

Hypothesis

Sequence homology between bacteriorhodopsin and G-protein coupled receptors: exon shuffling or evolution by duplication?

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Bacteriorhodopsin (BR) is a membrane protein of known structure, widely used for the homology modeling of G-protein-coupled receptors (GPCR). The observation of apparently transposed sequence similarities between some of the helical domains of BR and GPCR has led to the suggestion that exon shuffling may have occurred in the later evolution of GPCR, which would necessitate a different folding pattern for the seven transmembrane helices of GPCR. An alternate hypothesis is that duplication occurred in the evolution of an ancestral gene, such that helices 5–7 originated as duplicates of helices 1–3, leading to intragenic as well as intergenic similarities between helices 1–3 and 5–7 of BR and various GPCR. Analyses of GPCR and BR sequences suggest that such a duplication may have occurred; symmetry within the BR structure is also consistent with homology between these two regions. The hypothesis of evolution by duplication is consistent with the conventional, unshuffled homology model, which is also supported by the obvious conservation of the retinal binding Lys moiety on helix 7 in both BR and the mammalian opsins.

Bacteriorhodopsin; G-protein-coupled receptor (GPCR); Homology; Exon shuffling; Evolution; Duplication

The bacteriorhodopsin (BR) structural prototype [1] has been widely used as a template for the homology modeling of membrane proteins in the G-protein-coupled receptor superfamily [2–9], however, many investigators engaged in such modeling studies have acknowledged that the actual sequence similarity between the transmembrane helical domains of BR and G-protein-coupled receptors (GPCR) is rather low. Structural similarities have been inferred primarily by hydrophobicity analyses (which consistently predict seven hydrophobic helical domains in GPCR), and various lines of biochemical and molecular biological evidence (for recent reviews see [9,10]). This low degree of sequence similarity between BR and GPCR is not at all surprising, considering that their divergence must have occurred approximately at the time of that between prokaryotes and eukaryotes, which took place at least 1.5 billion years ago [11]. It has been noted, however, that for distant homologies, structure is often more highly conserved than sequence [12].

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An extremely interesting and provocative result is the recent report by Pardo et al. [13] that the greatest sequence similarities between certain individual BR transmembrane helices and the predicted GPCR helical regions appear to be out of order, e.g. the seventh BR helix (H7) is most homologous to the third GPCR helix (H3). These similarities led Pardo et al. to propose that exon shuffling may have occurred in the later evolution of the GPCR, necessitating an alternate folding pattern for the seven transmembrane helices of GPCR (Fig. 1). This would also imply that the BR structural prototype has been and is being misapplied by virtually all researchers working in this area, leading to incorrect models of GPCR and their modes of ligand binding, as well as calling into question the basis upon which a great number of molecular biology experiments have been designed and interpreted [5,9,10,14,15]. Clearly, then, this is a question that must be further investigated and resolved.

In this report, we present an alternate and possibly simpler hypothesis to explain the out-of-sequence helical homologies described by Pardo et al. At least part of their results and suggested alternate BR–GPCR homology model (see Fig. 4 in [13]) – specifically, a suggested homology between GPCR helices 1–3 and BR helices 5–7 (see Fig. 1) – can immediately be accounted for if one postulates that a duplication occurred in the evolution of an ancestral gene, such that helices 5–7 had

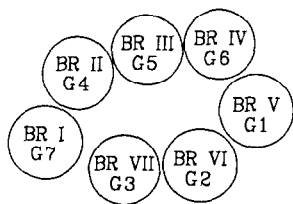
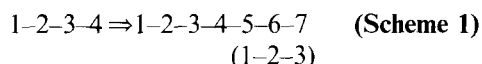


Fig. 1. Schematic of the 7 helices in the transmembrane domain of BR (BR I–BR VII), showing a hypothetical alternate homology to the 7 transmembrane helices of GPCR (G1–G7), as suggested by Pardo et al. [13]; the organization of the seven helices in GPCR is proposed to be somewhat different (compare Fig. 4 of Pardo et al.). Note the proposed homology between G1–G3 and BR V–BR VII, which immediately suggests a possible duplication of helices 1–3 as helices 5–7.

originated as duplicates of helices 1–3, or vice-versa (scheme 1).



By this interpretation, the apparently anomalous observations of Pardo et al. could be explained as having been due to residual sequence similarities between homologous helices within the GPCR and BR genes; in this case, one would expect to see some evidence of *intragenic* as well as *intergenic* similarities between helices 1–3 and 5–7 of BR and various GPCR.

Since, like exon shuffling, evolution by duplication and subsequent divergence of duplicated domains is a well established evolutionary mechanism, this possibility clearly warrants serious investigation. Pairwise comparisons of the sequences of helices 1, 2 and 3 to helices 5, 6 and 7 (i.e. H1 vs. H5, H2 vs. H6 and H3 vs. H7) from various GPCR, as well as BR H1 vs. BR H5, are shown in Fig. 2. The sequence similarities shown are between different numbered helices from a single gene, or from related sets of genes of monoamine neurotransmitter receptors; the latter involve comparisons of regions of multi-alignments for the relevant helical domains of various GPCR. Note that in GPCR both H2 and H6 contain a conserved proline, and both H3 and H7 contain a conserved serine; simply by aligning these highly conserved residues, one can produce the alignments shown in Fig. 2 for these pairs of helices.

One of the most striking matches (Fig. 2A) is in BR itself, between H1 and H5, which have an impressive 43% identity and 70% similarity, with one deletion*. As the multi-alignments of Fig. 2 demonstrate, there are

distinct similarities between the pairs of helices in the H1–H3 and H5–H7 segments of GPCR. These are intragenic similarities, suggesting that a duplication may have occurred in the evolution of an ancestral gene. Given these apparent similarities, it seems natural to ask how well they fit into the context of a larger alignment between the two regions of the gene encompassing H1–H3 and H5–H7. The BR gene is most suitable for such a comparison, since it lacks the large hydrophilic loop between H5 and H6 found in the mammalian GPCR (the cytosolic G-protein binding domain), which was probably incorporated in the gene sometime after the divergence with the bacterial gene.

As shown in Fig. 3, the BR precursor gene (from which the first 13 residues are proteolytically cleaved to form the mature protein) can be divided into two pieces which can be aligned with only a few small gaps, in such a way as to bring the helical regions into alignment as expected: H1 vs. H5, H2 vs. H6, and H3 vs. H7. In addition to the helical domains being 'in register', there is sufficient conservation of sequence that the alignment is statistically significant at the 3 S.D. level, relative to the same sequences randomized. This is about as much sequence similarity as one could realistically expect to find, considering that if such a duplication did take place in the evolution of an ancestor of BR and GPCR, it must have predated the divergence between prokaryotes and eukaryotes. It is also noteworthy that the sequence similarities between these two halves of the BR gene are fairly evenly distributed throughout the sequence, rather than being confined to the helical regions. Not only is this what one would expect based upon a uniform mutation rate following a gene duplication: it also suggests that the significance level of the alignment cannot simply be ascribed to inherent similarities between the amphipathic helices that typically characterize such membrane proteins.

The alignment shown in Fig. 3 could be used to support several possible scenarios for the origin of the 7-helix protein. One possibility would be that shown above as Scheme 1, and would suggest that H4 was already in place, and that only helices 1–3 were copied and added on to create helices 5–7 (or vice versa). The other possibility suggested by Fig. 3 would involve a different origin for H4. Since the gene can be divided almost exactly in half, with the homologous helical pairs of H1–H3 and H5–H7 in register, the origins of the BR gene from an ancestral transmembrane protein or protein module having only three helices is suggested; in this case, H4 would have arisen from loop regions forming the connection between the duplicate domains (Fig. 4, top panel). This would be a direct consequence of the need to maintain the correct transmembrane orientation of the two equivalent 3-helix transmembrane domains (H1–H3 and H5–H7), which would each have had dipole moments that would dictate the correct out-to-in orientation for odd numbered helices [16,17]. The

*The significance of this H1–H5 match can be appreciated by comparing it to the match of H1 with the other BR helices. Using the GCG GAP program [22] with a gap weight of 4 and gap length weight of 0.5, pairwise alignments of H1 with H2, H3, H4, H6 and H7 had only 10–16% identity (average: 14%), with an average quality score of 10.1 ± 1.15 . By comparison, the H1–H5 match had 43% identity and a quality score of 14.4, which is 3.7 standard deviations above the average score of the matches to the other BR helices.

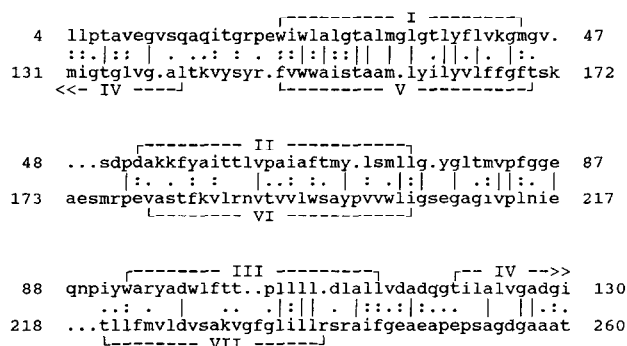


Fig. 3. Intragenic homology (21% identity, 46% similarity) at the protein sequence level between the N- and C-terminal portions of the BR precursor gene (residues 1–130 vs. 131–260). Note that the homologous helical pairs H1–H5, H2–H6 and H3–H7 are 'in register' with only a few small gaps; the aligned Asp (D) residues in H3–H7 are those shown in Fig. 4, bottom panel, which act as counterions in retinal binding; they are at identical depths within the lipid bilayer. The alignment shown is a composite of several slightly different alignments produced by the GAP program [22], using different gap and length weights. The alignment is statistically significant at > 3.0 S.D. over the same alignment randomized. Specifically, with a gap weight of 2.0 and a gap length weight of 0.5, the alignment quality score is 52.1; the random quality score of 100 randomized runs is 45.1 ± 2.3 , so the actual alignment scored 3.04 S.D. over random. Also note that the residue numbering scheme for this alignment differs from the conventional numbering for the mature BR protein, in which the first 13 residues have been removed in post-translational processing.

They both covalently bind retinal, the binding of which has been visualized in the BR electron diffraction structure ([1]; also see Fig. 4, bottom panel). This expected similarity presents one of the most significant problems for the shuffled homology model proposed by Pardo et al. [13], since multi-alignments clearly demonstrate that the critical Lys involved in retinal attachment is located on H7 in the mammalian opsins, as it is in BR. Thus, there is no reason to expect that in the mammalian opsins the structural arrangement of the helices with respect to retinal binding is radically different from that observed in BR. This, combined with the fact that multi-alignments clearly show that the mammalian opsins have substantial homology with the rest of the GPCR superfamily [3,18–20], suggests that the conventional (unshuffled) model for BR–GPCR homology is probably correct. The case for the exon shuffled homology model would be much more convincing if the retinal-binding Lys was observed on H3 of the mammalian opsins, since that is the helix suggested by Pardo et al. to be most homologous to H7 of BR; however, the homologous Lys is clearly observed on H7, in precisely the location predicted by the conventional, unshuffled homology model.

It should also be noted that according to the conventional homology model, the counterionic Asp-212 on BR H7 (Fig. 4) aligns with an Asn (N) on GPCR H7 that is conserved in the β -adrenergic and 5-HT_{1A} receptors [3]. This Asn on H7 has been shown to be critically

important for the binding of certain antagonists [21], as it interacts with the phenoxy oxygen of propranolol and related compounds, which is very near to the amino group that is known to bind to the conserved Asp on H3. Again, the conventional homology model accounts well for these similarities in terms of the three-dimensional BR structure, since the two homologous Asp residues on H3 and H7 are very near each other in BR (Fig. 4, bottom panel).

Nonetheless, the question still remains: even if a common ancestor of the BR and GPCR genes had evolved by duplication as we have proposed, why should GPCR H3 appear *more* similar to BR H7 than to BR H3? Much of this question involves the relative degree of sequence similarity, and unfortunately Pardo et al. do not give any information on the (unshuffled) alignment

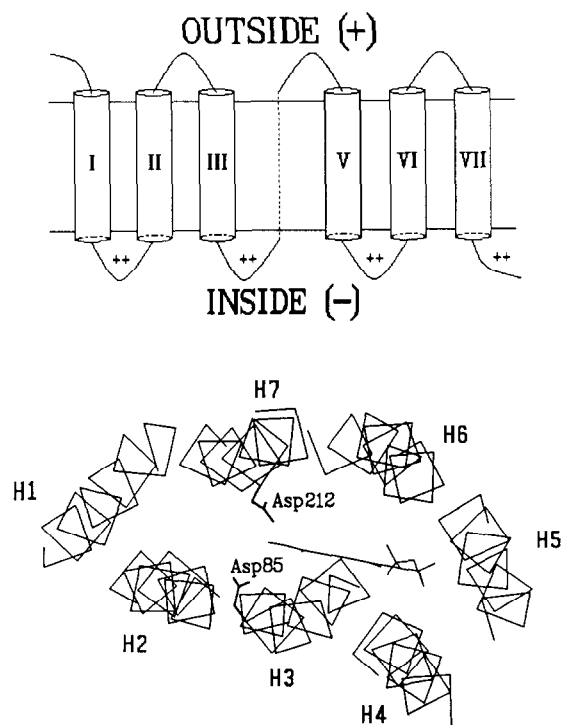


Fig. 4. (Top panel) Schematic diagram showing that if a 3-helix transmembrane module was duplicated and joined together, in order to maintain the correct orientation of each module with respect to the transmembrane potential [16,17], the connecting segment would have to cross the membrane. This suggests the possibility that helix 4 of BR could have originated in this manner, consistent with the duplication suggested by the alignment in Fig. 3. (Bottom panel) The actual structure of the transmembrane domain of BR [1], with the 7 helices shown as an α -carbon trace, with retinal visible in the cleft. Also depicted are the two equivalent Asp residues on H3 and H7, which serve as counterions to the protonated Schiff base covalently linking retinal to Lys-216 (not shown). Intramolecular symmetry between H1–H2–H3 and H5–H6–H7 is apparent, with H4 as an extra helix on one side of the cleft – see text for discussion. The residue numbering scheme used in this Fig. follows the conventional numbering for the mature BR protein (add 13 to obtain the numbering used in Fig. 3 for the BR precursor sequence), and is the same as that used in structure BRD1 from the Brookhaven Protein Data Bank, which has been visualized using Sybyl 6.0. Tripos Associates, St. Louis.

of BR H3 vs. GPCR H3, etc., which would be of some interest for the sake of comparison. It is certainly possible that convergent evolutionary forces might lead to (or at least act to conserve) sequence similarities between helical pairs such as H3 and H7, perhaps due to their symmetric locations in the proteins, as well as between similarly located helices in BR and GPCR (e.g. H3 vs. H3). One possible explanation of the superior match of BR H7 to GPCR H3 over that of BR H3 to GPCR H3, however, arises from the duplication hypothesis that we have proposed based in part upon the alignment of Fig. 3. This alignment suggests that several deletions may have occurred in H3 during the later evolution of BR, since the homologous BR H7 apparently still aligns with H3 of GPCR fairly well [13]. If GPCR H3 sequences are compared to BR H3, maintaining the same pattern of gaps shown for the latter in Fig. 3, a reasonable alignment is obtained (Fig. 5A). This could explain the difficulty that previous investigators have had in aligning BR H3 to GPCR H3, since such alignments are usually performed with a high gap penalty.

Finally, it is significant that the other apparent homologies actually reported by Pardo et al. (BR H3 to GPCR H5, and BR H1 to GPCR H7) are also matches between odd numbered helices, which could be accounted for by a second hypothetical duplication step, preceding that postulated above for 1-2-3 $\Rightarrow$ 5-6-7. If the gene had evolved by *successive* duplications (e.g. 1-2 $\Rightarrow$ 1-2-3-4 $\Rightarrow$ 1-2-3-4-5-6-7), there could be a residual general similarity between *all* odd numbered helices ('out-to-in' helices). Although it might seem unlikely that two successive duplication steps could have occurred in the evolution of the BR gene, there are at least moderate similarities visible across all the odd numbered helices of BR, consistent with such a scenario (Fig. 5B). In any case, two duplication steps are no less plausible per se than the two 'shuffles' that would be required to produce the alternate GPCR arrangement of the BR helices by exon shuffling as proposed by Pardo et al. (Fig. 1). Proteins such as triose phosphate isomerase provide clear and ample evidence that the successive duplication of domains has been a significant factor in protein evolution.

In summary, the results reported here provide an alternate interpretation for the observations of Pardo et al., and, combined with the arguments given above, support the conventional homology model that has been and is being utilized by a large number of research groups. Our results suggest that the reported sequence similarity between BR H7 and GPCR H3, and the proposed homology between GPCR helices 1-3 and BR helices 5-7, probably arise from an inherent intragenic similarity between the H1-H3 and H5-H7 regions that is observable in both BR and many genes in the GPCR superfamily (Fig. 2).

A: ALIGNMENT OF BR H3 AND BR H7 WITH GPCR H3

		* * * * *
BR H3	WARYADWLFTT..PLLLL.DLA	
5HT2 H3	IWIYLDVLFSTASIMHLCALSL	
$\alpha 2$ H3	IYLALDVLFTCTSSIVHLCALSL	
M1 H3	LWLALDYVASNASVMNLLLSF	
D2 H3	IFVTLDDVMCTASILNLCALSI	
BR H7	<u>LFMVLDVSAKVGFGLLILRSRA</u>	

B: SIMILARITY OF ODD NUMBERED HELICES IN BR

BR H1	WIWLALGTALMGLGTLVFLVKGMGV
BR H3	WARYADWLFTT..PLLLL.DLALLV
BR H5	FVWWAISTAAM.LYILYVLFVGFTS
BR H7	LFMVLDVSAKVGFGLLILRSRAIFG

Fig. 5. (A) Multi-alignment of a set of GPCR helix 3 (H3) sequences with BR H3 and BR H7, maintaining the pattern of gaps in BR H3 suggested by the alignment between BR H3 and BR H7 shown in Fig. 3. Matches between BR H3 and GPCR H3 are shown in bold and indicated by asterisks at the top of the alignment; matches between BR H7 and GPCR H3 are highlighted by underlining. Both BR H3 and BR H7 have nearly the same number of matches with this set of GPCR (8 and 9 identities, respectively). This suggests that part of the reason investigators have had difficulty in aligning BR H3 with GPCR H3 is that deletions in H3 may have occurred in the later evolution of BR, making alignment without gaps (the usual approach) difficult. The alignments in Figs. 2 and 3 suggest that, rather than exon shuffling [13], an inherent intragenic similarity between H3 and H7 in both BR and GPCR may underlie the similarity between BR H7 and GPCR H3. (B) A moderate degree of similarity is detectable across all four odd-numbered helices of BR, in addition to the matches shown previously for H1-H5 and H3-H7 (Fig. 3). Identities are shown in bold; an asterisk indicates chemically and evolutionarily similar residues in 3 out of 4 or all of the sequences in a given position. In particular, note the similarity in the central region of H1 and H7 (SA.VGFG with TA.MGLG); BR H1 to GPCR H7 was one of the matches reported by Pardo et al. [13].

We would like to emphasize that in all probability, the extreme evolutionary distance of these hypothetical events – whether exon shuffling or evolution by duplication – will make it impossible to prove either hypothesis simply by the analysis of sequence similarities. Molecular symmetry considerations (Fig. 4, bottom panel), however, support the hypothesis of an intragenic homology in BR. We have also argued that the obvious conservation of the retinal binding moiety, the Lys on H7 in both BR and the mammalian opsins, unambiguously supports the conventional homology model, consistent with the hypothesis of evolution by duplication, but difficult to explain if exon shuffling had occurred. Unfortunately, there is no accepted method for quantitatively assessing the significance of such structural correspondences and weighting them along with sequence similarities.

Thus, the alternate possibilities of exon shuffling or gene duplication in the evolution of this receptor superfamily must both remain hypotheses, until such time as more detailed information on the structure and function of GPCR becomes available.

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