It has been proposed that phosducin serves as a regulatory mediator between the phototransduction system and the light/dark-mediated molecular processes of the photoreceptors. A number of genes whose products interact within the phototransduction cascade, such as opsin, the β subunit of phosphodiesterase, and phosphokinase C have already been identified as causing retinal degenerations. Any

[☆] The nucleotide sequence data of the coding region of the cat phosducin cDNA have been submitted to the EMBL/GenBank Data Libraries as CATPHOSDUC under the accession number L35314.

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          10          20          30          40          50          60
          *          *          *          *          *          *
AA ATG GAA GAA GCC AAA AGC CAA AGT TTG GAG GAA GAC TTT GAA GGA CAG GCT ACC CAT ACA GGA
Met Glu Glu Ala Lys Ser Gln Ser Leu Glu Glu Asp Phe Glu Gly Gln Ala Thr His Thr Gly >

          70          80          90          100          110          120          130
          *          *          *          *          *          *          *
CCC AAA GGA GTA ATA AAT GAT TGG AGA AAG TTT AAA TTA GAG AGT GAA GAC AGT GAT TCA GTT CCA
Pro Lys Gly Val Ile Asn Asp Trp Arg Lys Phe Lys Leu Glu Ser Glu Asp Ser Asp Ser Val Pro >

          140          150          160          170          180          190
          *          *          *          *          *          *
CCA AGC AAG AAG GAG ATT CTC AGA CAA ATG TCT TCT CCT CAG AGT AGA GAT AAC AAA GAC TCA AAA
Pro Ser Lys Lys Glu Ile Leu Arg Gln Met Ser Ser Pro Gln Ser Arg Asp Asn Lys Asp Ser Lys >

          200          210          220          230          240          250          260
          *          *          *          *          *          *          *
GAA AGA TTC AGC AGA AAG ATG AGC ATT CAA GAA TAT GAA CTA ATC CAC CGG GAC AAA GAA GAT GAA
Glu Arg Phe Ser Arg Lys Met Ser Ile Gln Glu Tyr Glu Leu Ile His Arg Asp Lys Glu Asp Glu >

          270          280          290          300          310          320
          *          *          *          *          *          *
AAT TGC CTT CGT AAA TAC CGG AGA CAG TGT ATG CAG GAT ATG CAC CAG AAG CTG AGT TTT GGG CCT
Asn Cys Leu Arg Lys Tyr Arg Arg Gln Cys Met Gln Asp Met His Gln Lys Leu Ser Phe Gly Pro >

          330          340          350          360          370          380          390
          *          *          *          *          *          *          *
AGA TAT GGG TTT GTG TAT GAG CTG GAA ACT GGG GAG CAA TTC CTG GAA ACT ATT GAA AAG GAG CAG
Arg Tyr Gly Phe Val Tyr Glu Leu Glu Thr Gly Glu Gln Phe Leu Glu Thr Ile Glu Lys Glu Gln >

          400          410          420          430          440          450          460
          *          *          *          *          *          *          *
AAA ATC ACC ACA ATT GTT GTT CAC ATT TAT GAA GAT GGC ATT AAG GGC TGT GAT GCT CTA AAC AGT
Lys Ile Thr Thr Ile Val Val His Ile Tyr Glu Asp Gly Ile Lys Gly Cys Asp Ala Leu Asn Ser >

          470          480          490          500          510          520
          *          *          *          *          *          *
AGC TTT ACA TGC CTT GCA GTT GAA TAC CCT ATG GTC AAG TTT TGT AAA ATA AAA GCT TCT AAT ACA
Ser Phe Thr Cys Leu Ala Val Glu Tyr Pro Met Val Lys Phe Cys Lys Ile Lys Ala Ser Asn Thr >

          530          540          550          560          570          580          590
          *          *          *          *          *          *          *
GGT GCT AGG GAC CGC TTT TCC TCA GAT GTA CTC CCC ACA CTG CTT GTC TAC AAA GGT GGG GAA CTC
Gly Ala Arg Asp Arg Phe Ser Ser Asp Val Leu Pro Thr Leu Leu Val Tyr Lys Gly Gly Glu Leu>

          600          610          620          630          640          650
          *          *          *          *          *          *
ATA AGC AAT TTT ATT AGT GTT TCT GAA CAG TTT GCA GAA GAA TTT TTT GCT GGG GAT GTG GAG TCT
Ile Ser Asn Phe Ile Ser Val Ser Glu Gln Phe Ala Glu Glu Phe Phe Ala Gly Asp Val Glu Ser >

          660          670          680          690          700          710          720
          *          *          *          *          *          *          *
TTC CTA AAT GAA TAT GGG TTA CTA CCT GAA AGA GAG ACA CAT GCC CTA GAT CAG ACC AAC ATG GAG
Phe Leu Asn Glu Tyr Gly Leu Leu Pro Glu Arg Glu Thr His Ala Leu Asp Gln Thr Asn Met Glu >

          730          740          750          760          770          780          790
          *          *          *          *          *          *          *
GAA GAT ATT GAA TAA AGA GTC ACT ATG TCA ACA TCT CAT ATT TCT CCT TTA CAA GTT GAG CAT TGA
Glu Asp Ile Glu ***

          800          810          820          830          840
          *          *          *          *          *
TTT TCA TAG TAT CTA TAT TCC TTT AGA GAA CAC CAA GTA TGG CCA TGG CT

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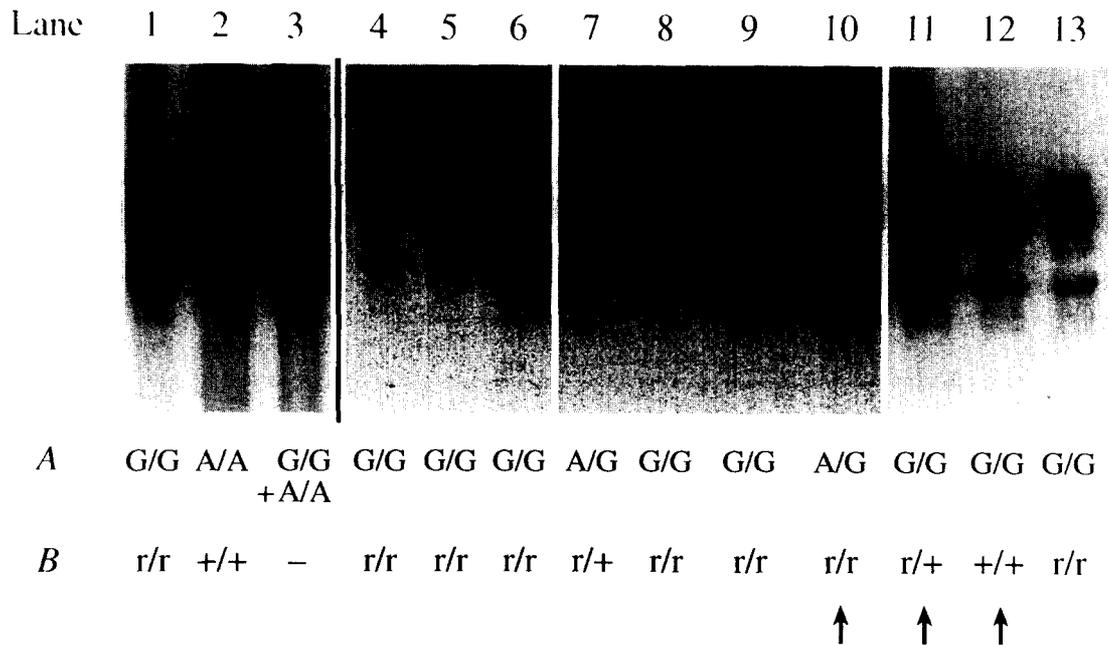


Fig. 2. Single-strand conformation analysis of the RT/PCR and genomic PCR. Lanes 1 and 2 are amplified products from the RT/PCR of normal and rdAc/rdAc retinal RNA. Lane 3 is a mixture of the PCR products from lanes 1 and 2. The remaining lanes are the PCR products from genomic DNA templates of cats within the colony. Line A indicates the allele typing based upon the phosducin SSCP. G = the SSCP allele corresponding to the G substitution in Gln-122 while A indicates the SSCP allele corresponding to the A in the third codon position; Line B specifies the genotypes of the cats based on phenotype as well as known matings (r = rdAc allele, + = wild type allele). The arrows indicate the samples which demonstrate discordance of the rdAc alleles with those of the phosducin SSCP.

retina-specific gene that participates in this pathway could be a candidate gene for the retinal degeneration in the Abyssinian cat. We selected the phosducin transcript for detailed studies because reverse transcription (RT)-polymerase chain amplification (PCR) experiments of transcripts from 4-week-old cat retinas had shown a selective reduction of the phosducin transcript in the rdAc/rdAc retina as compared to other photoreceptor-specific transcripts (data not shown).

RNA was purified from retinas of 4-week-old normal and affected cats [9]. Each retina was isolated and extracted individually and care was taken to keep the samples isolated from each other. The extraction of RNA from the normal and affected cats was carried out on separate days to minimize the possibility of any cross contamination.

The primers were selected based upon the published nucleic acid sequences for the mouse gene. The primer sequences were constrained to the coding region to maximize cross-species conservation. The primers were chosen to be 25 nucleotides in length in order to account for potential mismatches. Codon usage and third-position codon variations were considered in the selection of the primer sequences. All oligonucleotides were synthesized using the Cruachem PS250 synthesizer and were either

used directly or were purified using NENSorb columns (DuPont NEN Research Products, Boston, MA).

RT/PCR was performed according to the Perkin Elmer-Cetus protocols. 50 μ l PCR reactions were routinely performed using 30 cycles consisting of the following parameters: initial denaturation, 95°C, 3 min; annealing, 55°C, 1 min; synthesis, 72°C, 2 min; cycle denaturation, 95°C, 1 min. For some primer sets, the annealing temperature was raised to 60°C in order to reduce nonspecific synthesis. The 3' end of the coding region was initially amplified using 3' RACE (Gibco/BRL, Gaithersburg, MD) and then further enriched by nested PCR.

The PCR fragments were purified by agarose gel electrophoresis and ligated into the TA cloning vector (Invitrogen, San Diego, CA) according to the manufacturer's protocol. The chimeric plasmids were transformed into INV α F' cells and were selected on LB plates supplemented with ampicillin (50 μ g/ml) and X-gal (1.0 mg per 35 ml plate). Chimeric plasmid colonies were purified and the presence of the correct insert was established using a rapid PCR assay.

Double-stranded nucleotide sequencing was performed using plasmid-based primers as well as the primers used to generate the RT/PCR fragments. When necessary, additional sequencing primers were synthesized to complete

Fig. 1. Nucleotide and amino acid sequences of the coding region of the cat phosducin transcript. The first 25 underlined nucleotides were converted to the mouse sequence by the 5' PCR primer. The A to G silent polymorphism in Gln-122 that was detected by SSCP analysis is noted in italics.

the sequencing. All sequencing reactions were done using standard protocols (Sequenase[®], United States Biochemical, Cleveland, OH) [10] and each fragment was sequenced on both strands from at least two different start positions. Multiple clones from both normal and affected animals were sequenced to establish that observed nucleotide differences were not the result of PCR-induced alterations. Direct sequencing of PCR products, including the 3'-terminus of the coding region, was done using a double-stranded DNA cycle sequencing protocol (Gibco/BRL, Gaithersburg, MD).

The PCR products obtained by RT/PCR and those amplified directly from genomic DNA were briefly reamplified in the presence of ³²P-labeled oligonucleotide primers and then used for single-strand conformation analysis (SSCP). The forward primer, 5'-ACCAGAAGCTGAGTTTTGGGC-3', and the reverse primer, 5'-GGAGTACATCTGAGGAAAAGCG-3', flank the nucleotide alteration and are within a single genomic exon [11]. The DNA was alkali-denatured and electrophoresed on nondenaturing 5% acrylamide gels containing 5% glycerol at room temperature. The gels were dried and the bands were detected by autoradiography.

The cat phosducin sequence was obtained by sequencing several cDNA clones derived from the RT/PCR products using normal and rdAc/rdAc retinal RNA templates. Overlapping fragments were generated so that a complete cat sequence could be constructed except for the 23 nucleotides at the beginning of the coding region that are replaced by the 5' oligonucleotide primer. Repeated efforts to amplify and clone the 5' terminus of the transcript with primers from the 5' untranslated region and by 5' RACE (Gibco/BRL, Gaithersburg, MD) were unsuccessful.

The nucleotide sequences for the rdAc/rdAc and normal cat phosducin transcripts were identical except for two nucleotide substitutions. A silent A to G substitution was identified in the third position of Gln-122. A T to C change in the first position of Phe-167 altered the predicted amino acid in the normal cat phosducin transcript to a Leu in the corresponding rdAc transcript. The coding sequence for the cat phosducin transcript is presented in Fig. 1; note that the first 23 nucleotides (underlined) are derived from the mouse phosducin sequence. In order to eliminate the possibility that the base differences between the rdAc/rdAc and the normal phosducin sequences were the result of Taq polymerase-induced artifacts, direct nucleotide sequencing of the PCR products was performed. The nucleotide difference associated with Phe-167 was not observed by direct PCR sequencing and most likely was an artifact that arose during PCR amplification and then perpetuated in the cloned fragment. The nucleotide substitution in the Gln-122 codon was identified in both RT/PCR products and amplification fragments generated from genomic DNA.

SSCP confirmed that the polymorphism was detectable by RT/PCR and by PCR of the genomic DNA from other

cats within the colony (Fig. 2). Despite the large size of the amplified fragment, the altered mobilities were clearly identifiable. While the majority of the affected animals (which are highly inbred) are homozygous for the G substitution, the DNA in the lanes marked by arrows indicate nonlinkage of the polymorphism with the rdAc alleles. Direct nucleotide sequencing confirmed that the SSCP polymorphisms in these animals reflect the predicted nucleotide sequence difference (data not shown).

A comparison of the cat phosducin nucleotide sequence with those of other mammals demonstrates that the gene product is highly conserved [4,12–14]. Cross species comparisons of nucleotide and amino acid substitutions can define conserved regions of the phosducin peptide. In order to avoid a biased assessment of these differences, these comparisons are founded upon congruence analysis [15], which employs existing data sets to obtain robust phylogenetic evidence. While there are a number of proposed phylogenies that relate these five mammalian species, the only consensus regards the close relationship of the rat and mouse. While there is some evidence that the cat (as a member of carnivora) is more closely related to rodentia [16], most models are unable to distinguish the evolutionary points of bifurcation of carnivora, artiodactyla (cow) and primates (human) along the phylogenetic tree [16–18]. The minimum nucleotide and amino acid substitutions along the phosducin coding region are based upon the simplest consensus phylogeny.

A subset of the observed nucleotide and amino acid substitutions can be attributed to the evolution of rodentia from a common ancestral sequence. This group of sequence alterations provide the most convincing evidence for the conserved regions of peptide sequence. At these positions, the nucleotides from the cat, human, and cow, are identical and there is a high degree of certainty that the substitutions in the mouse and/or rat sequences reflect a

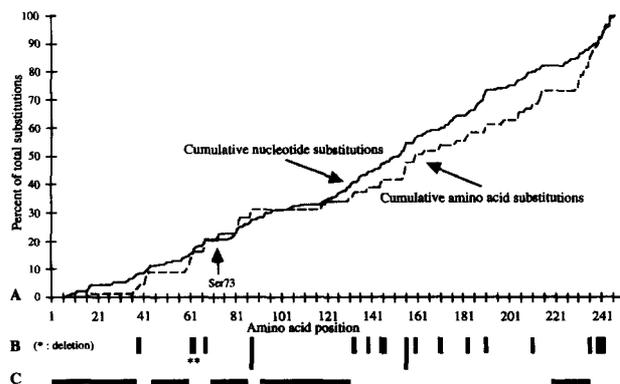


Fig. 3. Distribution of nucleotide and amino acid substitutions along the phosducin coding sequence. The nonlinearity of the cumulative amino acid substitutions indicates regions of relative conservation as compared to the almost even distribution of nucleotide substitutions along the coding region. Amino acid substitutions specifically attributed to rodentia are shown below the main graph with bars indicating the major regions of amino acid conservation.

change from an established ancestral condition. The cumulative distribution of all nucleotide substitutions along the phosducin coding region as well as the cumulative amino acid substitutions are shown in Fig. 3. Of the 193 nucleotide substitutions, 88 could be specifically localized to the rodentia branch. This is consistent with the reportedly higher rate of mutations that have been observed in the evolution of rodentia [19,20]. The distribution of this selected group of nucleotide substitutions is not shown in Fig. 3 but it is virtually identical to the distribution of all of the nucleotide substitutions identified among the different species. The amino acid substitutions for the rodentia branch of the phylogenetic tree are shown in Fig. 3B. The distribution of these substitutions provides strong evidence for five major conserved regions of the phosducin peptide. Note that the Ser-73 that is phosphorylated by protein kinase A [11] is located within the third conserved region of the phosducin peptide.

RT/PCR, combined with SSCP analysis and nucleotide sequencing, is an effective approach for candidate gene analysis. The nucleotide sequence provides a reliable means of genetic analysis and complements SSCP and other forms of mutation analysis. While we encountered difficulties in completing the nucleotide sequence of the 5' end of the phosducin coding region, we have definitively excluded phosducin as a candidate gene for this retinal degeneration by demonstrating genetic nonlinkage of a polymorphism within the phosducin gene with the rdAc allele. Analyses of coding regions do not exclude the possibility of mutations that modify transcription or RNA processing and create a population of altered peptides that would disrupt cellular function. This would be an unlikely mechanism for a recessive disorder such as rdAC, which is more likely a point mutation in a critical photoreceptor gene.

The authors express their appreciation towards Dr. Anthony Bledsoe, Department of Biological Sciences, University of Pittsburgh, for his proposal to use phylogenetic data to identify regions of protein conservation. Dr. Toshimuchi Shinohara graciously made the phosducin nucleotide sequences available prior to their publication. This work was supported by grants from The National Eye

Institute, EY09192, and Research to Prevent Blindness, New York, NY. A portion of this work was previously presented at the Association of Research in Vision and Ophthalmology, Sarasota, FL, 1991.

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