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Primary structure of frog rhodopsin

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Amphibians have been employed extensively to study the anatomy, physiology, biochemistry, and cell biology of the visual system for decades, yet there have been no reports concerning the primary structure of amphibian visual transduction components. Thus, we have determined the entire nucleotide sequence of frog (*Rana pipiens*) rhodopsin cDNA, including a putative transcription start point and poly A tail, by sequence analysis of PCR products and mRNA. The open reading frame predicts an opsin of 354 residues, six residues longer than the mammalian rod opsins, containing 11 potential phosphorylation sites in the C-terminal domain. RNA blot analysis revealed two transcripts of ca. 1.7 and 3.1 kb. Frog rhodopsin exhibits ~85% identity to mammalian rhodopsin at the amino acid level. Sequence analysis of additional components will produce the framework from which a more detailed understanding of amphibian phototransduction can emerge.

Rhodopsin; Frog (*Rana pipiens*); Photoreceptor; Polymerase chain reaction; RNA sequencing, Northern analysis; Transcription start point; Visual transduction

1. INTRODUCTION

Rhodopsin, the visual pigment of retinal rod photoreceptor cells, consists of an $M_r \approx 40$ kDa glycoprotein (opsin) to which a light-sensitive chromophore (11-*cis* retinaldehyde) is attached (reviewed in [1–4]). It is also the most extensively studied and prototype member of a superfamily of G-protein coupled membrane receptors, which share a common seven transmembrane helix domain structure [4,5]. The recent identification of over 30 mutations in the human rhodopsin gene [6–8] in well-defined cases of autosomal dominant retinitis pigmentosa (adRP), a hereditary blinding disease, has sparked intense interest in defining the relationship of rhodopsin's structure to its physiological function in the photoreceptor cell. Herein, we report the complete primary structure of rhodopsin from the common leopard frog, *Rana pipiens*, as determined exclusively by RNA sequencing and analysis of PCR products, and briefly compare our findings with some of the known structural features of rhodopsins from other species.

2. MATERIALS AND METHODS

2.1. *RNA isolation and cDNA synthesis*

Fifty retinas were dissected free of pigment epithelium under ice-chilled phosphate-buffered saline from dark-adapted frogs (*Rana pipiens*, Northern; Charles Sullivan Co., Nashville, TN), and frozen immediately in liquid nitrogen. Poly A^+ RNA was isolated directly from homogenized retinal tissue using the Fast Track mRNA isolation kit (Invitrogen). Prior to synthesis of the first strand of cDNA, RNA was precipitated at −20°C with 1 M LiCl and 7 vols. of ethanol to remove residual sodium dodecyl sulfate. Synthesis of cDNA was carried out with 2 μg of retinal RNA and 1 μg of a modified oligo-dT primer [9] using a cDNA synthesis kit (Promega). The cDNA was then diluted 1.10 with water and stored at −20°C prior to use.

2.2. *Oligonucleotide synthesis and PCR*

Oligonucleotides were synthesized with an Applied Biosystems Model 391 PCR-Mate DNA synthesizer. The oligomers were deprotected and cleaved from the column in 30% ammonium hydroxide at 55°C for at least 8–16 h. Ammonium hydroxide was removed by evaporation in a speed-vac and the oligomers were dissolved in water. PCR was performed in a total volume of 25 μl, or 400 μl for preparative purposes, as previously described [10]. The following primers were employed: A, 5′-ATGAACGGCACAGAGGGCCCCAA; B, 5′-TTCCGGAACTGCATGCTCAC; C, 5′-CCGCATGCGGCCGCAGATCTAGA, D, 5′-CGATGATCTTATGCAGGTGACACC; E, 5′-GCCAGGTAATACTGAGGGTAGTCG (see Fig. 1), in addition sequencing primers 5′-CTGGCCAGTTCATCTTGCTAAA, 5′-CAACCCTGTCATCTACATT, 5′-CTGGACCAGCCAACGAGTGG, 5′-AAATTGCCCTGTGGTCCTTGG, and 5′-CCTTGTTAAGGCAAAGAATGCCG were also used.

2.3. *DNA and RNA sequencing*

DNA sequencing of amplified PCR products was carried out as previously described [10]. RNA sequencing was performed using 6 μg of frog retina poly A^+ RNA and an internal frog opsin primer in a reverse transcription reaction as described [11].

2.4. *RNA blot analysis*

One to 2 μg of poly A^+ RNA was electrophoresed in a 0.8% agarose, formaldehyde-containing gel, transferred to nitrocellulose membranes [12], and hybridized [11] with a radiolabeled frog PCR product, PCR-BC (Fig. 1). Two initial washes were carried out in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate) at room temperature. Four 20 min high-stringency washes were performed at 60°C in 0.1 × SSC prior to autoradiography without intensifying screens.

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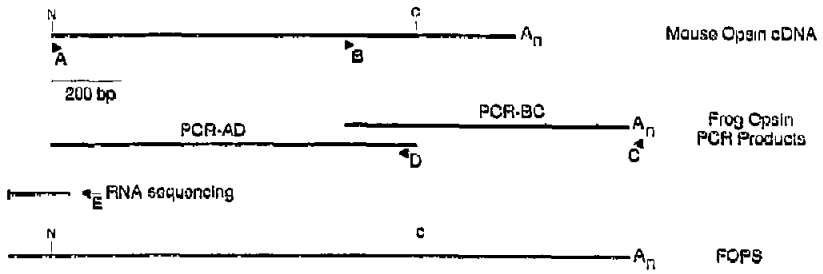

Fig. 1. Amplification and RNA sequencing of frog rhodopsin. Shown at the top is a schematic representation of a portion of mouse opsin cDNA. Primers A and B were designed from the mouse rhodopsin sequence [9]. Primers D and E were designed from the sequences determined from PCR products, PCR-AD, and PCR-BC. Inclusion of the results from RNA sequencing with Primer E results in the full length opsin mRNA designated FOPS.

2.5. *Computer analysis of DNA sequences*

Analysis was performed on microcomputers using Microgenie (Beckman) or on a minicomputer for multiple sequence alignments and database searches through the Molecular Biology Information Resource (Department of Cell Biology, Baylor College of Medicine).

3. RESULTS AND DISCUSSION

3.1. *PCR amplification of frog opsin*

We developed a general scheme for PCR amplification of the entire frog opsin mRNA that should be applicable for any moderate to high abundance transcript in which sequence information is available from another species. The relatively high degree of sequence conservation among the opsins from diverse species [3], while not essential for success, greatly simplified the use of cross-species PCR amplification. As shown in Fig. 1, in the first PCR reaction a primer specific for mouse opsin cDNA sequence (B) was used in conjunction with a universal 3′ end primer (C) [9] and frog first strand cDNA as a template, allowing amplification of frog opsin-specific sequence (PCR-BC) from the 3′ end of the mouse opsin primer to the poly A tail. The sequence determined from product PCR-BC was then used to construct a frog opsin-specific antisense primer representing the C-terminal region (D). This primer was used in a PCR reaction with a primer representing the N-terminal region of mouse opsin (A), yielding a PCR product covering the majority of the protein coding region (PCR-AD). A frog opsin-specific antisense primer (E) was synthesized according to the sequence determined near the 5′ end of product PCR-AD for direct sequencing of frog poly A^+ RNA, allowing determination of sequence up to a putative transcription start point of the frog opsin gene.

3.2. *RNA blot analysis*

Since several rhodopsin genes and cDNAs have been analyzed in the laboratory where the PCR was done, RNA blot analysis was used to confirm the origin of the PCR amplified material. Previously we showed that two transcripts of 3.0 and 1.7 kb are identified in frog retinal RNA using a mouse opsin cDNA probe [9]. A blot containing fish and chicken retinal RNAs was probed since the chicken [20] and frog opsin sequences are more closely related, and the fish opsin sequence has been amplified in the lab, but not yet sequenced. As shown in Fig. 2, using high stringency washing conditions, only RNA from frog retina showed hybridization to RNAs of 1.7 and 3.1 kb; lanes containing RNA from frog liver, chicken retina, or fish retina exhibited no hybridization signal, even after prolonged exposure times (not shown). We had also previously shown that the multiple mouse opsin transcripts identified by RNA blot analysis result from the use of alternate poly A sites in the 3′ end of the mouse opsin gene [9]. Since only a single 5′ end is indicated by RNA sequence analysis, it is likely that the frog opsin gene also produces its two transcripts by the same mechanism. The length of the sequence shown in Fig. 2 is consistent with it representing the shorter 1.7 kb mRNA species.

3.3. *Primary structure of frog opsin*

The sequences determined from the overlapping PCR products, PCR-AD, PCR-BC, and from the RNA sequencing with primer E, allowed a composite full length frog opsin sequence (see Fig. 1, FOPS) to be constructed (Fig. 3). The 1,730 nt sequence consists of 105, 563, and 1,062 residues of 5′, 3′, and protein coding segments, respectively. The open reading frame predicts a protein of 354 amino acids; six residues longer than the mammalian rod opsins sequenced to date, all of which are 348 residues in length. The additional residues are found in the C-terminal domain resulting in 11 potential phosphorylation sites, as opposed to the 6 or 7 found in mammalian opsins. With the exception of bovine [13,14] and ovine [15], which have 7, all other mammalian, *Drosophila* [16,17], and blowfly [18] opsins have 6 potential phosphorylation sites, while opsins from lamprey [19] and chicken [20] have 11 and 10 sites, respectively, in the C-terminal domain (see Fig. 3A). Additional phosphorylation sites may explain the faster rate of phosphorylation suggested for frog rhodopsin as compared to bovine [21].

As with other known vertebrate rhodopsins, frog rho-

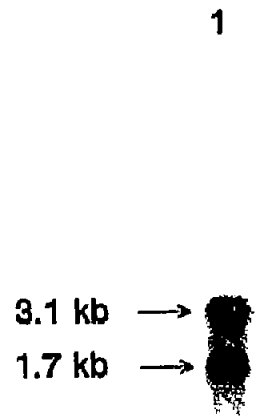

Fig. 2. RNA blot analysis of frog rhodopsin. Approximately 2 μg of poly A$^+$ from each tissue was electrophoresed onto a formaldehyde containing agarose gel. After blotting to nitrocellulose the blot was probed with a radiolabeled frog opsin PCR product representing the 3' end and washed at high stringency. Lanes 1–5 contain frog retina, frog liver, chicken liver, chicken retina, and fish retina RNAs, respectively.

dopsin contains two *N*-glycosylation consensus sequences near the N-terminus (Fig. 4A). Within the first 17 residues of the N-terminal domain including the glycosylation sites, there is 88% identity of the frog sequence with that of bovine and ovine rhodopsins, and 82% with the rhodopsin sequences of human [22], chicken and lamprey. The first *N*-glycosylation site (Asn^2-Gly^3-Thr^4) is identical in all vertebrate species, while the second site (Asn^{15}-Lys^{16}-Thr^{17}) is identical in most species examined thus far, except human and mouse, where Lys^{16} is replaced by an aliphatic residue (Ala or Val, respectively). This is consistent with the Asn-Xaa-Ser/Thr consensus sequence where Xaa can be any residue except proline [23]. The presence and structures of oligosaccharides at both glycosylation sites of frog rhodopsin have been confirmed recently [24].

The C-terminal domain of frog rhodopsin also contains two putative adjacent sites for palmitoylation (Cys^{322} and Cys^{323}, Fig. 4B), as found in all other rhodopsins sequenced thus far. The presence of a covalently attached palmitoyl moiety has been rigorously demonstrated for bovine rhodopsin [25,26]. It is presumed that the palmitoyl moieties insert into the lipid bilayer of the rod outer segment disc membrane, thereby anchoring a proximal region of the C-terminal domain to the membrane. The additional loop that would be predicted to exist (see Fig. 5) has been shown to be involved in interaction with rhodopsin's G-protein, transducin [27]. The C-terminal residue (Ala^{354}, Fig. 2) is also found at the C-terminus of all other rhodopsins thus far analyzed, including squid and octopus rhodopsins [28,29], which have long insertions near their C-termini (see Fig. 3). It is possible that the C-

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ACCTCTTCTGGAACATAAAGTTTTTCTTGGGGTTCCTGAGACGAGGGAGAAACACAGAAGTGGTTTCTGAGCCGAGGGGGGTGAGAACTTTCAAGAGCTGCCACCATGAACGGAACAGAA-120
                                                                                                    *  M  N  G  T  E -5

GGTCCCAATTTTTACATCCCCATGTCTAACAAGACTGGGGTAGTACGAAGCCCCTTCGACTACCCTCAGTATTACCTGGCAGAGCCATGGAAGTATTCAGTATTGGCCGCCTACATGTTC-240
 G  P  N  F  Y  I  P  M  S  N  K  T  G  V  V  R  S  P  F  D  Y  P  Q  Y  Y  L  A  E  P  W  K  Y  S  V  L  A  A  Y  M  F -45

TTGCTCATCCTCCTCGGTTTACCAATCAACTTTATGACCCTGTATGTCACCATCCAGCACAAGAAGCTCCGAACACCCTTAAACTACATCCTGCTAAATCTTGGCGTTTGCAACCACTTC-360
 L  L  I  L  L  G  L  P  I  N  F  M  T  L  Y  V  T  I  Q  H  K  K  L  R  T  P  L  N  Y  I  L  L  N  L  G  V  C  N  H  F -85

ATGGTCCTGTGTGGATTCACCATCACGATGTACACCTCCCTCCATGGATACTTTGTATTCGGACAGACTGGTTGCTACTTTGAAGGCTTCTTCGCTACCCTTGGTGGTGAAATTGCCCTT-480
 M  V  L  C  G  F  T  I  T  M  Y  T  S  L  H  G  Y  F  V  F  G  Q  T  G  C  Y  F  E  G  F  F  A  T  L  G  G  E  I  A  L 125

TGGTCCCTGGTGGTGTTGGCCATTGAGCGATACATTGTGGTCTGCAAGCCCATGAGCAATTTCCGATTTGGTGAGAACCATGCCATGATGGGTGTAGCATTCACCTGGATCATGGCCTTG-600
 W  S  L  V  V  L  A  I  E  R  Y  I  V  V  C  K  P  M  S  N  F  R  F  G  E  N  H  A  M  M  G  V  A  F  T  W  I  M  A  L -165

GCTTGCGCTGTTCCCTCCACTCTTCGGCTGGTCCAGATACATCCCTGAAGGAATGCAGTGCTCATGTGGAGTTGACTACTACACTCTGAAGCCCGAGGTCAACAATGAGTCCTTTGTCATC-720
 A  C  A  V  P  P  L  F  G  W  S  R  Y  I  P  E  G  M  Q  C  S  C  G  V  D  Y  Y  T  L  K  P  E  V  N  N  E  S  F  V  I -205

TACATGTTCGTTGTCCACTTCCTCATCCCTCTGATCATCATTTCCTTCTGCTACGGACGTCTGGTCTGCACTGTGAAAGAGGCTGCAGCCCAGCAGCAAGAATCAGCCACCACCCAGAAG-840
 Y  M  F  V  V  H  F  L  I  P  L  I  I  I  S  F  C  Y  G  R  L  V  C  T  V  K  E  A  A  A  Q  Q  Q  E  S  A  T  T  Q  K -245

GCCGAGAAAGAAGTCACCCGAATGGTTATCATTATGGTCATTTTCTTCCTGATTTGCTGGGTCCCCTACGCCTATGTTGCATTCTACATCTTCACCCACCAAGGCTCAGAGTTCGGCCCG-960
 A  E  K  E  V  T  R  M  V  I  I  M  V  I  F  F  L  I  C  W  V  P  Y  A  Y  V  A  F  Y  I  F  T  H  Q  G  S  E  F  G  P -285

ATTTTCATGACCGTGCCAGCTTTCTTTGCCAAGAGCTCTGCCATCTACAACCCTGTCATCTACATTATGCTGAACAAACAGTTCCGTAACTGCATGATCACCACCCTGTGCTGTGGCAAA-1080
 I  F  M  T  V  P  A  F  F  A  K  S  S  A  I  Y  N  P  V  I  Y  I  M  L  N  K  Q  F  R  N  C  M  I  T  T  L  C  C  G  K -325

AATCCCTTTGGAGATGACGATGCCTCCTCTGCTGCCACTTCCAAGACAGAAGCCACCTCTGTCTCTACCAGCCAGGTGTCACCTGCATAAGATCATCGTCCAAGCCTGTGTCAGGTGCCT-1200
 N  P  F  G  D  D  D  A  S  S  A  A  T  S  K  T  E  A  T  S  V  S  T  S  Q  V  S  P  A  *                              -354

CCTCACTCAGCTCCCGCTACCCCATCTTCCGCATCCTCCTTGTTAAGGCAAAGAATGCCGCCTATACCCTTATAAATACCTGAAACATATTTTGCCCAGAAGGGTAAGAGCCTCTCCCAA-1320
CCCCTTCGCTGCTGAAACCGACTGCATTAAATTGCTTTGCAATGCGATGGAGCCATTTATGGCAGCGAAGGGTTCGTTTCACCAACTAAACAAGGCAAGAATTTGCCAGAATTGTAAAT-1440
ACCTCAATGGCCGAGGAATAGCTGTTAGCCTGGAAACGCCTTCTGCACAAATAAAGACAAGAGACGAGACTTTAAGCAACAAATGTCCTAGAATCGGCAGCCCTTGGTGTTGATACCATG-1560
GCGCAAAGAGTGTCTGGCCAGTTCATCTTGCTAAATTTGTCTTTGTAATACAATGGAGGGTTTTTTGTAATACGTTATAAAAATTCCAGTTTTCTGCTTATTTATCTGAAGGAGGGGAAG-1680
ACAGCCCCTCTACCCCGGAAATAAACGGCCACTTGGCAGAGAAAAAAAA$_n$                                                                    -1730
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Fig. 3. Nucleotide and predicted amino acid sequence of frog rhodopsin. The complete nucleotide sequence of frog rhodopsin is shown. In-frame stop codons that delimit the open reading frame are marked by asterisks. The translation initiation codon is shown in boldface. Nucleotide and amino acid numbering are shown to the right of the sequence. A poly A site (AATAAA) in the 3'-untranslated region is underlined.

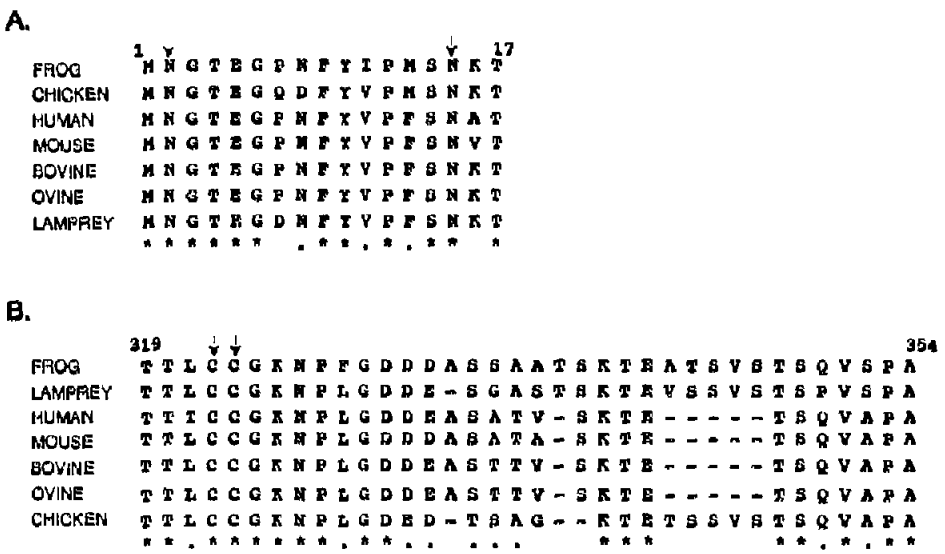

Fig. 4. Comparison of N- and C-terminal domains of rhodopsin in several species. (A) Sequence comparison of the N-terminal domain. Glycosylation sites are marked by arrows. (B) Sequence comparison of the C-terminal domain. Asterisks indicate identities, periods denote conserved amino acid differences. Gaps (–) were introduced to optimize alignments. The sequences shown were downloaded from the PIR database as referenced in the text.

terminal Ala is required for proper insertion and orientation of rhodopsin in the membrane.

Several other residues that have been shown to be essential for rhodopsin function in mammals are also maintained in frog rhodopsin (Fig. 5). Two histidine residues (His^{65}, His^{152}) required for the shift from the metarhodopsin I isomerization state to the metarhodopsin II state [1,30] are maintained in the frog sequence. Deletion analysis of cysteine residues revealed two cysteines (Cys^{110}, Cys^{187}) that form a disulfide bridge required for normal structure of bovine rhodopsin [31,32], both of which are maintained in the frog rhodopsin sequence. Seven residues (Phe^{115}, Ala^{117}, Glu^{122}, Trp^{126}, Ser^{127}, Trp^{265}, Tyr^{268}) thus far have been shown to interact with the chromophore [1–4] (see Fig. 5) in the retinal binding pocket, all of which are maintained in the frog rhodopsin primary structure. Additionally, charged residues, Glu^{134} and Arg^{135}, are present at the beginning of the second cytoplasmic loop (i2, Fig. 5) which are necessary for transducin binding. Glu^{113} and His^{211} which are the counterion of the Schiff base and a possible major site of metarhodopsin I/metarhodopsin II modulation, respectively, are also conserved.

3.4. *Predicted topography of frog opsin*

Fig. 5 shows a predicted topographical model for frog rhodopsin as it would be arranged in the rod outer segment disc membrane. Many of the residues shown to be essential for structure and function, non-conserved differences in comparison to other opsin sequences, and residues that are implicated as causative of adRP (see below) are shown. This model is strictly analogous to topographical models previously proposed by other investigators [33–35], based upon data obtained by chemical modification, protease and transglutaminase susceptibility, antibody binding, and theoretical physical-chemical considerations.

3.5. *Potential application to human disease studies*

Using the candidate gene approach [11], point mutations or small deletions in the human rhodopsin gene have been shown to segregate with adRP in several families studied throughout the world [6–8]. The altered residues identified are shown in Fig. 5. Overall, frog and human rhodopsin display 85% identity and another 10% similarity at the amino acid level. All but two (Gly^{89}, Phe^{220} in human rhodopsin) of the residues found to be mutated in patients with adRP are identical in the frog rhodopsin sequence, consistent with the essential nature of these residues. Furthermore, the majority of the residues are also identical in rhodopsins of more distantly related species such as squid, octopus, *Drosophila* and blowfly. The compilation of sequences from diverse species allows residues that may be essential for function to be identified, and the search for further mutations to be initially concentrated in these regions. This type of sequence comparison may be especially useful for the application of the candidate gene approach to much larger genes with more complex intron/exon organizations (i.e. rod phosphodiesterase α and β subunit genes) to focus on regions where significant sequence differences might be found.

These results represent the first molecular description of a frog phototransduction cascade component. Molecular analysis of other essential visual transduction genes in frog may yield the information necessary to explain the differences observed in biochemical and

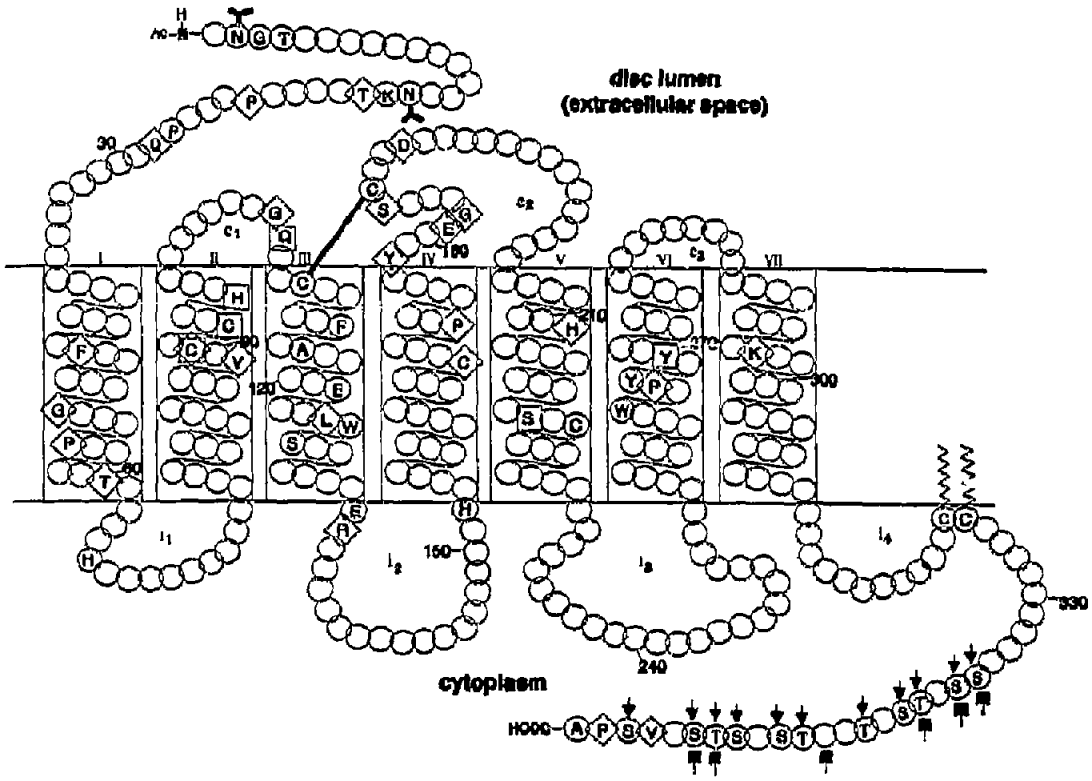

Fig. 5 Topographical model of the secondary structure of frog rhodopsin. The predicted topography (based on [3]) of frog rhodopsin from the anterior portion of a disc membrane is shown The N-terminus, as well as loops e_1, e_2 and e_3, are within the disc lumen, seven transmembrane domains (I–VII) are present, and loops i_1, i_2, i_3, i_4, and the C-terminal portion are located in the photoreceptor cytoplasm. Boxed residues indicate non-conserved sequence differences in comparison with mammalian, chicken, and lamprey opsin sequences. Encircled residues are known to be important for rhodopsin function as described in the text. Residues within a diamond are implicated as mutations responsible for certain forms of human adRP Only mutations involving single amino acid differences are shown. Arrows above the C-terminal sequence mark the positions of potential phosphorylation sites at the C-terminus; boxes below show the relative position of phosphorylation sites in human rhodopsin.

electrophysiological properties of phototransduction in mammals and amphibians, leading to a better understanding of visual transduction.

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