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REVIEW ARTICLE

The fourth intracellular domain of G-protein coupling receptors: helicity, basicity and similarity to opsins

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Abstract The minimal size of the fourth intracellular domain of heptahelical G-protein coupling receptors (GPCRs) is close to 15 residues, and a juxtamembrane 15-residue segment is predicted as helical (Helix-8) in most of the receptors. Sequences of opsins, non-visual opsin-like (family A) GPCRs and Taste-2 receptors correspond with bovine rhodopsin at four positions in this tract. This is especially evident in monoamine receptors. In most GPCRs, the conserved juxtamembrane segment also has a large fraction of basic sidechains, and a considerable excess of cationic over anionic residues. The conservation is not dependent on the preferred G-protein α subunit or the overall length of the domain, indicating an additive speciation. In rod opsins and some A-GPCRs this segment has been shown to associate with the bilayer and to interact with G-proteins. The segment could also be involved in precoupling of receptors and transducers. These interactions could be helped by both the structural propensities and the high content of cationic sidechains.

Keywords Basic sidechain abundance · Cytoplasmic helix · Juxtamembrane conservation · Receptor phylogeny · Transducer activation · Transducer recognition

Abbreviations

bRho	Bovine rod opsin (rhodopsin)
ci	Ciliary
rh	Rhabdomeric
GPCR	G-protein coupling receptor
ic1, ic2, ic3, ic4	The intracellular domains 1,2,3,4
H8	Helix-8
RGS	Regulator of G-protein signaling

Introduction

Signal transduction in response to homeostatic messengers (e.g. catecholamines and hormonal peptides) is in many cases initiated by heptahelical plasma membrane receptors (for general reviews see Fredriksson et al. 2003; Milligan and Kostenis 2006; Sprang et al. 2007). These receptors couple to, and activate, transducers powered by GTP (and especially the α subunits of heterotrimeric G-proteins (Gilman 1987), and usually are referred to as G-protein coupling receptors (GPCRs). Activated G-proteins trigger the effector enzymes, initiating the biochemical cascades such as those based on the generation of cAMP. Several families of heptahelical GPCRs are defined, based on sequence alignments and taxonomic relations (Fredriksson et al. 2003; Horn et al. 2003; Fredriksson and Schioth 2005). The most numerous group of GPCRs, family A (Horn et al. 2003) or the rhodopsin family (Fredriksson et al. 2003), has significant sequence similarity with opsins

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(the receptors activated by light signals), which could reflect derivation from common ancestor(s).

Despite large efforts in deciphering ways of regulation of transducer activity by GPCRs, many basic questions still need to be answered (see e.g. Oldham and Hamm 2007). This especially concerns the formation of GPCR-transducer complexes. Common elements and motifs have been identified in transmembrane domains of opsins and the like GPCRs (Palczewski et al. 2000; Ballesteros et al. 2001b; Miura et al. 2003; Madabushi et al. 2004; Lehmann et al. 2007; Parker et al. 2008b). These elements are not found in other GPCR families, and the transduction by these receptors would depend on currently unidentified motifs that have interactive similarities with A-GPCRs. However, all GPCR classes use quite similar major transducers, and the competitive adequacy of intracellular sequences of these receptors should be supported by common elements able to assume similar structure, and to sufficiently engage the transductional partners.

In the course of signal transduction, the association of GPCRs with transducers proceeds mainly through intracellular domains of the receptors. Among these, the ic4 domain in bovine rhodopsin is known to form a short helical loop (Palczewski et al. 2000) via palmitoylation of a cysteine pair (Papac et al. 1992). This loop interacts with transducin (Marin et al. 2000) and is also used in activation of the Gq α subunit by the protease-activated receptor 1 (PAR-1) (Swift et al. 2006). Other studies indicate the importance of this segment in ER processing and acceptance of GPCR molecules (Thielen et al. 2005; Yasuda et al. 2009), and in tertiary structure of the transductional unit based on the seventh transmembrane helix (Katragadda et al. 2004; Choi et al. 2005; Li et al. 2007). As will be reviewed here, the segment is predicted as highly helical, and is rich in basic residues in all families of visual and non-visual GPCRs. In a large majority of receptors, the segment is also conserved in several aligned positions. This is discussed in relation to possible common roles of the tract.

Materials and methods

Sequences of opsins were collected from the US National Center for Biotechnology Information (NCBI) Entrez (http://ncbi.nlm.nih.gov/database/entrez) and from Swiss-Protein (http://expasy.org/sprot) databases, and the respective sequence identifiers and receptor groupings are listed in the Supplementary Table S-Lists. All opsins are functional visual opsins. The 207 human A-GPCRs examined comprise most of the 'canonical' non-sensory A-GPCRs (i.e., receptors identified with agonists) currently included in the G-protein coupling receptor database, GPCRDB (Horn et al. 2003) and the IUPHAR database (Harmar et al. 2009). The human olfactory A-GPCRs (384 receptors), Taste-2 GPCRs (24), family B receptors for peptides (peptide B-GPCRs; 15), Frizzled/Smoothened receptors (11), and family C glutamate receptors and calcium-sensing receptors (C-GPCRs; 13 receptors) were retrieved via GPCRDB, and represent most of the currently verified human receptor sequences in the respective groups. The olfactory A-GPCRs and Taste-2 family receptors are at present largely not assigned to agonists. Also, agonist definitions for these receptors are much broader than with the canonical GPCRs. However, the olfactory receptors (and to a degree also the Taste-2 receptors, see the Supplementary Table S-Motifs-3) share similarities to opsins found in the canonical A-GPCRs. Receptors with less than 15 residues in the fourth intracellular (ic4) domain (as defined in the topology programs listed below) were not examined. These receptors, however, represent less than 1% of the human canonical A-GPCRs (2 of 209; gonadotropin-releasing hormone receptor (s-p P30968) with 2, and the proteinase-activated receptor 3, PAR-3 (s-p O00254) with 13 residues), less than 2% of the olfactory A-GPCRs (7 of 391), and one of 25 human Taste-2 receptors. No ic4 domains with less than 15 residues were found in human GPCRs from other families or in any opsins.

Bovine rhodopsin (bRho) was used as the alignment standard, since the sequences of its intracellular domains are very close to the pattern of vertebrate opsins (see Figs. 1, 2, 3; Supplementary Tables S1, SA1.1-8), and are shared with other opsins and with A-GPCRs of all phyla in a large number of positions in transmembrane segments (see Carleton et al. 2005; Parker et al. 2008b; Supplementary Table S-Motifs-2). It should be noted that mammalian and vertebrate rod opsins produce profiles very similar to bovine rhodopsin in comparisons with the ic4 domain of A-GPCRs (Fig. 1), and three A–GPCRs can also serve as templates (Supplementary Figure S1; Table SA2.1-18). The boundaries of domains in bRho represent consensus from three topology programs (see below).

Comparisons of sequence alignments were done by ssearch3 program (Pearson 2000; available at http://bioch.virginia.edu), and also by blast programs (available at the expasy website). Estimates in the ssearch3 and blast programs use points of accepted mutation (PAM) matrices to evaluate non-identical residues (see e.g. Frommlet et al. 2004). Extraction and quantitation of sequences and motifs were done by Microsoft Visual Basic macros. From various tests using intracellular domains of unrelated receptors and channels, identity to bovine rhodopsin at any intracellular position is $\leq$5%, and the random similarity $\leq$10%.

All compared sequences of human A-GPCRs (canonical or olfactory) align with bovine rhodopsin in transmembrane domains 1–3, 6 and 7, allowing definition of segment boundaries that fit well with the rhodopsin model

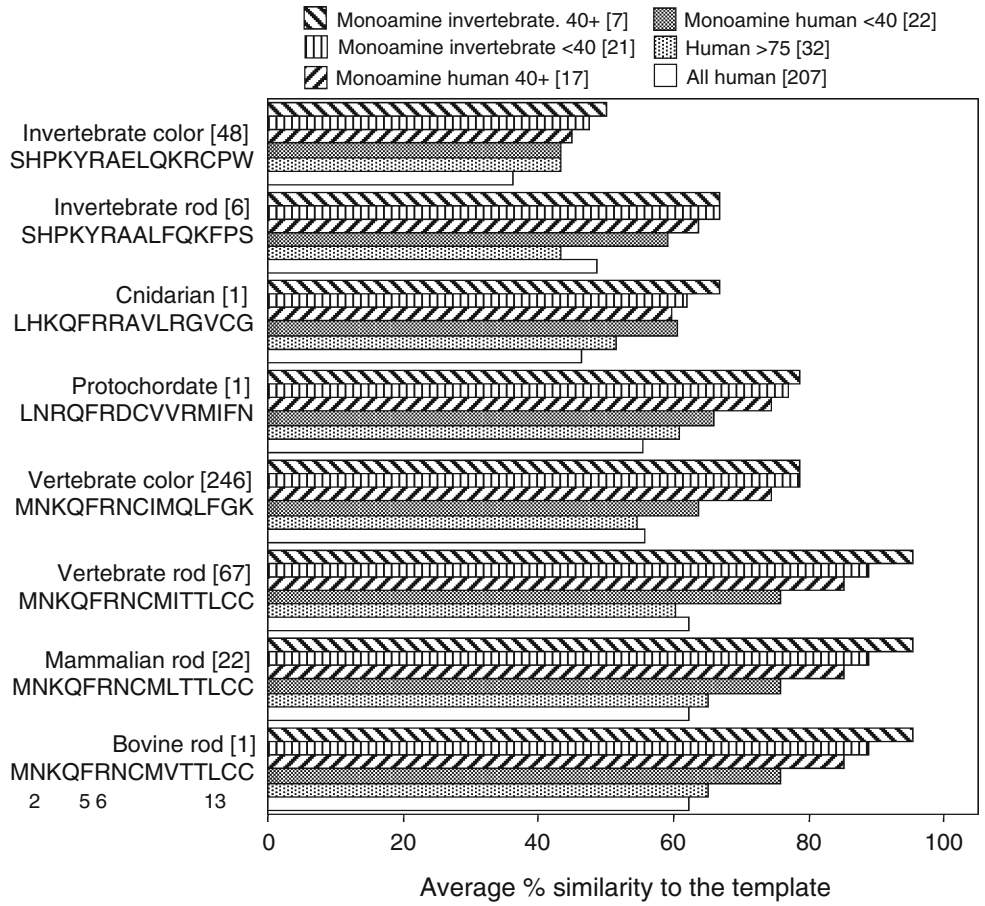


Fig. 1 Similarity to different opsin patterns for the most conserved residues in the fourth intracellular domain of family A-GPCRs. The most conserved positions in any group of opsins or A-GPCRs correspond to residues 2, 5, 6 an 13 in bovine rhodopsin (see the graph inscriptions and Table 1, Supplementary Table SA1). The testing included all human A-GPCRs with 15 or more residues in the domain (*All human*), human A-GPCRs with 75 or more residues in the domain (*Human* > *75*), human monoamine A-GPCRs with less than 40 (*Monoamine human* < *40*) and with more than 40 residues in the domain (*Monoamine human 40+*), invertebrate monoamine A-GPCRs with less than 40 (*Monoamine invertebrate* < *40*), and with 40 or more residues in the domain (*Monoamine invertebrate 40+*). The templates employed were bovine rod opsin (s-p P02699), a protochordate opsin (*Ciona intestinalis* larval eyespot opsin NCBI N001027727), a cnidarian opsin (*Carybdea restonii* green opsin NCBI BAG80696), and the most frequent residues at ic4.1–15 positions of mammalian rod, vertebrate rod, vertebrate wavelength-selective (*color*), invertebrate rod and invertebrate color opsins (inscriptions in this figure and Supplementary Table SA1.2-8; derived from the numbers of receptors shown). Comparisons with four individual A-GPCRs are shown in the Supplementary Figure S1 and Table SA2.1-18

(Palczewski et al. 2000). Up to 75% of these boundaries also correspond to those predicted by tmhmm, tmpred and sosui programs (at the expasy website). Human glucagon receptor, human Frizzled-1 receptor and human metabotropic glutamate receptor 1 were used as templates in, respectively, families B, Frizzled and C. Positions in the fourth intracellular domain are numbered from N-termini of the respective alignments. In comparisons based on bRho, ic4.15 thus refers to a residue corresponding to the 15th residue (Cys323) in the ic4 domain of bRho. This, largely, also would correspond to residue 7.71 in the notation based on transmembrane pivots (Ballesteros et al. 2001b), which, however, cannot accommodate mutational shifts relative to the pivot.

Estimates in the sspro program (Pollastri et al. 2002; baldig@genome.ics.uci.edu), the porter program (Pollastri and McLysaght 2005; http://distill.ucd.ie/porter) and the GOR V program (Kloczkowski et al. 2002; http://gor@iastate.edu) in most cases agreed within 25% in prediction of residues with helical, extended coil and random coil conformation. For uniformity, the presented estimates were made in the sspro program.

Domain polarity was approximated by summing the percent abundance of Asp, Glu, Lys, Arg, His, Asn and Gln in the intracellular domains (Supplementary Tables SC). These residues are considered as the most polar among those of protein amino acids (e.g. Zimmerman et al. 1968; Grantham 1974).

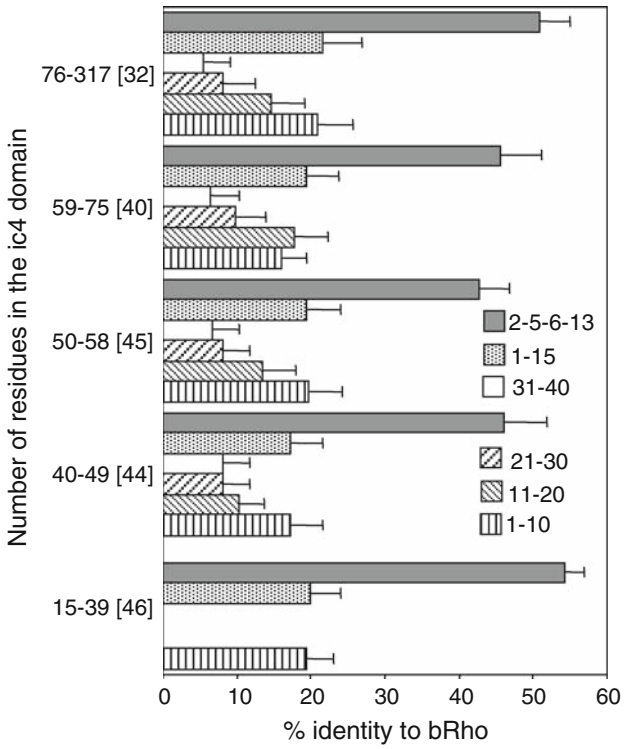


Fig. 2 Identity to bovine rhodopsin in segments of the fourth intracellular domain of human A-GPCRs shows no clear relation to the overall domain length. Average identities (with the respective standard errors) are shown for the successive 10-residue bins, for the N-terminal 1-15 tract, and for the four most conserved residues (positions 2, 5, 6 and 13) in the domain. *Numbers* of receptors are shown in brackets after the length ranges on the *y* axis. The random identity to bovine rhodopsin in a moving-window $n/n + 3/n + 4/n + 12$ test is $\leq 5\%$ [based on a 60-step run with tachykinin-1 receptor (s-p P25103; ic4 domain of 100 residues; the non-shifted ic4 identity at positions 2, 5, 6 and 13 of 75%)]

Surveys

Diversity of GPCRs and choice of template sequences

The ic4 domain shows similar sequence patterns for a contemporary non-bilaterian or a protochordate opsin and the vertebrate rod opsins (Tables 1, Supplementary SA1). Some of the strongly conserved intracellular residues of opsins (Carleton et al. 2005) are also shared with A-GPCRs (Table 1, Supplementary Table SA; Figs. 1, Supplementary Figure S1). The most conserved residues in the ic4 domain of A-GPCRs (positions 2, 5, 6 and 13 as aligned with bRho) correspond equally well with bovine rhodopsin and with the most frequent residues in mammalian or vertebrate rod opsins. The similarity is slightly less with other chordate *ci* opsins, and appreciably lower with a cnidarian *ci* opsin, and with rhabdomeric invertebrate opsins (Fig. 1).

Differences across opsin templates are much more evident in comparisons involving monoamine receptors. As seen in Fig. 1, both human and invertebrate monoamine receptors correspond strongly with all vertebrate rod opsins in the most conserved ic4 positions, and this is especially prominent for monoamine receptors with long ic4 domains. The level of similarity drops appreciably in comparisons of monoamine receptors with color-selective *ci* opsins, vertebrate or invertebrate, and especially relative to invertebrate *rh* opsins. The fourth intracellular domain of family A-GPCRs shows similar conservation patterns in comparisons with individual A-GPCRs, but bovine rhodopsin affords better correspondence across the intracellular domains (supplementary Figure S1). These analyses indicate that bovine rhodopsin, beside high similarity to other opsins (Carleton et al. 2005), is also an appropriate template for comparisons involving intracellular domains of A-GPCRs.

Size, constituents and conservation cin the fourth intracellular domain

This domain shows a large variation in size especially among A-GPCRs (Table 1). In opsins, the size of this domain is rather disperse in the invertebrate, and is not uniform (although the span is narrow) even in the vertebrate (Table 1). Sequences in this domain show considerable differences between rod and color opsins, including the most frequent residues (Table 1, Supplementary Table SA1.1-5). However, the first 15 residues of both opsin types show more similarity. Positions 2, 5 and 6 are conserved above 90% in all vertebrate opsins, and position 13 above 57% (Table 1, see Supplementary Table SB regarding identities with bRho). These four positions are the most conserved in the ic4 domain for any group of opsins (Table 1, Supplementary Table SA1.1-8). The ic4 domains of invertebrate opsins differ considerably from the vertebrate, but the most similar positions are again 2, 5, 6 and 13. Position 6 is the most correspondent, with Arg found in 90% of all opsins. In *ci* opsins, there is a strong decrease in similarity to bRho with distance from the seventh transmembrane domain (Table 1, Supplementary Table SA1.2-5, 8).

There is a large conservation of sequence at the N-terminus of the ic4 domain in A-GPCRs either relative to bovine rhodopsin (Table 1, Supplementary Table SB; Fig. S1B) or as compared to discrete A-GPCR sequences (Fig. S1A). Positions 2, 5, 6 and 13 are $\sim 50\%$ identical to bRho in A-GPCRs (Supplementary Table SB). Positions 2, 5, 6 and 13 in the canonical A-GPCRs, 2 and 13 in the olfactory, and 2, 6 and 13 in Taste-2 receptors are similar to bRho above 50% (Table 1, Supplementary Table SA1). As could be expected, the average similarity to bRho at positions 2, 5, 6 and 13 is $\sim 70\%$ in 1218 canonical A-GPCR sequences from all phyla (Supplementary Table SA1.9), and in 199 mammalian Taste-2 receptors (Supplementary

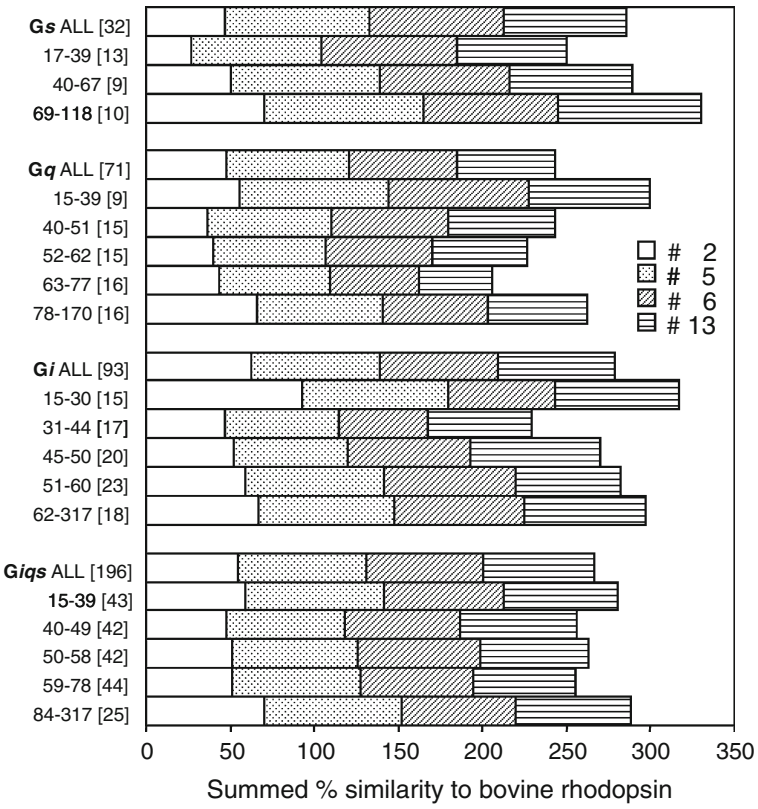


Fig. 3 Similarity to bovine rhodopsin at positions 2, 5, 6 and 13 of the fourth intracellular domain in human A-GPCRs with different lengths of the domain and preferring different major G-protein α subunits. (The maximum is 400) The comparison included 196 receptors with known α subunit preferences. *Numbers* of receptor species are indicated in brackets after specification of the range of sequence length. For positions 5 and 6, significant similarities to vertebrate rod opsins were found in Scheffé *t* tests

Table SA1.10). The conservation is thus very widely shared among orthologs of A-GPCRs and Taste-2 receptors. Positions 5 and 6 (usually with FR motif) are similar in more than 67% human A-GPCRs. In receptors possessing the ic4.14–15 Cys pair, this segment can form a loop (Palczewski et al. 2000) and attach to the bilayer via palmitoylation of cysteine (Papac et al. 1992).

Receptors from families B, C and Frizzled all have large ic4 domains, with less dominant cationic character than A-GPCRs (Table 1, Supplementary Table SC1), and with much less similarity to bRho at the four positions most conserved in A-GPCRs (Table 1). The Taste-2 receptors have a high similarity to bRho at three of these positions (Table 1) and the similarity to bRho appears non-trivial at positions 2 and 5 of family B receptors, and at position 2 of Frizzled and C-GPCRs. The four internally most similar ic4 positions in these families are also always in the 1–13 section, and show average similarities to the corresponding templates of 86, 94, 99 and 90% in Taste-2, B, Frizzled and C-GPCRs, respectively (Supplementary Table SA3). As with family A, this points to an additive speciation of the fourth domain, and again indicates a special status for the juxtamembrane segment.

The bulk composition of the ic4 domain is shown in Supplementary Table SC1. All GPCRs, and especially the family A receptors show larger basic and aromatic residue fractions and much lower cysteine than the vertebrate rod opsins. This could support a more dynamic reactivity. However, many longer ic4 domains show large oligocationic motifs that could serve in membrane anchoring/retention (e.g. McCabe and Berthiaume 1999), as well as in interactions with transducers and effectors. The 3–14 segment has essentially no proline (except in family C), and cysteine is significantly represented only in *ci* opsins and Frizzled receptors (Supplementary Tables SC3, S-Cysteine). The ic4 juxtamembrane segment was shown to be important for activity of G-protein α subunits with bovine rhodopsin (Marin et al. 2000) and with an A-GPCR (Swift et al. 2006) in connection to cysteine pair at positions 14–15. In vertebrate wavelength-selective opsins and in invertebrate opsins, the segment does not contain significant cysteine in these positions. The canonical A-GPCRs with long ic4 domains (76–317 residues) show the ic4.14–15 CC pair in 30% of the sequences (Supplementary Table SA1.33), while only 17% of the receptors with the 15–30 residue length range have this pair (Supplementary Table SA1.29).

All opsins and almost all GPCRs (see "Materials and methods") show a minimum of 15 residues in the fourth intracellular domain. Conservation in the domain is not significantly connected to its length, viewed against either identity to bRho (Fig. 2; Supplementary Table SB) or vs. PAM similarity to the opsin (Fig. 3; Table 1, Supplementary Table SA1.11–19). In A-GPCRs, there is a clear

Table 1 Conservation relative to bovine rhodopsin in the fourth intracellular domain of GPCRs

Group	*N*#	Average length	Length range	Most frequent 2–5–6–13	Similarity to Rho at position				Pool or segment similarity to bRho					
					% #2	% #5	% #6	% #13	% # 2–5–6–13	% # 1–10	% # 11–20	% # 21–30	% # 31–40	KR–DE 1–15
Opsins														
Bovine rhodopsin	1	40	40	NFRL										2
Vertebrate rod opsins	67	43	40–46	NFRL	100	97.8	100	94	97.9	91.3	94.9	69	60.5	1.99
Vertebrate color opsins	246	39	33–47	NFRF	100	100	91	58	87	83.3	28.7	38.4	30.3	2.24
Protochordate color opsin[a]	1	46	46	NFRI	100	100	100	0	87.5	80	40	50	45	2
Cnidarian color opsin[b]	1	43	43	HFRV	50	100	100	50	75	60	15	35	25	4
Invertebrate rod opsins	6	74	43–137	HYRF	50	67	100	50	66.7	29.2	12.5	29.2	27.5	2.34
Invertebrate color opsins	48	44	39–105	HYRC	51	52	96	46	61.2	30.9	8	40.3	26.9	2.67
Family A-GPCRs														
All A-GPCRs														
<40	46	27	15–39	NFRL	58	84	72	66	69.8	41.2	26.5			2.41
40+	161	65	40–317	SFRL	53	75	67	64	64.7	41	22.8	21.9	23	2.34
Gi-preferring														
<40	21	24	15–37	NFRL	81	83	60	67	72.6	44.8	26.7			2.71
40+	72	60	41–317	GFRL	57	75	73	70	68.8	43.1	21.8	22.4	24.2	2.46
Gq-preferring														
<40	9	29	15–39	DFRL	56	89	83	72	75	40	24.4			2.67
40+	62	70	40–170	SFRL	47	70	62	56	58.7	38.5	23.1	20.9	22.1	2.34
Gs-preferring														
<40	13	29	17–39	SFRL	27	77	81	65	62.5	35	22.5			2.31
40+	19	72	40–118	NFRL	61	92	79	79	77.6	45.3	2.5	24.2	22.1	2.21
Gx	11	52	32–91	SFQA	41	77	45	36	50	35.9	21.4	19.5	25	1.09
Monoamine														
<40	22	25	15–35	NFRL	68	98	75	84	81.3	4.1	29.1			2.91
40+	17	106	40–317	NFRL	79	97	88	88	88.2	48	28	29	21	2.29
Invertebrate monoamine														
<40	21	23	16–32	NFRL	71	90	86	76	90.5	48.3	27.6			2.43
40+	7	78	52–102	NFRL	86	100	100	100	96.4	479	25.7	20	18.6	3.14
A-olfactory														
15+	384	23	15–69	NVKL	92	22	48	59	55.3	42	17.7			3.69
Other GPCR families														
Taste-2	24	26	17–38	NLKL	88	46	69	79	70.3	37.3	28.6			3.21
Peptide family B	15	74	43–133	EAER	50	67	37	0	23.3	25.3	12.7	23.3	25.3	2.13

Table 1 continued

Group	*N#*	Average length	Length range	Most frequent 2–5–6–13	Similarity to Rho at position				Pool or segment similarity to bRho					
					% #2	% #5	% #6	% #13	% # 2–5–6–13	% # 1–10	% # 11–20	% # 21–30	% # 31–40	KR–DE 1–15
Frizzled	11	92	25–242	SLQT	45	32	32	13	31.8	24.1	1.2	31.8		2.73
Family C	13	139	33–386	KRNS	46	13	4.2	4.2	16.7	0.8	2.1	29.2		2.08

The length ranges included are indicated after group labels. The receptors are human, unless noted otherwise. The fourth intracellular domain of bovine rhodopsin is defined as residues 309–348 in s-p P02699, MNKQFRNCMVTTLCCGKNPLGDDEASTTVSKTETSQVAPA. In 207 human A-GPCRs, the most frequent residues at ic4.1–15 positions are LSKNFRRAFRRLLCC (for details see Table SA1)

% # mean *%* similarity to bRho at the position, *KR-DE* the difference in numbers of cationic and anionic sidechains per domain

[a] *Ciona intestinalis* larval eye spot opsin (NCBI NP_001027727)

[b] *Carybdea restonii* green opsin (NCBI BAG80696)

decrease across successive 10-residue bins in either identity (Fig. 2) or similarity to bRho (Table 1), regardless of the overall domain size. No large differences in the similarity are found at positions 5 and 6 for A-GPCRs preferring different major types of Gα subunits, and this again does not relate to the overall domain size (Table 1; Fig. 3). However, A-GPCRs with unclear Gα preference ("Gx") show only ~50% similarity (Table 1). Interestingly, the canonical A-GPCRs with longer ic4 domains show higher ic4.1–15 similarity to bRho than those with shorter domains (Table 1).

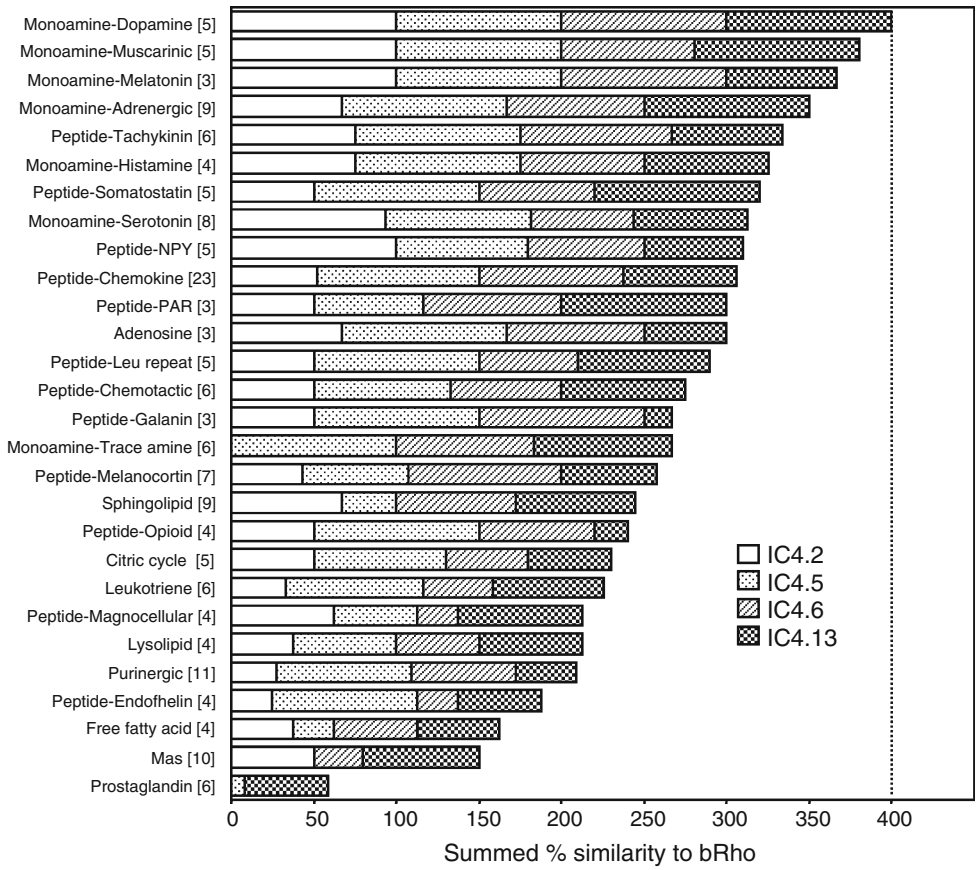
Positional conservation in the fourth intracellular domain and agonist types

Monoamine receptors show the highest conservation in the juxtamembrane section of the ic4 domain (Fig. 4; Table 1, Supplementary Table SA1.19–21; see also Fig. 1). This is found in all phyla, and in invertebrates is near identity to bRho/*ci* opsins at positions 5, 6 and 13. These invertebrate positions are identical to bRho in receptors with ≥40 ic4 residues (Table 1). This is the more striking bearing in mind that the invertebrate receptors examined are a highly heterogeneous collection of six ortholog types across seven taxonomic species from three phyla, with the domain size in the range of 16–102 residues, and with G-protein preferences varying across all major α subunits (see the Supplementary Table S-Lists).

Similarities in the segment 1–15 of this domain were also examined in 172 human A-GPCRs grouped by the agonist category (Fig. 4). Full identity with bRho at the four most conserved positions is found for dopamine receptors. Other subgroups of monoamine receptors also show large similarities (especially the receptors with long ic4 domains; see Supplementary Table SA1.39–41). Low positional conservation is detected for most groups of A-GPCRs responding to lipophilic agonists (see also Supplementary Table SA1.34), among which several also have modifications in the seventh transmembrane (tm7) NPxxY motif (not shown). Mutations in this motif are present as well in some endothelin receptors, which also have a low conservation of the ic4 segment (Fig. 4). The two prokineticin receptors, with heavily mutated tm7 domains, have a poor correspondence in the ic4 domain to either bRho (Supplementary Table SA1.42) or to a monoamine A-GPCR (not shown).

Structural propensities in the fourth intracellular domain

For the entire fourth intracellular domain, predictions for any opsins and canonical A-GPCRs indicate less than 40% of helical organization, and similar is found for families B, C and Frizzled (Fig. 5a). The generally short ic4 domains



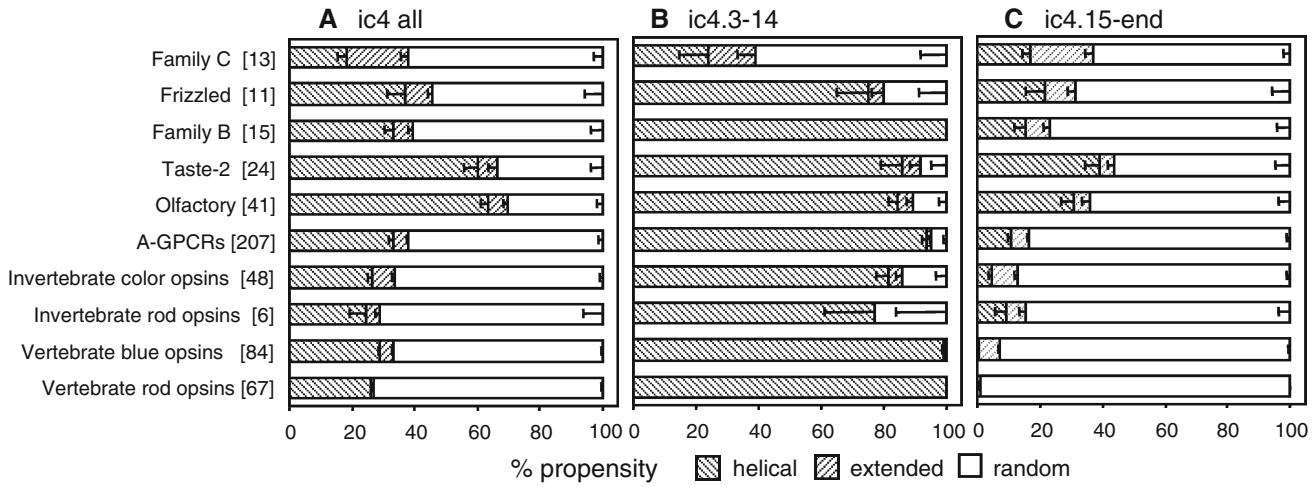


Fig. 4 Similarity to bovine rhodopsin at the most conserved positions in the fourth intracellular domain of human family A-GPCRs grouped by agonist type. Groups of three or more receptors responding to similar agonists were compared (172 of 207 (83%) of the receptors). The data shown are average percentages for numbers of receptors indicated in brackets after group names. The maximum level of similarity for the four positions is indicated by the dashed line. For identification of receptors included in the respective groups, see the Supplementary Table SA

Fig. 5 Profiles of structural propensity in the fourth intracellular domain of G-protein coupling receptors. Sequences of the indicated *numbers* of receptors were evaluated for helical, extended-coil and random-coil character in sspro program. Shown are average predictions for the entire domains (**a**), for residues 3–14 (**b**), and for residues from 15 to end of the respective domain (**c**), with the respective standard errors. At positions 1–2 in the ic4 domain, helical predictions were 10.4% in A-GPCRs, <1% in vertebrate opsins, and ~26% in invertebrate opsins

of the olfactory A-GPCRs and the Taste-2 receptors, however, show a much larger overall helical tendency (Fig. 5a). The first two bRho-aligning residues of this domain are very frequently predicted as unstructured in opsins and A-GPCRs (see the caption of Fig. 5). The following 12 residues in vertebrate opsins are computed as almost entirely helical (Fig. 5b). A more than 90% helical structure is indicated for this segment in the canonical A-GPCRs, and complete helicity is expected for family B receptors (Fig. 5b). The ic4.3–14 tract in other families

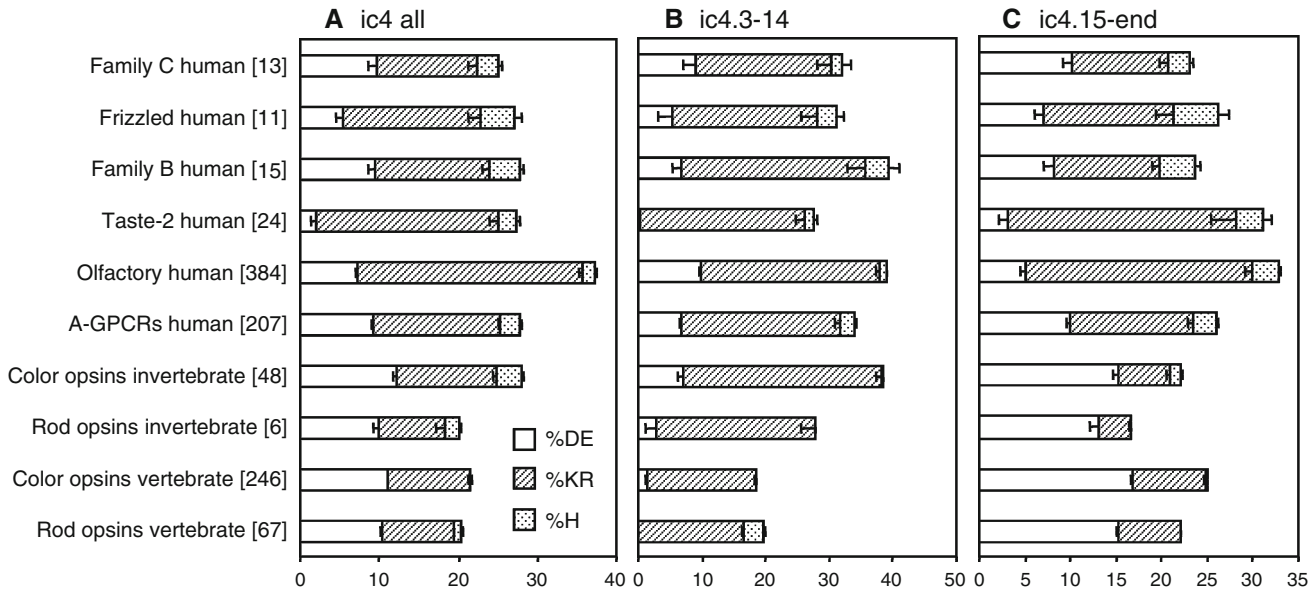


Fig. 6 Percentage fractions of ionic and histidine residues in the fourth intracellular domain of opsins and GPCRs. The *numbers* of receptors examined are in brackets following the group labels. Shown are the averages with the respective standard errors. **a** The entire fourth domain. **b** Residues 3–14 in the domain. **c** Residues from position 15 to end of the domain

should be >80% helical, with the exception of family C (Fig. 5b). However, most of C-GPCRs show significant helical propensities in locations adjacent to, or overlapping the, juxtamembrane 15-peptide in the domain. These receptors have large ic4 domains (averaging 139 residues; Table 1) transducing in cooperation with satellite proteins (Shin et al. 2003), and a looser structuring may have evolved in that connection. The structuring of residues past position 14 is predicted as very low in *ci* rod opsins, and as low in other opsins, and in canonical A-GPCRs (Fig. 5C).

The above surveys indicate, as expected, an overwhelmingly helical organization for the N-terminal 3–14 part of the ic4 domain, a tract that in A-GPCRs also contains three of the four positions most conserved either internally or relative to opsins. The large helicity expected for the same stretch of B-GPCRs does not strongly connect to a positional similarity with opsins, but could be related to a high ionic content, found in all GPCRs (see Table 1, Supplementary Table SC3).

Ionic and polar residues of the fourth intracellular domain

The fraction of strongly polar residues in the ic4 domain ranges from 28 to 36% in opsins, and from 34 to 48% in non-visual GPCRs, and is between 35 and 37% for all canonical GPCRs (Supplementary Table SC1). Basic residues tend to be less, and acidic more represented in opsins relative to non-visual groups (Fig. 6a). All groups of non-visual GPCRs have a substantially larger fraction of cationic residues than the vertebrate (ciliary) opsins in the juxtamembrane 1–15 segment (Table 1, Supplementary Table SC2). This is even more pronounced in the N-terminal 3–14 segment (Fig. 6b; Supplementary Table SC3). In this helical tract (Fig. 5b), the surplus of cationic sidechains is narrowly in the range of 2–3 for all families (Supplementary Table SC3), and the basic residues outnumber the acidic in most cases by a large factor (Supplementary Table SC3). In *ci* opsins, this segment shows very few acidic residues (Fig. 6b; Supplementary Table SC3). The non-visual GPCRs (excluding Taste-2) usually have one acidic residue in this segment. Residues past position 14 in all opsins include more anionic than cationic sidechains (Fig. 6c; Supplementary Table SC4). However, with the exception of family C, the non-visual GPCRs retain an excess of cationic residues in this part of the ic4 domain (Fig. 6C; Supplementary Table SC3).

Addition of histidine, a sidechain that can be substantially protonated even in the physiological pH range (Perutz et al. 1985), and in protein sequences is a frequent conservative substitute for fully protonated Lys and Arg residues (and also has polarity similar to these sidechains), would significantly increase the cationic surplus of non-visual GPCRs, but not of opsins (Fig. 6).

The larger basic fraction in non-visual GPCRs compared to opsins is accompanied by a smaller percentage of amide sidechains (Supplementary Table SC). This difference is pronounced for the 3–14 segment (Supplementary Table SC3), but is not seen consistently past residue 14 (Supplementary Table SC4).

Discussion

For A-GPCRs and opsins, comparisons employing opsin standards should be justified in terms of molecular evolution, as both groups could have evolved from similar precursor(s) (Teller et al. 2003; Carleton et al. 2005; Fredriksson and Schioth 2005). Opsins apparently retained large inter-clade similarity due to their specific role and lower evolutionary diversification. In all opsin groups, as well as in A-GPCRs and Taste-2 receptors, there is conservation of several transmembrane residues and motifs (Palczewski et al. 2000; Ballesteros et al. 2001b; Miura et al. 2003; Madabushi et al. 2004; Lehmann et al. 2007; Parker et al. 2008b) and the Supplementary Table S-Motifs) and of many intracellular residues (Carleton et al. 2005; Table 1, Supplementary Table SA; Figs. 1, S1 in this review). The presence of both ciliary (*ci* type) and rhabdomeric (*rh* type) opsins in quasi-bilaterian cnidarians, in mollusks, and among mammalian brain non-visual opsins (Nilsson and Arendt 2008), indicates that molecules similar to the two types of opsins could be expressed and used in non-visual functions by both contemporary vertebrates and invertebrates. Evaluation of these subjects, however, requires more sequences of both opsins and GPCRs from various taxonomic groups.

Twelve residues at positions 3–14 in the fourth intracellular domain are uniformly forecast as helical. This length would correspond to about three turns of an α-helix (e.g. Cornette et al. 1987). The high conservation of Leu and a large expression of Phe at ic4.13 (see Supplementary Table SA1), which both are residues with large helical potentials (Chou and Fasman 1974), could be instrumental in maintenance of such a structure. Helix-8 in intact cells should be parallel to the bilayer and perpendicular to transmembrane helix 7 (Palczewski et al. 2000; Katragadda et al. 2004; Choi et al. 2005) and shows conservation of both structure and sequence across opsin-like GPCRs, and in Taste-2 receptors. Strong internal conservation of sequence is also found for a similar juxtamembrane tract of the fourth intracellular domain in other GPCR groups. This tract could generally interact with G-proteins in the course of signal transduction (Marin et al. 2000; Nie and Lewis 2001; Okuno et al. 2003; Tetsuka et al. 2004; Thielen et al. 2005; Swift et al. 2006; Scheerer et al. 2008).

Disruption of the helix could lead to a constitutive activation of G-proteins (Okuno et al. 2003), which points to a direct interaction of H8 and the transducer. If this interaction occurs at the C-terminus of the Gα subunit (e.g. Conklin et al. 1996; Marin et al. 2002), the loss of receptor dimers in conditions blocking this region of cognate α subunits (Parker et al. 2008c) could also implicate the H8 segment in formation of pre-transductional ensembles of GPCRs and G-trimers (Baneres and Parello 2003; Philip et al. 2007), possibly at the level of ER (Dupre et al. 2006; Herrick-Davis et al. 2006). Removal of H8 [which results in failure of receptor maturation (Yasuda et al. 2009)] may prevent association with G-proteins at the level of ER/Golgi, which would expose the receptor to proteasome activity, resulting in decrease of dimeric fraction and receptor density, as after disabling of Gi subunits by pertussis toxin (Parker et al. 2007, 2008c). Complexes of receptors and G-proteins could bypass the proteolytic processing by being too large to fit into a proteasome cavity (Wang et al. 1998). In epithelial cell types, at least the neuropeptide Y (NPY) receptors are known to be largely dimeric (Berglund et al. 2003; Dinger et al. 2003) and heteropentameric (Parker et al. 2007) or even heterooctameric (Parker et al. 2008a), and heteropentamers also can predominate in vivo (Estes et al. 2008). That would involve extensive and prolonged contacts with G-protein subunits. Helix-8 appears to interact with all major Gα subunits (Marin et al. 2000; Feng et al. 2003; Okuno et al. 2003; Tetsuka et al. 2004; Delos Santos et al. 2006; Swift et al. 2006). The coupling through H8 could be dynamic and short-term (Okuno et al. 2003; Scheerer et al. 2008), since disruption of the helix could result in a constitutive activation (Okuno et al. 2003). The reviewed evidence could support interactions of H8 segments in GPCRs with G-protein α subunits either at the level of receptor—$G\beta\gamma$ complexes (Dupre et al. 2006), or involving precoupled $\alpha\beta\gamma$ trimers.

The stringent conservation of length of the fourth intracellular domain of GPCRs at an apparent minimum of 15 residues should also be connected to helicity. The association of H8 with transducers could be stabilized through anchoring via acylation of cysteine (Papac et al. 1992). However, cysteine in the cytoplasmic helix is absent from a majority of GPCRs (Supplementary Tables S-Cysteine, SA1.11–19). The abundant basic residues may interact with the bilayer via acylation (e.g. Basar et al. 1999; Masin et al. 2005), but thus far not documented for GPCRs), or could have a more dynamic ionic association with phospholipid headgroups (Perides et al. 1987; Bentham et al. 2006). The interaction can also use aromatic residues near cationic sidechains (Shi et al. 2002; Aliste et al. 2003), as in the highly conserved FR motif in the H8 helix. This and similar aromatic–basic pairs (fairly frequent at ic4.5–6) could participate in the activation of transducers, as reported for motifs from other intracellular domains (Ikezu et al. 1992), or serve as switches, in an analogy with the DRY/ERW motif (Ballesteros et al. 2001a). The juxtamembrane helix would start after position 2 (where the poor helix formers or strong helix breakers Asn, Ser and Gly (Chou and Fasman 1974) represent about 70% of residues in A-GPCRs). At positions 1–2 of the ic4 domain, A-GPCRs are predicted as ∼90%, and vertebrate opsins as >99% random-coiled (the legend of Fig. 5).

In Helix-8, replacement of basic residues by acidic produces a constitutive activation of the β1-adrenergic receptor (Delos Santos et al. 2006), which is consonant with the idea that the cation-sponsored interactions are dynamic, while acidic residues could engage in stronger links, as in integrins (Zhang et al. 2004). Cannabinoid CB1 receptor also depends on a H8-containing segment for association with the Gi3 α subunit (Mukhopadhyay et al. 2000). In PACAP-1 receptor, RS motif in the helical juxtamembrane segment is critical for G*s* coupling (Lyu et al. 2000). This loop could be responsible for the decrease of receptor affinity which occurs upon activation of the α subunit (Okuno et al. 2003). A very low structuring of residues past H8 in opsins and A-GPCR, and a generally low structuring of the C-terminus across GPCRs (Fig. 5c) may also relate to interactions with RGS proteins (Riddle et al. 2005) and effectors. In this respect, it should be noted that 75% A-GPCRs have ic4 domains with 41–317 residues (Supplementary Table SA.1.12–18), longer than that found in mammalian rod opsins (Supplementary Table SA1.2). This is also found for most B- and C-GPCRs (Supplementary Tables SA1.26–28, SA3) These segments have considerable, and largely unexplored, potentials for partnering with transducers and effectors.

The conservation of sequence in the fourth intracellular domain of all GPCRs, either relative to bovine rhodopsin or to internal templates, is independent of domain length, and is concentrated in the 2–13 segment. Vertebrate rod opsins mainly use the Gt_1 α subunit, vertebrate cone opsins the Gt_2, invertebrate opsins the Gq, Taste-2 receptors the Gt_3 (gustducin), olfactory receptors the Golf, B-GPCRs with peptide agonists generally prefer the Gs, Frizzled receptors apparently use the Go, C-GPCRs couple either the Gq or the Gi α subunit types, and the canonical A-GPCRs variously Gi, Gq and Gs subunits (see Supplementary Table S-Lists, with designations mainly from Alexander et al. (2007). These assignations add up to indicate the general lack of dependence of sequence conservation in this juxtamembrane section of the ic4 domain on the overall length of the domain or the principal transducer used.

Basic motifs in intracellular domains have been extensively identified in activation of transducers by GPCRs, including those in the first (Wu et al. 1997; Yamashita et al. 2008), the second (Xie et al. 1997; Saito et al. 2005), the third (Ikezu et al. 1992; Okamoto and Nishimoto 1992; Lee et al. 1996; Wade et al. 1999; Kohen et al. 2001; Couvineau et al. 2003; Granier et al. 2004; Langer et al. 2005) and the fourth (Lyu et al. 2000; Marin et al. 2000; Couvineau et al. 2003; Tetsuka et al. 2004) domain. Motifs comprising basic and large neutral residues, e.g. of the BBXXB (B basic, X neutral, in most cases large sidechain) type (Okamoto and Nishimoto 1992), were found to also serve in Gα activation of single-pass GPCRs (Sun et al. 1995; Murthy and Makhlouf 1999). The high basicity of intracellular domains in GPCRs can also be viewed as an interactive signature reflecting competition for multiple transducers and effectors. These findings will be reviewed separately.

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