Comparison of the retinitis pigmentosa mutations in rhodopsin with a functional map of the C5a receptor

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Abstract

We compare the known retinitis pigmentosa (RP) mutations in rhodopsin with mutational data obtained for the complement factor 5a receptor (C5aR), a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs). We have performed genetic analyses that define residues that are required for C5aR folding and function. The cognate residues in rhodopsin are not preferentially mutated in RP, suggesting that the predominant molecular defect in RP involves more than simple misfolding or inactivation. Energy calculations are performed to elucidate the structural effects of the RP mutations. Many of these mutations specifically disrupt the environment of the retinal prosthetic group of rhodopsin, and these do not correspond to essential residues in C5aR. This may be because a retinal group is present in rhodopsin but not in C5aR. Another subset of RP mutations is more generally important for receptor structure, and these mutations correlate with essential residues of C5aR.
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1. Introduction

G protein-coupled receptors (GPCRs) are cell-surface receptors containing seven transmembrane helices separated by intra- and extra-cellular loops. Upon ligand binding, GPCRs trigger a cascade of downstream events (Neves, Ram, & Iyengar, 2002; Hamm, 2001). The estimated 948 GPCRs in the human genome (Takeda, Kadowaki, Haga, Takaesu, & Mitaku, 2002) include sensors for endogenous polypeptide and small-molecule hormones, environmental chemicals such as odorants, and light. As GPCRs cannot readily be reverse-engineered to determine their precise mechanism of action, a wide variety of structural and genetic studies have been used to explore this important class of proteins (see for example Wess (1999)).

The functioning of GPCRs relies upon a complex chain of events. The polypeptide must be synthesized and translocated into the endoplasmic reticulum, where it must be folded into the appropriate tertiary structure. For at least some receptors, including the C5a receptor (Floyd et al., 2003), GABA-A/B receptors (Balasubramanian, Teissere, Raju, & Hall, 2004), and angiotensin 2 type I receptor (Hansen, Theilade, Haunso, & Sheikh, 2004), oligomerization at this stage appears to be essential for further processing. Receptors must be transported to their site of action: the plasma membrane in most cases, or the outer segment discs in photoreceptors. The expressed receptor must exhibit a physiologically reasonable level of basal and pharmacologically triggered activity, and must also undergo appropriate downregulation, usually by phosphorylation and endocytosis, in order to terminate the signal

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(Tsao, Cao, & von Zastrow, 2001; Luttrell & Lefkowitz, 2002). Each of these steps has presented an opportunity for evolution to fine-tune the signaling cascades mediated by GPCRs, but each also presents an opportunity for failure or dysregulation. Therefore, analysis of nonfunctional or hyperfunctional receptors can provide insight into the mechanism of GPCR signaling. On the other hand, it is always difficult to discern a specific reason why a particular GPCR mutant shows aberrant function.

1.1. Mutational data from the congenital retinal dystrophies

The inherited retinal dystrophies provide a rich natural data set for understanding how receptors act as molecular switches. Retinitis pigmentosa (RP) is a hereditary progressive blindness syndrome with an incidence of 1 in 3500 individuals (Phelan & Bok, 2000). The mode of Mendelian inheritance of the condition—autosomal dominant or autosomal recessive—depends on the specific causative mutation. Retinitis pigmentosa is a genetically heterogeneous condition that has been linked to mutations in numerous components of the phototransduction cycle, including rhodopsin (Dryja et al., 1990a, 1990b), cGMP phosphodiesterase subunits (Huang et al., 1995; McLaughlin, Sandberg, Berson, & Dryja, 1993), the cGMP-gated cation channel (Dryja et al., 1995), and visual arrestin (Nakamachi, Nakamura, Fujii, Yamamoto, & Okubo, 1998; Nakazawa, Wada, & Tamai, 1998). The most common locus of mutation is, however, rhodopsin, which accounts for 1/3 of autosomal dominant cases (Phelan & Bok, 2000).

We became interested in investigating the structural information that can be derived from the rhodopsin mutations that give rise to retinal dystrophies. More than 100 different mutations in the opsin gene have been associated with RP (Farrar, Kenna, & Humphries, 2002; Phelan & Bok, 2000) or related milder diseases such as congenital stationary night blindness (CSNB) (Lem & Fain, 2004) and Leber congenital amaurosis (Woodruff et al., 2003). There have been multiple attempts to classify these mutations according to the behavior of the mutant rhodopsin; see for example Sung, Schneider, Agarwal, Papermaster, and Nathans (1991), Sung, Davenport, and Nathans (1993), Kaushal and Khorana (1994), Mendes, van der Spuy, Chapple, and Cheetham (2005) and references therein. Some classes of mutant rhodopsins are retained in the endoplasmic reticulum (ER), either because of failure to fold properly or because they are not transported to the outer segment. Other mutants have aberrant endocytosis, aberrant stability or post-translational modification, or enhanced coupling to transducin (Mendes et al., 2005). Importantly, the largest class of rhodopsin mutants is still the "unclassified" category, underscoring the fact that mutant classification relies on experimental data. Furthermore, the classes are not mutually exclusive, since a single point mutation could have complex effects on the protein's behavior.

Despite the genetic heterogeneity of RP, it is felt that many of the RP-associated alleles of rhodopsin are gain-of-function mutations (Rao & Oprian, 1996; Lem & Fain, 2004). Although formally this claim would have to be tested for each mutant on a case-by-case basis, some general remarks can be made. Opsin is constitutively active in the absence of retinal (Surya, Foster, & Knox, 1995; Woodruff et al., 2003), so mutants that fail to form a chromophore may activate transducin at a low (Melia, Cowan, Angleson, & Wensel, 1997) but unremitting level. This has been verified explicitly for several RP and CSNB mutants (Gross, Rao, & Oprian, 2003a; Dryja, Berson, Rao, & Oprian, 1993; Rao, Cohen, & Oprian, 1994; Robinson, Cohen, Zhukovsky, & Oprian, 1992). Certain RP mutants that associate poorly with retinal (Mendes et al., 2005) are retained in the ER (Stojanovic, Hwang, Khorana, & Hwa, 2003; Rajan & Kopito, 2005; Sung et al., 1993; Sung et al., 1991) or Golgi apparatus (Zhu et al., 2006), and at least some of these may show a certain level of basal signaling even though they fail to reach the cell surface. Although the ER is not a canonical site of GPCR signaling, the recently described membrane estrogen receptor is a GPCR that natively signals from the ER (Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005), so this mechanism may become more widely appreciated in the future. In some mutant rhodopsins, the functional defect appears to be an alteration in the cascade of photointermediates or in the light sensitivity of the receptor (Bosch, Ramon, Del Valle, & Garriga, 2003; Ramon, del Valle, & Garriga, 2003). As further evidence for constitutive activity in the retinal dystrophies, we note that when retinal degeneration results from non-rhodopsin mutations in the photosignaling cascade, the mutation often mimics a state of constant light activation. For example, RP can occur when rod cGMP-gated Ca^{2+} channels are constitutively closed (Dryja et al., 1995; Lisman & Fain, 1995).

1.2. Random saturation mutagenesis of the C5a receptor

Complement factor 5a (C5a), a component of the mammalian complement system, serves as a chemotactic factor for neutrophils in the inflammatory response (Kohl, 2001). Its receptor, the C5a receptor (C5aR) (Gerard & Gerard, 1991), has been investigated as a target for pharmacotherapy in inflammatory states (Allegretti et al., 2005). Besides its intrinsic pharmacologic interest, the C5a receptor (C5aR) serves as a good model for family A GPCRs, which also include rhodopsin. Human C5aR and rhodopsin exhibit 19% amino acid identity, which is average for GPCRs, and there are several common points between these receptors that strongly suggest that they employ a similar mechanism.

These similarities are both structural and functional. The intracellular and extracellular loops of the two receptors are similar in length, and both contain a disulfide bond between the third transmembrane helix (TM3) and second extracellular loop (EC2). Both possess the canonical DRY

motif of class A GPCRs, in the form of a DRF sequence in C5aR and ERY in rhodopsin. The role of EC2 appears to be similar in these two receptors. Mutational data showed that in C5aR, EC2 serves to cap the transmembrane bundle and hold the receptor in the off state; when the EC2-TM binding is disrupted, the receptor becomes constitutively active (Klco, Wiegand, Narzinski, & Baranski, 2005). Similarly, the 11-*cis*-retinal group of rhodopsin lies in an interhelical crevice that is capped by EC2 (Palczewski et al., 2000; Teller, Okada, Behnke, Palczewski, & Stenkamp, 2001; Okada et al., 2002, 2004; Li, Edwards, Burghammer, Villa, & Schertler, 2004). More significantly, it appears that the activation of both receptors involves an increase in the distance between TM3 and TM6. In rhodopsin, conformationally constrained mutants show that activation requires the cytoplasmic ends of these helices to move apart (Sheikh, Zvyaga, Lichtarge, Sakmar, & Bourne, 1996; Meng & Bourne, 2001), whereas in C5aR, random saturation mutagenesis (Baranski et al., 1999) and mutant cycle analysis (Gerber, Meng, Dotsch, Baranski, & Bourne, 2001) both support an activation-induced reorientation of TM3 and TM6.

Our laboratory has performed extensive genetic studies of C5aR (Baranski et al., 1999; Geva, Lassere, Lichtarge, Pollitt, & Baranski, 2000; Klco, Nikiforovich, & Baranski, 2006; Klco et al., 2005) using random saturation mutagenesis of each TM helix and interhelical loop. The mutagenesis is performed by replacing the coding sequence of the region of interest with doped oligonucleotides to generate, for each receptor domain, a library of approximately 10^5 mutant receptors. The term "saturation" is used to indicate the entire region of interest is simultaneously and uniformly mutagenized, not that every combinatorially possible mutant is included in the library. We have found that a nucleotide mutation rate of 20% leads to an optimal level of mutational pressure in the screen. The mutant library is screened in a yeast strain that coexpresses the C5a ligand, which signals in an autocrine manner. In these yeast, the presence of a functional receptor causes expression of a selectable marker. The cDNAs encoding functional receptors are isolated and sequenced.

In our screens, the vast majority of mutant receptors are nonfunctional because their random mutations are injurious to receptor folding or signaling. Patterns of amino acid conservation are apparent in those receptors that do retain ligand-dependent signaling ability despite their random mutations. When residues are preserved in the random mutagenesis screen, we hypothesize that they may be essential for the receptor to be functional. This hypothesis can be tested by point mutation. For example, residue W102 of C5aR was unmutated in all functional mutants isolated from our scan of the EC1 loop (Klco et al., 2006), strongly suggesting that this residue is essential for the functioning of the C5aR, either for the structural integrity of the receptor or for its switch mechanism. In support of this prediction, a W102A point mutation severely impaired ligand-dependent signaling by the

C5aR. In contrast, residues that are highly tolerant of changes, such as G106 of EC1, are inferred to be nonessential for receptor folding and signaling. One caveat of this analysis is that essentiality is unlikely to be a black-or-white characteristic of individual residues. The importance of residues for signaling is in reality likely to fall along a continuum, and the essentiality of any given position may be dependent upon the modifying effects of mutations at other positions. However, we have generally found there to be a strong association between a residue's being preserved under random saturation mutagenesis and its essentiality for receptor signaling.

The random mutagenesis studies of C5aR and the naturally occurring RP mutations in rhodopsin provide complementary views of the receptor activation mechanism. In this study, we first consider the significance of overlaps between the two data sets in terms of proposed activation mechanisms and known receptor-activating hot spots. To give our analysis a firmer structural grounding, we next present a computational model for the structure of inactive C5aR. This structure contains an internal network of interacting essential residues. Although this network is also likely to be present in rhodopsin, it does not appear to be the target of RP mutations. Finally, we use energy calculations to identify possible structural consequences of the RP mutations.

2. Methods

2.1. Multiple sequence alignment

The amino acid sequences of human C5aR and human and bovine rhodopsin were aligned by using the most conserved residue(s) in each helix as a reference point (Mirzadegan, Benko, Filipek, & Palczewski, 2003). These are shown in bold in Fig. 3. Although these residues were not perfectly conserved between C5aR and rhodopsin for all TMs, the subset that was conserved allowed each TM to be unambiguously aligned without gaps. This method has previously been used by, for example, Baldwin, Schertler, and Unger (1997).

For Fig. 1, the extracellular and intracellular loops and amino- and carboxy-termini of C5a and rhodopsin were also aligned so that mutations in rhodopsin could be mapped onto corresponding positions in C5aR. Because the loop regions are divergent, the alignment was performed by hand with the goal of minimizing gaps while aligning charged residues and the conserved disulfide-forming cysteine in EC2. We note that changes to the alignment in the loop regions would have no impact on our analysis. The complete alignment is shown in the Supplementary Data.

2.2. Molecular modeling

The 3D structure of the TM regions of C5aR was modeled by the same energy calculation procedure that was earlier employed for rhodopsin (described in detail elsewhere by Nikiforovich & Marshall (2003)). Briefly, in the first step, the TM helical fragments of C5aR were aligned with those of rhodopsin. Boundaries of the aligned TM regions were defined as follows: TM1, I38–A63 (the first and last residue, respectively); TM2, N71–Q98; TM3, A107–V138; TM4, A150–F172; TM5, E199–F224; TM6, R236–F267; and TM7, L281–Y300. Then, each individual TM helix was subjected to energy minimization starting from backbone dihedral angles corresponding to those in the rhodopsin X-ray structure (the PDB entry 1F88 (Palczewski et al., 2000)). The TM helices were packed together according to a previously described procedure

(Nikiforovich, Galaktionov, Balodis, & Marshall, 2001). The packing procedure consisted of minimizing the sum of all intra- and inter-helical interatomic energies in the multi-dimensional space of parameters that included "global" parameters (those related to movements of individual helices as rigid bodies, namely, translations along the coordinate axes X, Y, Z and rotations around these axes T_X, T_Y, and T_Z) and "local" parameters (the dihedral angles of the side chains for all helices). The starting values of those angles were optimized before and after energy minimization by an algorithm developed by us earlier (Nikiforovich, Hruby, Prakash, & Gehrig, 1991). To decrease the number of variables in this complicated system and to avoid problems of local convergence, the approximation of "hard helical cores" (backbones) and "soft shells" (side chains) for each helix was applied; the dihedral angles of backbones (but not those of side chains) were fixed at the values obtained by energy calculations for the individual TM helices.

The "global" starting point for the TM bundle corresponding to the resting state was selected by spatial alignment of TM helices onto the X-ray structure of rhodopsin. Energy minimization that started from the global parameters corresponding to the PDB entry 1F88 yielded a 3D structure differing from 1F88 by the rms value of 2.40 Å (Cα-atoms only) using the energy convergence criterion of $\Delta E \leqslant 1$ kcal/mol. Energy calculations for RP mutants of rhodopsin were performed exactly as for rhodopsin itself, including modeling of the presumed light-activated state (Nikiforovich & Marshall, 2006). The ECEPP/2 force field with rigid valence geometry (Dunfield, Burgess, & Scheraga, 1978; Nemethy, Pottle, & Scheraga, 1983) was used for all energy calculations; a distance cut-off of 8 Å was used for non-bonded interactions. Residues of Arg, Lys, Glu and Asp were present as charged species with the macroscopic dielectric constant set at the ECEPP/2 standard value of 2.0. One run of energy minimization for a typical TM bundle required ca. 6 h on a single-node PC with a 2.8 GHz processor running under the Linux operating system.

3. Results and discussion

3.1. Essential residues of C5aR are not preferentially mutated in RP

In our C5aR mutagenesis studies, we screened libraries of mutant receptors for clones that retained the ability to signal to downstream effectors in the presence of ligand. A separate screen was performed for each helix and intra- or extra-cellular loop of the receptor. Seventy-eight out of 350 positions in C5aR were identified as being "preserved"; that is, in the selected functional mutants, these positions did not show nonconservative mutations, but rather changed only to similar amino acids. (We included positions that exhibited one nonconservative change but no other mutations in order to account for the possibility that a highly dissimilar amino acid might occasionally be permissible in the context of other compensatory changes.) These positions have been highlighted in blue or yellow in Fig. 1.

Our interpretation of the preserved positions is that they are required for receptor folding and stability or for the activation switch mechanism. Receptors with mutations at the preserved positions either are unstable or are unable to switch into the "on" conformation, thus explaining their failure to be selected in our screen. The screen does not
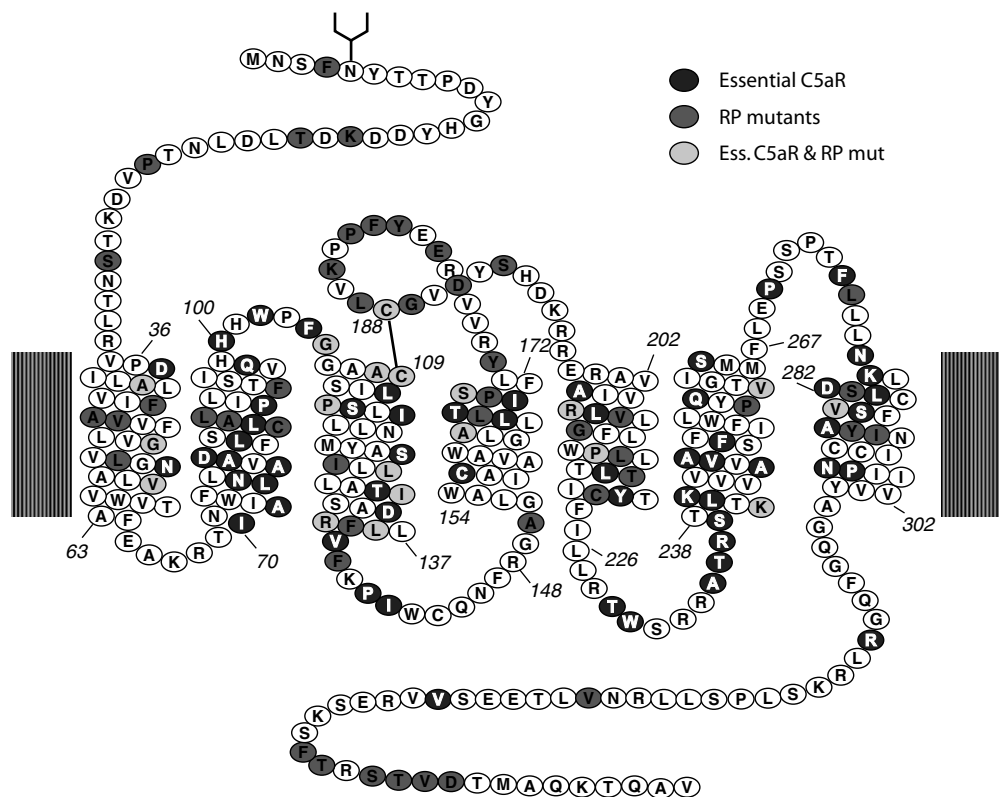

Fig. 1. Schematic diagram of C5aR with the amino terminus (extracellular) at the top and the carboxyl terminus (intracellular) at the bottom. Amino acid positions are colored to reflect mutational data: blue denotes a residue found to be essential in random mutagenesis of the C5aR; red denotes a residue whose homologue in rhodopsin has been implicated in retinitis pigmentosa (Mendes et al., 2005); yellow denotes positions falling into both categories. Selected residue numbers are marked for reference purposes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

select against constitutively active receptors. To test for constitutive activity, all functional mutants were reassessed for signaling in the absence of ligand.

Fig. 1 also shows the reported retinitis pigmentosa mutations, colored in red or yellow, mapped onto the corresponding positions of the C5aR primary sequence. Yellow color has been used to highlight residues that are both preserved in C5aR and mutated in RP.

Within the TM regions, each of the seven helices has been implicated in both RP disease and C5aR activation, as judged by the presence of both RP mutations and preserved residues in each helix. If RP were caused mostly by inactivating or misfolding mutations in rhodopsin, then one would expect good overlap between these two sets of positions. The pattern of overlap is, instead, uneven. Some helices (particularly TM3) show abundant overlap between the data sets, whereas others (particularly TM2) show less overlap than would be expected by chance. We believe that the preserved positions of C5aR are important for the receptor's expression and folding and for its ability to serve as a functional switch; receptors with mutations at these positions presumably have defective folding or function since they are not identified in our functional screens. In other words, mutations at preserved positions are normally loss-of-function mutations. The less than complete red/blue overlap in Fig. 3 shows that mutation of a position that is essential for C5aR folding or function is not always, in rhodopsin, sufficient to produce the RP phenotype. This finding supports the hypothesis that RP disease is caused by (possibly small) gain-of-function changes, rather than by loss of function, in addition to any folding or localization defects of the mutant rhodopsin.

3.2. Hot spots for constitutive activation of C5aR

Random mutagenesis of the C5aR produced several mutants that were constitutively active in yeast. These receptors were distinguished by the fact that they were able to signal through the reporter pathway even in the absence of a coexpressed C5a ligand. Since some RP mutants have been shown to exhibit a gain of signaling function (Dryja et al., 1993; Govardhan & Oprian, 1994; Jin, Cornwall, & Oprian, 2003; Keen et al., 1991), and there may be many others that also have this property, we hypothesized that naturally occurring RP mutations would occur in the same regions as activating mutations in our C5aR screens.

Constitutively active mutants, or CAMs, were identified in the screens of TM1, TM3, TM6, and EC2 (Baranski et al., 1999; Geva et al., 2000; Klco et al., 2005). These CAMs do not all operate by the exact same mechanism, because there is no mutational motif that is common to all of the CAMs. The single CAM in the TM1 screen does not supply enough information to deduce an activating pattern. However, distinct patterns were noted in other activating regions. In the TM3 screen, four out of five CAMs included at least one of the mutations I124N or L127Q. These two mutations alone are sufficient to create constitutive activity (Baranski et al., 1999) in a mechanism that would seem to involve TM6,

since both side chains point towards that helix and in particular towards F251. In support of this hypothesis, a F251A point mutation created a CAM (Whistler et al., 2002). The most parsimonious explanation of these findings is that hydrophobic interactions between I124, L127, and F251 maintain the off state of the receptor, and mutations that reduce this hydrophobic interaction allow constitutive switching to the on state. Activation of the C5aR therefore appears to involve a change in the relative position of TM3 and TM6, most likely an increase in the distance between these two helices, as was shown for rhodopsin (Altenbach et al., 1996; Dunham & Farrens, 1999; Farrens, Altenbach, Yang, Hubbell, & Khorana, 1996).

To compare these data to the naturally arising mutations in rhodopsin, we mapped the RP mutations onto their cognate positions in the C5aR (Fig. 1). The random mutagenesis screen of EC2 was unusual in that 23 out of 29 functional receptors were ligand-independent. Constitutive activity arose when multiple mutations were present at C188 or in the ranges 181–184 and 190–192. Taken together these data suggested that EC2 serves as a "lid" capping the interhelical cleft, making multiple weak interactions to stabilize the off state. Disruption of several of these interactions releases the EC2 lid and allows the receptor to switch to the on state (Klco et al., 2005).

EC2 is also a hot spot for RP mutations. Of the 27 residues in the rhodopsin EC2, 11 have been found to be mutated in RP (Fig. 1). We suggest two possible mechanisms that would account for constitutive activity in rhodopsin EC2 mutants—although we note that these mutants' constitutive activity has not yet been explicitly demonstrated. First, by capping the TM bundle, EC2 may serve as a linchpin to maintain the receptor-inactive TM3/TM6 orientation; removing the linchpin allows the helices to snap into the active conformation. Second, the β4 sheet of EC2 (residues 186–189) forms a lid that covers and stabilizes the retinal prosthetic group (Palczewski et al., 2000). Mutations in this region (including at C187, G188, and D190) inhibit 11-*cis*-retinal incorporation, thereby destabilizing the inactive state, since retinal serves as an inverse agonist.

The fact that CAMs were found by mutagenizing EC2 of C5aR, coupled with the fact that RP mutants are found in EC2 of rhodopsin, supports the hypothesis that RP involves a gain of rhodopsin function. However, as we show below, energy calculations that we have performed to model the RP mutant rhodopsin molecules suggest that the retinal group confers some unique structural features on rhodopsin as compared to C5aR.

3.3. Essential residues of C5aR form a network of interactions in the TM region

We used computational modeling of C5aR to rationalize the existence of essential positions as well as their role in the receptor switch mechanism. A 3D model of the TM region of inactive C5aR was built by using as a starting point the X-ray structure of dark-adapted rhodopsin (PDB entry

1F88), as described in Section 2. Random saturation mutagenesis previously identified 57 essential positions in the TM helices of C5aR, as shown in Table 1. The side chains of these residues form a specific network of interactions when mapped onto the 3D model of the TM region of C5aR (Fig. 2). Most of the essential residues are in close

Table 1
Residues in the TM regions of C5aR found to be preserved by random mutagenesis (Baranski et al., 1999; Geva et al., 2000)

Helix	Preserved residues
TM1	A40, G51, N55, V58
TM2	I70, A72, L76, N77, A79, A81, D82, L84, L87, P90, Q98
TM3	A108, C109, L112, S114, I116, S123, L126, T129, I130, D133, R134, L136, L137
TM4	C157, A164, L166, T168, I169, S171
TM5	A203, R206, L207, P214, L218, Y222
TM6	R236, S237, K239, L241, K242, F251, Q259, V260, S266
TM7	K280, D282, L284, V286, S287, A289, N296, P297

contact with at least one of the other essential residues, where contact is defined as a distance of less than 5 Å between at least one pair of atoms belonging to the side chains of corresponding residues. Eleven residues are not in contact with any other preserved residue, namely A40, G51, A72, L84, Q98, L136, P214, K239, A246, S266 and A289.

The interaction network among the essential residues can be roughly divided into two main domains, one closer to the extracellular face of C5aR and the other closer to the intracellular face. With the exception of the disulfide-forming C109, all residues that are both preserved in C5aR and conserved in the rhodopsin-like family of GPCRs (underlined and italicized in Fig. 2) are located closer to the intracellular face of C5aR. In turn, this part of the network can be divided into two clusters of residues in close contact. The first cluster contains residues N55, V58 (TM1), L76, N77, A79, A81, D82 (TM2), N296 and P297 (TM7), and the second cluster contains residues S123, L126, T129, I130, D133,
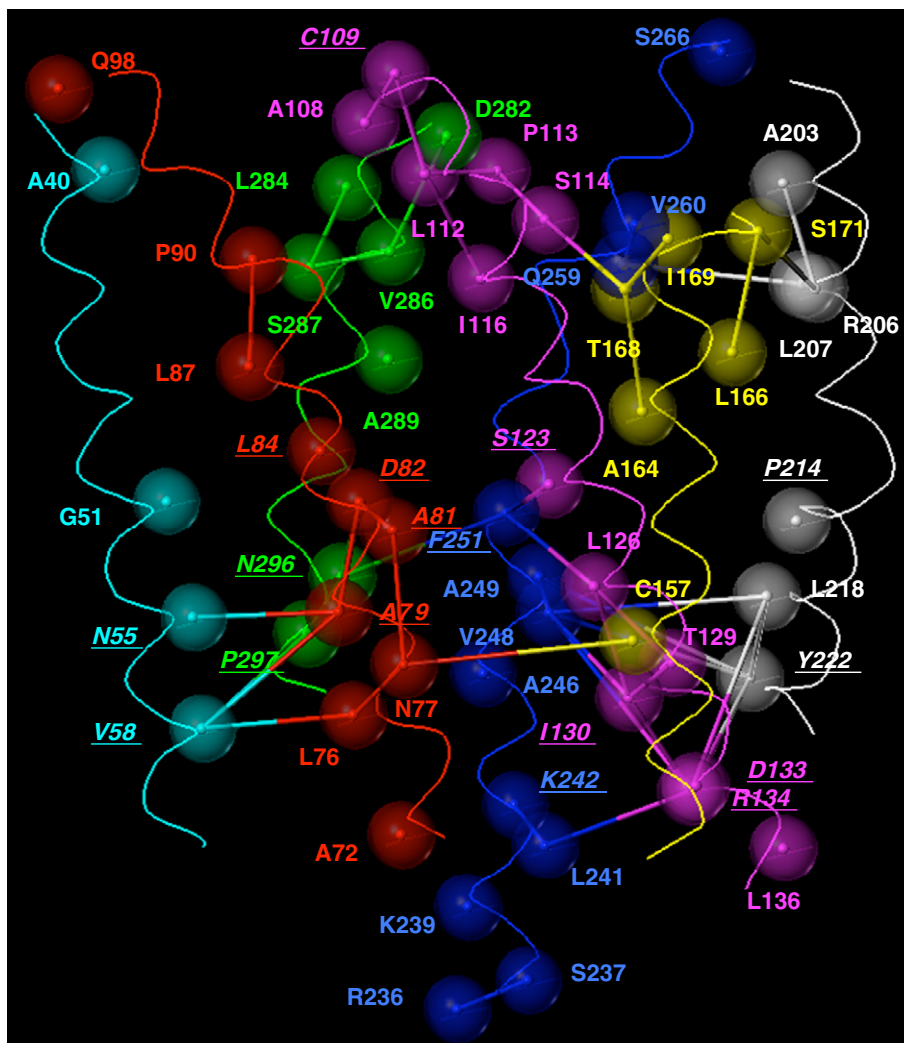

Fig. 2. Network of interactions between the preserved residues in C5aR. Residues are shown as semi-transparent balls. TM helices are shown as one-line ribbons. Close contacts between the side chains are shown as sticks connecting the balls. Residues that are conserved in the rhodopsin family of GPCRs are labeled in underlined italics. Transmembrane helices are color-coded: TM1 in cyan, TM2 in red, TM3 in magenta, TM4 in yellow, TM5 in white, TM6 in blue, and TM7 in green. The extracellular face of the receptor is at the top. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

R134 (TM3), C157 (TM4), L218, Y222 (TM5), L241, K242, V248, A249 and F251 (TM6). The two clusters are connected through a weak interaction between N77 and C157 and by much more strong interactions of F251 (TM6) with S123 and L126 in TM3 as well as with N296 in TM7.

The network depicted in Fig. 2 represents a system of inter-residue interactions necessary for maintaining proper functioning of the C5a receptor; any changes in the network lead to either impaired function or hyper-functionality, such as in constitutively active mutants. Indeed, Fig. 2 highlights the unique role of the side chain of F251 in maintaining the network of interactions between the preserved residues in C5aR. The F251A mutant is known to be a strong constitutively active mutant (Whistler et al., 2002). Moreover, the side chain of F251 is in close contact not only with the preserved S123, L126 and N296, but also with the side chains of residues L127 (TM3), F254 (TM6) and N292 (TM7). Placement of a polar side chain at L127 can give constitutive activity as in the I124N/L127Q ("NQ") mutant (Whistler et al., 2002); also, modifications of F254 lead to constitutively active mutants (Nikiforovich, Sen, & Baranski, 2006). Combined, these findings suggest that residues in the vicinity of F251 form a hot spot for designing new constitutively active mutants of C5aR.

3.4. The network of essential residues is not preferentially mutated in RP

We chose to focus on the TM regions of C5aR because these are highly conserved between GPCRs. Multiple sequence alignments of 270 members of GPCR family A allowed the most conserved residues to be identified (Mirzadegan et al., 2003); these positions, highlighted in bold in Fig. 3, are largely conserved from rhodopsin to C5aR and allow us to align the TM helices. In addition, as described, random saturation mutagenesis data allowed us to identify a network of essential residues in C5aR. It is reasonable to suppose that rhodopsin contains a similar network, because 32 of the 35 TM residues most highly conserved in family A GPCRs are conserved between rhodopsin and C5aR (Fig. 3).

When we mapped the known RP mutations onto the structure of rhodopsin, we found that they did not preferentially coincide with the presumed network of essential positions. On the contrary, out of 39 TM RP mutations in rhodopsin, only 13 are in positions that correspond to essential residues of C5aR (see Fig. 3). Mapping of the RP mutations onto the 3D structure of the TM region of rhodopsin (Fig. 4, right) shows that there are much less close contacts between the side chains of RP mutant residues than there were between the preserved residues. Only a few inter-helical contacts are observed between the RP positions in the X-ray structure of rhodopsin, specifically L40-T289 (TM1-TM7), M44-T94 (TM1-TM2), F44-K296 (TM1-TM7), L125-P215/L216 (TM3-TM5), R135/Y136-C222 (TM3-TM5), and C167-M207/H211 (TM4-TM5).


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                40          50          60
                 |           |           |
C5AR_HUMAN   ILALVIFAVVFLVGVLGNALVVWVTA
OPSD_BOVIN   SMLAAYMFLLIMLGFPINFLTLYVTV
                 |           |           |
                40          50          60


                70          80          90
                 |           |           |
             TINAIWFLNLAVADFLSCLALPILFTSIVQ
             TPLNYILLNLAVADLFMVFGGFTTTLYTSL
                 |           |           |
                70          80          90


               110         120         130
                 |           |           |
             AACSILPSLILLNMYASILLLATISADRFLL
             TGCNLEGFFATLGGEIALWSLVVLAIERYVV
                 |           |           |
               110         120         130


               150         160         170
                 |           |           |
             AGLAWIACAVAWGLALLLTIPSF
             ENHAIMGVAFTWVMALACAAPPL
                 |           |           |
               150         160         170


               200         210         220
                 |           |           |
             ERAVAIVRLVLGFLWPLLTLTICYTF
             NESFVIYMFVVHFIIPLIVIFFCYGQ
                 |           |           |
               200         210         220


               240         250         260
                 |           |           |
             RSTKTLKVVVAVVASFFIFWLPYQVTGIMMSF
             AEKEVTRMVIIMVIAFLICWLPYAGVAFYIFT
                 |           |           |
               250         260         270


               280         290         300
                 |           |           |
             KLDSLCVSFAYINCCINPIIY
             FMTIPAFFAKTSAVYNPVIY
                 |           |           |
               290         300
```
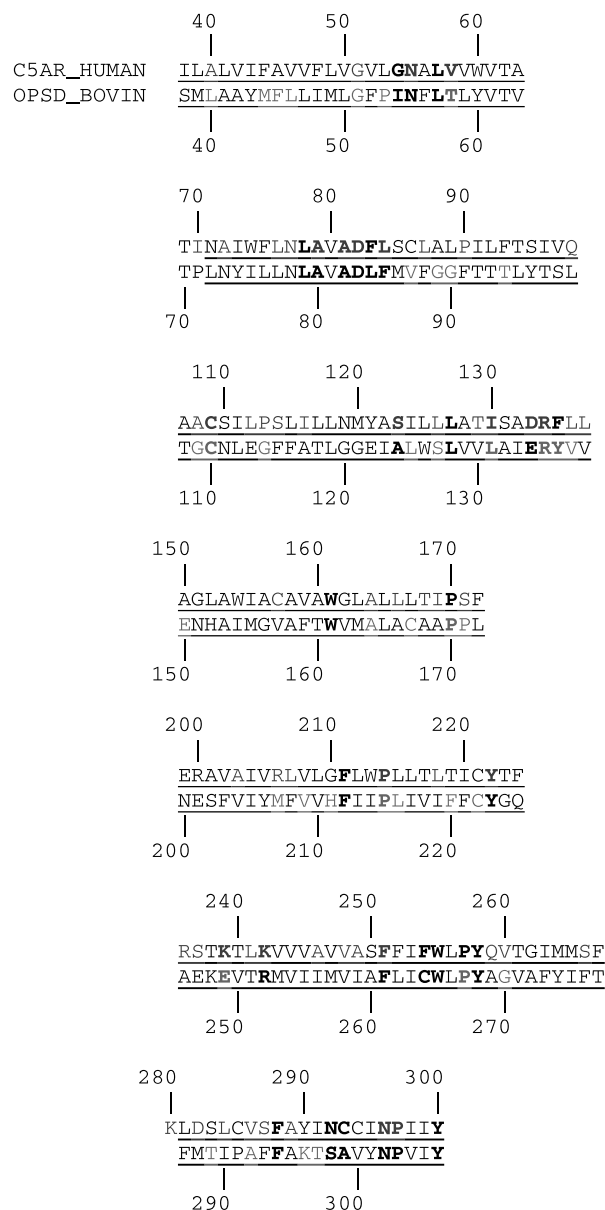
Fig. 3. Sequence alignment showing essential residues in C5aR (blue) and positions of RP mutations in rhodopsin (red). Residues conserved in the rhodopsin family of GPCRs are in bold (Mirzadegan et al., 2003); TM helices are underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

3.5. Energy calculations define four different classes of ionogenic RP mutations

3.5.1. Outwardly directed ionogenic RP mutations

We have shown that RP does not arise from simple disruption of the receptor-stabilizing network of inter-residue interactions. We asked whether we could deduce any other common structural ground for the mutations that are observed in RP. According to the database maintained at http://www.retina-international.com, most of these mutations (23 out of 39) are replacements by potentially charged residues, namely R, K, D, or E (shown in bold in Fig. 5). For some of these positions (L46, P53, L216, and G270),
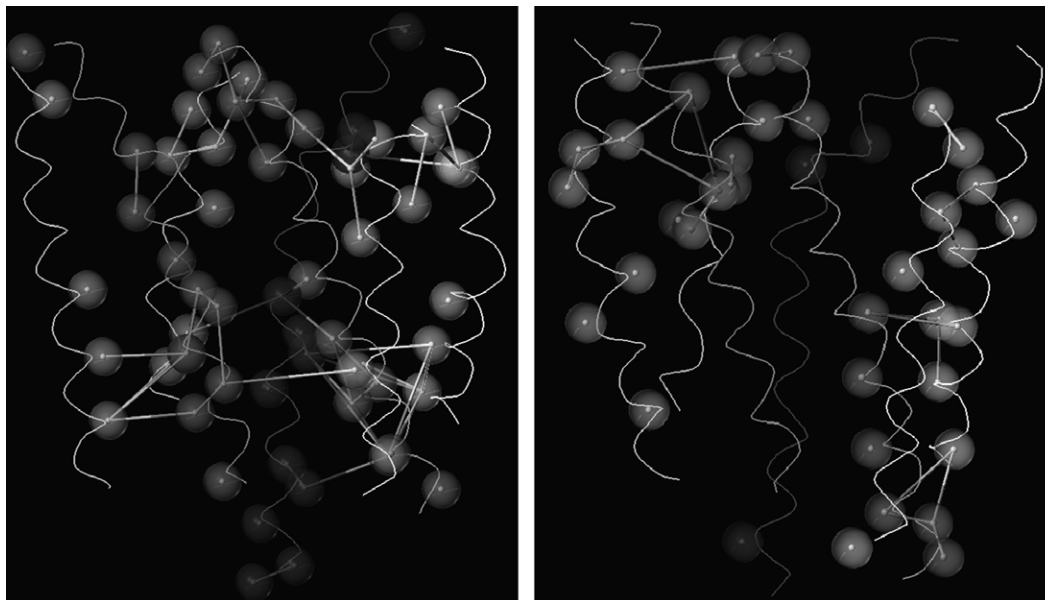
Fig. 4. Preserved residues in C5aR (left, the same as in Fig. 2) and positions corresponding to RP mutations in rhodopsin (right) mapped onto the 3D structure of rhodopsin. Color codes, sticks showing contacts and projections are the same as in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Fig. 5. RP mutations in the TM regions of rhodopsin; bold type indicates mutations to the charged residues R, D, E, or K. At positions where bovine and human rhodopsin are nonidentical, the bovine residue is indicated before the human one. When a preserved residue of C5aR was present at the corresponding position, that residue is indicated. Boxes are used to categorize the RP mutations: red, mutations affecting interactions with retinal; blue, general structural mutations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

adis et al. (2006, 2003, 2004)) has suggested that rhodopsin dimers or oligomers are the functional unit and that signaling by monomers occurs less efficiently; the tightness of receptor packing appears to be important for determining the rate of phototransduction (Calvert et al., 2001). In some studies, dilution of wild-type rhodopsin into detergent micelles, so that only monomeric rhodopsin is present, results in receptors that signal at a rate close to that of wild-type (Hofmann, 2006), arguing against an essential role for rhodopsin dimerization in signaling; but in other studies, rhodopsin monomers signal more slowly than oligomers (Jastrzebska et al., 2004). Thus it appears likely, but not certain, that rhodopsin density is important for signaling.

One interesting possibility is that rhodopsin–lipid or rhodopsin–rhodopsin contacts might help to maintain the off conformation. A packing-related rhodopsin regulatory mechanism could explain the rod outer segment's unusual membrane composition (Boesze-Battaglia & Schimmel, 1997). It seems reasonable to suppose that dense rhodopsin packing places inwardly directed, stabilizing forces on each receptor monomer and reduces the basal activity of the receptor. Outwardly directed charged side chains would electrostatically repel adjacent receptors or lipids and reduce these stabilizing forces, thus giving rise to a weakly constitutively activated receptor. If this mechanism were operative, then RP mutations might arise at positions that interact directly with membrane lipid molecules. In a recent simulation run on the IBM Blue Gene supercomputer, phospholipids containing both stearic acid and the ω-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) were allowed, along with cholesterol, to assume their preferred orientations around rhodopsin (Grossfield, Feller, & Pitman, 2006). P53 of rhodopsin was found to be one of the residues that preferentially interacted with cholesterol and

the side chains are directed out of the TM bundle, toward the hydrophobic membrane lipids. Our modeling procedure does not specifically address interactions with lipids, but in general, charged side groups should not be tolerated in the lipid bilayer and could potentially destabilize the receptor.

Alternatively, outward-pointing charged residues could interfere with oligomerization of receptors. Work by the Palczewski group (for example Filipek et al. (2004) & Foti-

G270 was found to preferentially interact with DHA. Although amino acids L46 and L216 did not preferentially interact with membrane lipids, mutations at these positions could conceivably act by a longer-range mechanism.

3.5.2. RP mutations repositioning the charged side chain of E113

After examining outwardly directed ionogenic mutations, we performed energy calculations to derive 3D models of the resting state of the 14 other ionogenic RP or CSNB mutants, namely L40R, G51R, T58R (TM1); V87D, G89D, G90D (TM2); G109R, G114D, L125R (TM3); A164E, C167R (TM4); M207R, H211R (TM6); and A292E (TM7); we also considered here the non-ionogenic mutation T94I in TM2 since this mutation is closely allied with G90D (Gross et al., 2003a, Gross, Xie, & Oprian, 2003b). In each of these receptors, the mutant side chain is directed inward toward the helical bundle and lies in the vicinity of retinal, in most cases closer than 10 Å (Fig. 6). Our calculations were intended to determine whether these mutations had any effect on the retinal group's environment, which in turn is known to be important for rhodopsin activation.

Side chains of the residues in positions 90, 94, and 292 are in the near vicinity of the unprotonated side chain of
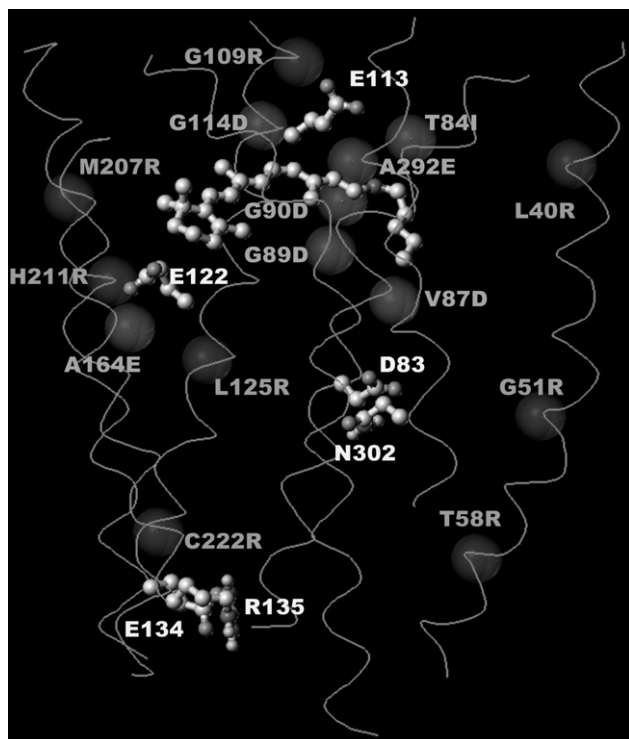

Fig. 6. Mapping of RP mutations on the 3D model of the TM region of rhodopsin. Mutations are shown as semi-transparent balls colored either in red (for replacements by negatively charged side chains) or in blue (positively charged side chains). Side chains of residues D83, E113, E122, E134, R135, N302 and K296 with *cis*-retinal attached are shown as ball-and-stick models. TM helices are shown as one-line ribbons. The extracellular face of the receptor is at the top. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

E113. This residue is involved in a crucial salt bridge with the positively charged nitrogen atom of the Schiff base connecting the side chain of K296 to *cis*-retinal. This interaction is believed to stabilize the dark-adapted state of rhodopsin (Cohen, Oprian, & Robinson, 1992; Robinson et al., 1992). According to energy calculations, the side chain of E113 may nearly equally populate conformations featuring either the salt bridge or a hydrogen bond between the γ-carboxyl of E113 and the hydroxyl of the side chain of T94. The latter, in turn, may switch to form a hydrogen bond with the backbone carbonyl of G90. Previous work with rhodopsin mutants at T94 (including T94D) supports an important role for the hydroxyl group of T94 in stabilizing the electrostatic network around the Schiff base linkage in rhodopsin (Ramon et al., 2003). In mutants G90D and A292E, the electrostatic repulsion between the negatively charged side chains of E113 and D90/E292 forces the side chain of E113 away from retinal, which disrupts the interaction stabilizing the resting state of rhodopsin. Similarly, the side chain of E113 is driven away from retinal in the T94I mutant; in this case, however, the cause is steric hindrance due to replacement of the hydroxyl in T94 by a bulky aliphatic group in I94. All three mutants are known as constitutively active mutants of opsin and are associated with CSNB (Dryja et al., 1993; Gross et al., 2003a, 2003b).

A similar spatial position of the E113/Q113 side chain was found by energy calculations of two known pronounced constitutively active mutants of rhodopsin, namely G90D/M257Y and E113Q/M257Y (Nikiforovich & Marshall, 2006). The same changes of spatial orientation of the negatively charged E113 side chain may occur also in mutants V87D, G89D, G114D and G109R. Movement away from retinal is facilitated in the former three mutants by electrostatic repulsion between negatively charged side chains, and in the latter one by electrostatic attraction between the oppositely charged E133 and R109 (Fig. 6). Though an alternative conformation of the E113 side chain is not excluded in these mutants (as in the constitutively active mutants discussed above), interaction of E113 with retinal is effectively weakened.

3.5.3. RP mutations strengthening the TM3–TM5/TM4 interaction

Another group of mutants is composed of L125R, C167R, M207R, H211R and A164E. According to energy calculations, the main difference between these mutants and wild-type rhodopsin is in strengthened interactions between TM3 and TM5/TM4 because of the additional electrostatic attraction between the positively charged residues in TM5/TM4 and the negatively charged E122 (Fig. 6). Repulsion between E164 and E122 also effectively strengthens interaction between TM3 and TM5. This, in turn, may weaken interactions between TM3 and TM6, facilitating conformational transition to the activated state of rhodopsin. It should be noted, however, that our energy calculations for all RP mutants did not find a significant energetic preference for the presumed activated state of the TM region of

the mutants (such as that deduced for rhodopsin from spectroscopic data (Hubbell, Altenbach, Hubbell, & Khorana, 2003)), as was found earlier for the strong constitutively active mutants G90D/M257Y and E113Q/M257Y (Nikiforovich & Marshall, 2006).

The 12 RP mutants described above were produced by replacement of residues in positions close to retinal, namely 87, 89, 90, 94 in TM2; 109, 114, 125 in TM3; 164 and 167 in TM4; 207, 211 in TM5; and 292 in TM7. Since the retinal group and its charged Schiff base are features unique to rhodopsin, there is no reason to expect that other receptors will be activated or, on the contrary, injured by mutations in this same spatial domain. These positions may be regarded as those important for stabilization of the off state specifically in rhodopsin; mutations in the corresponding positions may not be of the same importance in other GPCRs. Indeed, only five out of the 12 corresponding residues in C5aR were found to be preserved in our random mutagenesis screen, namely A108, P113, A164, R206 and V286 (see Fig. 5, red boxes).

3.5.4. Helix-destabilizing RP mutations

A fourth set of RP mutations appear to cause overall structural instability of the seven-transmembrane bundle. The RP mutations G51R and T58R target positions that are located far from retinal but close to the residue D83 that is highly conserved throughout the rhodopsin-like family (Mirzadegan et al., 2003). Our energy calculations show that electrostatic attraction between R51/R58 and D83 influences the balance of interactions between conserved residues D83 in TM2 and N302 in TM7; this pair was shown to interact in many GPCRs (Cook et al., 1993; Donnelly et al., 1999; Flanagan et al., 1999; Perlman et al., 1997; Sealfon et al., 1995; Zhou et al., 1994). The importance of this cluster is independent of any contribution from the 11-*cis*-retinal group. One can assume that residues in the corresponding positions of other GPCRs would be likely to be detected in random mutagenesis as the preserved ones; indeed, this was the case for C5aR (residues G51 and V58). Destabilization of the resting state in this group of the RP mutants may be, therefore, of a more general nature and not specific for visual pigments. The same may be true for the non-ionogenic RP mutants with replacements of proline residues within TM helices by non-prolines and vice versa—since proline is noted for its helix-breaking properties—or for mutations disrupting the conserved disulfide bridge (C110Y, L131P, P171L, P215T, and T289P). Essential residues were found in or near the corresponding positions of C5aR in six out of seven of these cases (Fig. 5, blue boxes).

The RP mutation C222R occurs in the vicinity of the conserved ERY fragment, but our energy calculations for this mutant did not find significant changes in the spatial orientation of the side chains of E134 and R135. Also, the results of molecular modeling did not suggest why mutation L40R (corresponding to the preserved residue A40 in C5aR) leads to functional impairment. Generally, however, one may conclude that RP mutations influencing interactions close to retinal, and, therefore, specific for rhodopsin, are unlikely to correspond to the preserved residues of other receptors such as C5aR, as determined by random mutagenesis. On the other hand, RP mutations targeting residues with a more general structural role in the receptor are likely to correspond to essential residues of other GPCRs—as we observed in our screens of the C5aR.

4. Conclusions

In this article we have compared two independent sets of GPCR mutations: one from the naturally arising retinitis pigmentosa and night blindness mutations in rhodopsin, another from random saturation mutagenesis of the C5a receptor. These data sets indicate, in different ways, residues that are essential for receptor function. Structural analysis of both sets, performed by molecular modeling, allowed the mutational data to be rationalized in several ways. EC2 of C5aR is the site of many activating mutations, and retinitis pigmentosa mutations are also common in EC2. When the essential residues of C5aR are mapped onto a structural model, they form an interacting network that stabilizes the receptor. To the extent that C5aR and rhodopsin are structurally related, this network should also be present in rhodopsin; however, the RP mutations do not preferentially map to the network, suggesting that RP does not arise from wide-ranging structural destabilization of the receptor.

Many RP mutations introduce residues with ionizable side chains. Computational modeling of individual charge-introducing RP mutations was performed to identify their structural consequences for rhodopsin. Most of the RP mutations cause disruption of interactions between retinal and the rest of the molecule in the dark-adapted state, and, therefore, are specific for rhodopsin. In general, these positions were not found to be preserved in our screens of the C5aR. In our calculations, however, some mutations appear to be important for overall receptor structural integrity, including G51R, T58R, and mutations involving proline such as Ll31P, P171L, P215T, and T289P. The corresponding positions were preserved in the genetic screens of C5aR, suggesting that these components of the receptor-stabilizing network are shared between C5aR and rhodopsin.

Taken together, our findings suggest that GPCRs, including rhodopsin, have considerable overlap in their mechanisms of action. However, rhodopsin has evolved additional molecular mechanisms, centered on the retinal, that may serve to minimize the basal activity of the receptor. The need for this extra safeguard becomes evident when one considers that slight increases in activity can lead to retinitis pigmentosa or congenital night blindness.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.visres.2006.07.010.

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