Amino Acids (2011) 40:261–268
DOI 10.1007/s00726-010-0616-1

REVIEW ARTICLE

Two intracellular helices of G-protein coupling receptors could generally support oligomerization and coupling with transducers

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Received: 21 April 2010 / Accepted: 27 April 2010 / Published online: 23 June 2010

Abstract For many G-protein coupling receptors (GPCRs), the upkeep of receptor dimers could depend on association with functional Gi α subunits. This is known for Y1, Y2 and Y4 neuropeptide Y receptors [presented in the companion paper (Estes et al., Amino Acids, doi:10.1007/s00726-010-0642-z, 2010)]. Interactions with transducers use mainly intracellular domains of the receptors. Intracellular loops 1 and 2 in GPCRs are short and lack extensive helicity that could support transducer anchoring. Interaction with G-proteins is known to use the juxtamembrane Helix 8 in the fourth intracellular domain, for which we document a helix-stabilizing $n/(n + 4)$ pattern of large hydrophobic sidechains. Another intracellular helix located in the C-terminal portion of the third intracellular loop does not display a strong stabilizing pattern, and is found in many studies to serve dynamically in association and activation of transducers and effectors. We show that these tracts share features across metazoan phyla not only in opsins and opsin-like receptors (including the Y receptors), but also in Taste-2 and Frizzled receptors. Similarities of these helices across GPCR groups could have both phylogenetic and functional roots.

Keywords G-protein coupling receptor · Neuropeptide Y · Receptor dimer · Receptor heteropentamer · Y1 receptor · Y2 receptor · Y4 receptor

Abbreviations

bRho	Bovine rhodopsin
GIR	Glucocorticoid-induced receptor (GPCR83)
GPCR	G-protein coupling receptor
H8	Helix 8
NPY	Human/rat neuropeptide Y
PTX	Pertussis toxin (EC 2.4.2.-)
ic3, ic4	The third and fourth intracellular domain
tm1…tm7	The respective transmembrane domains 1–7 of GPCRs

Introduction

Many heptahelical G-protein-coupling receptors (GPCRs) interact with G-protein α subunits using residues in two juxtamembrane intracellular tracts, one abutting the sixth transmembrane (tm6) domain, and another following the seventh transmembrane (tm7) domain (Helix 8; Palczewski et al. 2000). As reviewed here, both tracts show a large internal (consensus) conservation, significant similarity to opsins, abundance of ionic and especially basic sidechains, and large proven or predicted helicity. These features are also found across receptors responding to neuropeptide Y (NPY) and related peptides, which were surveyed in detail (as the group of GPCR to date characterized the most in regard to oligomerization). The two helices could be generally important in interactions of GPCRs with transducing

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0616-1) contains supplementary material, which is available to authorized users.

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GTPases. Assemblies of dimers with G-proteins via the two helices could be the primary and renewable form of organization for Y receptors (including the heteromerization documented in the companion paper (Estes et al. 2010), and likely for many other (especially peptide) GPCRs.

Methods used in surveying

The boundaries of domains in opsins and A-GPCRs were defined by alignment with those of bovine rhodopsin [PDB files 1F88, 1HZX, 3DQB, 3C9L, 1U19 and consensus from *tmhmm*, *tmpred* and *sosui* programs (available at the *expasy* website)], and in about 75% of the cases also correspond with boundaries from the above topology programs. The boundaries for other receptor groups are from the topology programs, aided by alignments with consensus profiles of the respective groups (Frommlet et al. 2004) in FASTA (Pearson and Lipman 1988) or CLUSTAL W (Thompson et al. 1994) programs (expasy website).

Local alignments with fixed residue positions were employed for transmembrane domains and for sections of these and intracellular domains. In A-GPCRs, the pivots (such as an asparagine in tm1; Palczewski et al. 2000) and ubiquitous motifs in transmembrane segments [LAxxD in tm2 (Parker et al. 2008a), D[E]RY[W] in tm3 (Flanagan 2005; Scheerer et al. 2008), WxP in tm6 (Holst et al. 2009) and N[D]PxxY in tm7 (Palczewski et al. 2000)] are highly correspondent by both approaches. Length of the transmembrane segments was fixed for domains 1, 2, 4 and 5 to 23, for tm3 to 27, for tm6 to 24, and for tm7 to 25 residues. These lengths correspond to topologically and crystallographically defined lengths of the transmembrane domains in bovine rhodopsin, and in many cases also to topological boundaries for non-visual A-GPCRs. Bovine rhodopsin (bRho) was used as template based on the finding that vertebrate opsins align better with GPCRs than invertebrate opsins (see Parker and Parker 2010, esp. Figs. 1, S1; Supplementary Tables S2.2, S3.2 in this review).

Comparative surveying of 210 human non-olfactory A-GPCR sequences (listed in the Supplementary Table S1) finds all to share proline pivot with opsins in the sixth transmembrane (tm6) domain at position 6.15, and more than 80% (including all Y and Y-like receptors) also to have proline pivot in the fifth transmembrane (tm5) domain, at position 5.14 (Tables S1 and S4). About 18% receptors do not have proline at or near tm5.14 (Tables S1 and S4), and most of these have lower similarity with ciliary opsins in intracellular domains (Tables S2.2, S3.2), and thus far were only identified in the vertebrate.

Helicities were predicted in the *porter* program (Pollastri and McLysaght 2005). The predictions are in good agreement with published structural characterization, where available.

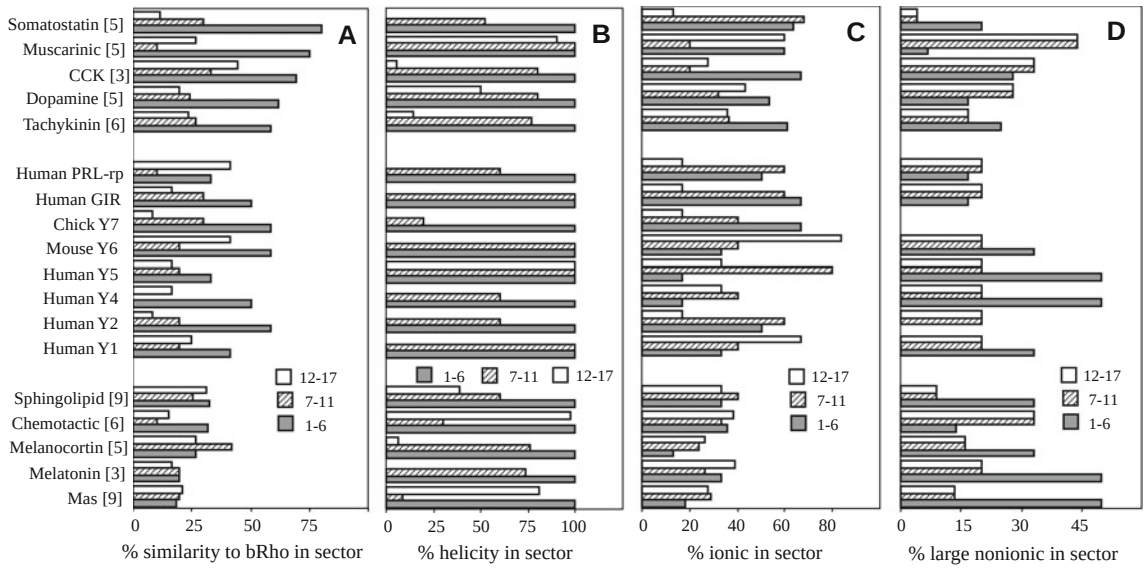

Fig. 1 A comparison of constituents in the C-terminal section of the third intracellular domain of Y receptors and other A-GPCRs grouped by agonist type. The receptors are human unless otherwise noted. All data are averages of the respective parameters for juxtamembrane positions 6-1, 11-7 and 17-12 (as counted toward the C terminus). The receptors groups represent the five highest (*top*) and least (*bottom*) scores for similarity to bRho at the juxtamembrane positions 6-1 in 30 receptor groups responding to similar agonists (see the Supplementary Table S2.1). Numbers of receptors in the respective groups are shown in *brackets* after the group labels. **a** Similarity to bovine rhodopsin, **b** predicted helicity, **c** ionic sidechains (His, Lys, Arg, Asp and Glu), **d** large non-ionic sidechains (Phe, Trp, Tyr, Ileu, Leu, Met and Val, "FWYILMV"). For other details, see "Methods" and Table S2

Surveys

Size, helicity and ionic character in intracellular domains 1 and 2 of GPCRs

As seen in Tables 1 and S1, the first two intracellular loops of GPCRs are quite short and do not present significant helicity. In loop 1, an unstructured centerpiece represents more than 50% of residues in most groups, and separates short helical termini that are contiguous with the adjacent transmembrane domains. Abundance of ionic residues in vertebrate opsins and the like receptors, however, suggests considerable potential for short-term electrostatic interactions with transducers and effectors. Fractions of the large hydrophobic residues (F, W, Y, I, L, M and V, "FWYILMV") are in most groups below the random value of 35%. In loop 2 the disordered centerpiece constitutes again above 50% of all residues in many groups. Ionic residues are less abundant in ic2 than in ic1. The large hydrophobes are above random in several groups, and most of these residues are in the less organized center of ic2.

Size, helicity and similarity to opsins in intracellular domains 3 and 4 of GPCRs

Intracellular domain 3 shows considerable variation in size and ionic sidechain content within and across GPCR groups (Table 2; Fig. 1; Supplementary Table S2). The ic3 domain across canonical GPCRs has at least 13 residues, but shows 400 or more in invertebrate A-GPCRs. In ic3 domains of non-visual A-GPCRs that have more than 30 residues, internal conservation (consensus) is higher at the C terminus relative to the N terminus (Tables 2, S2), and especially relative to the mid-section (Table S2). The comparison in Table 2 was made with eight residues for each end, which have very largely helical prediction in non-sensory A-GPCRs (corresponding to about two turns of an α helix (Pauling et al. 1951). A more detailed comparison in Table S2 considers positions (as counted from the C terminus) 6-1 (which are entirely helical in opsins and non-sensory A-GPCRs), 11-7 and 17-12. It should be noted that the two octads overlap for family C receptors.

The fourth intracellular domain also has at least 13 residues, with a completely helical prediction for residues 3-13 (Helix 8; see Table 1 in Parker and Parker 2010). A detailed assessment of residues in the ic4 domain is given in Parker and Parker (2010) and for Helix 8 in Fig. 3 in this review.

The C-terminal juxtamembrane helix in the third intracellular domain of GPCRs

Participation in signal transduction of residues and motifs in the C-terminal part of the third intracellular (ic3) domain was shown in a large number of studies (see "Discussion"). These tracts in Y receptors and other GPCRs were therefore examined for similarities in sequence, structure (especially helicity; Palczewski et al. 2000) and residue

Table 1 Intracellular loops 1 and 2 in GPCRs: domain size and fraction of ionic and hydrophobic residues

Group and number of receptors	Intracellular loop 1					Intracellular loop 2				
	Length	% in cpc	Basic (%)	Acidic (%)	lhph (%)	Length	cpc (%)	Basic (%)	Acidic (%)	% lhph
Opsins										
Bovine rhodopsin	11	54.6	36.4	0	27.3	14	7.4	21.4	7.1	28.6
Vertebrate rod [67]	11	54.6	36.1	4.1	27.3	14	71.4	21.4	6.9	28.5
Vertebrate color [241]	11	55.2	38.9	0.26	31.7	14	69.1	22.1	3.7	31.9
Invertebrate rod [8]	11	54.6	18.2	0	19.3	14.5	74.1	28	0	27
Invertebrate color [48]	11	54.6	18.9	0	19.1	13.7	69.8	18.4	3.7	29.5
Non-visual GPCRs										
Human A-GPCRs [210]	11.2	53	25.9	1.7	32.5	14.9	71	26.1	1.7	34.9
Invertebrate A-GPCRs [39]	11.4	53.8	23.3	3.8	39.7	14.7	72.3	26.5	4.4	29.9
Olfactory [391]	11	58.6	17.9	7.7	36	15	37.6	15	0.8	44.5
Taste-2 [25]	14	29.4	20.9	6.9	34.9	18	21.9	23.3	0.7	48.4
Peptide B-GPCRs [15]	12	50	38.2	0.53	23.2	13.9	45.9	4.8	7.6	47.8
Adhesion B-GPCRs [14]	10.2	48.9	22.3	4.8	29	13.5	51.2	28.2	8.4	36.2
Frizzled [11]	10.4	84.1	29.1	12.6	26.6	13.1	61.2	17.4	12.6	23.6
C-GPCRs [17]	15	43.2	22.2	8.1	26.1	17.2	47.6	22.5	4.2	28.3
Taste-1 [3]	14.3	35	21	4.8	26.7	21.7	48.1	15.3	3.2	36.7

% cpc the percentage of residues in the non-helical centerpiece, *% lhph* the percentage of large hydrophobic sidechains (F, W, Y, I, L, M, V). The receptors not otherwise identified are human

Table 2 Consensus, similarity to bovine rhodopsin and helicity in N-terminal and C-terminal octads of intracellular domain 3 in GPCRs

Group	Length of ic3	N-terminal octad			C-terminal octad		
		cons. (%)	sim. (%)	hel. (%)	cons. (%)	sim. (%)	hel. (%)
Opsins[a]							
Vertebrate rod [67]	28 ± 0	82.7	82.9	100	86.8	93.4	100
Vertebrate blue [80]	28 ± 0	78.1	71.2	91.5	90.2	90.6	100
Invertebrate rod [8]	41 ± 0.5	75	53.9	100	89.1	59.4	100
Invertebrate color [48]	41 ± 1	75	53.9	100	78.4	54.3	100
Non-visual A-GPCRs							
Human "P5P6" no monoamine [131]	31 ± 1.8	34.7	14.5	98.9	29	43.8	94.4
Human "P5P6" monoamine [40]	97 ± 62	46.6	13.8	100	54.4	46.9	98.8
Human "P6" [39][a]	38 ± 9	23.3	16	93.1	27.4	31.1	94.4
Invertebrate monoamine [28][a]	199 ± 113	36.2	10	100	62.1	50.5	97.3
Human olfactory [60]	18 ± 0	42.2	23.8	100	57.2	24.6	27.5
Other GPCR families							
Taste-2	20 ± 0	48.4	13.8	31.3	55.9	41.7	96.9
Peptide B (B1) [15]	21 ± 1.2	48.3	12.1	80.8	40	25	80.8
Adhesion B (B2) [9]	23 ± 9.9	30	18.8	87.5	36.3	22.5	42.5
Frizzled [11]	26 ± 2	53.4	21.2	98.4	77.6	52.6	99.4
Family C [13]	13 ± 0	63.5	19.2	26.9	77.9	24	61.5

cons. (%), % internal consensus; *sim.* (%), similarity to bRho; *hel.* (%), % helical content as predicted in the porter program. The number of receptors in a group is shown in brackets after the group label

[a] All surveyed opsins and all invertebrate monoamine GPCRs except s-p Q19007 have proline pivots at both tm5.14 and tm6.15 ("P5P6" type). The "P6" receptors have proline pivot only at tm6.15

types [in particular, basic (Okamoto et al. 1991) and helix-stabilizing large hydrophobic residues].

Excepting the C5a anaphylatoxin receptor (s-p P21730), the ic3 domain is defined with 17 or more residues in canonical A-GPCRs. Motifs that activate G-proteins are frequently found in the C-terminal helix of ic3 (11 residues in bRho, and 9-11 in Y receptors and other A-GPCRs; Table S2.2). In Y and similar receptors, the correspondence with bRho template and the internal consensus are invariably larger for C-terminal residues 6-1 compared to sections 11-7 and 17-12 (Fig. 1a; Table S2), indicating a larger functional conservation for the C-terminal hexad. The low N-terminal correspondence with bRho does not improve by shifting the frame of comparison for up to three residues, and the C-terminal correspondence is decreased by such shifts. It should be noted that the C-terminal similarity to bRho is quite low for a number of groups across A-GPCRs, and especially for receptors lacking tm5.14 proline (Fig. 1a; Tables 2, S1, S2.2 and S4).

Helicity forecasts for the ic3 domain are supported crystallographically for opsins (PDB files 1F88, 1HZX, 3DQB, 3C9L, 1U19) and by NMR for the vasopressin V2 receptor (PDB 2JX4). This helicity appears to be contiguous with the sixth transmembrane (tm6) domain, and is predicted as complete for the hexad abutting the tm6 domain (Fig. 1b). Residues 17-12 (counted toward C terminus) have usually no helicity forecast in ic3 domains with 20–40 residues (which are found in most Y receptors and other A-GPCRs, and in all opsins; Tables 2, S1 and S2). Similar to opsins, in A-GPCRs this could be an unstructured hinge assisting movements of the C-terminal ic3 helix, and of the N-terminal extension of the tm5 helix.

The ionic (mainly basic) sidechains in Y receptors are abundant in ic3-Ct.6-1 and especially in Ct.11-7 part (Fig. 1c; Table S2.2), with a considerable decrease in Ct.17-12. This is pronounced for the most conserved compared to the least conserved A-GPCR groups (Fig. 1c; Table S2.1), and is found in all ciliary opsins (Table S2.2). The large hydrophobic/non-ionic sidechain content is however at or somewhat above the random in the Ct.6-1 of the Y receptors, but much below random in other parts, and similar is found across A-GPCRs (Fig. 1d; Table S2).

C terminus of the ic3 segment also has a good correspondence with opsins in Taste-2 [gustducin (Gt_3)-preferring], and especially in Frizzled (Go-preferring) receptors (Tables 2, S2.2), both transducing through PTX-sensitive α subunits with highly similar C termini (Table S5). The Taste-2 receptors also show a large correspondence with bRho in Helix 8 (Table S3.2).

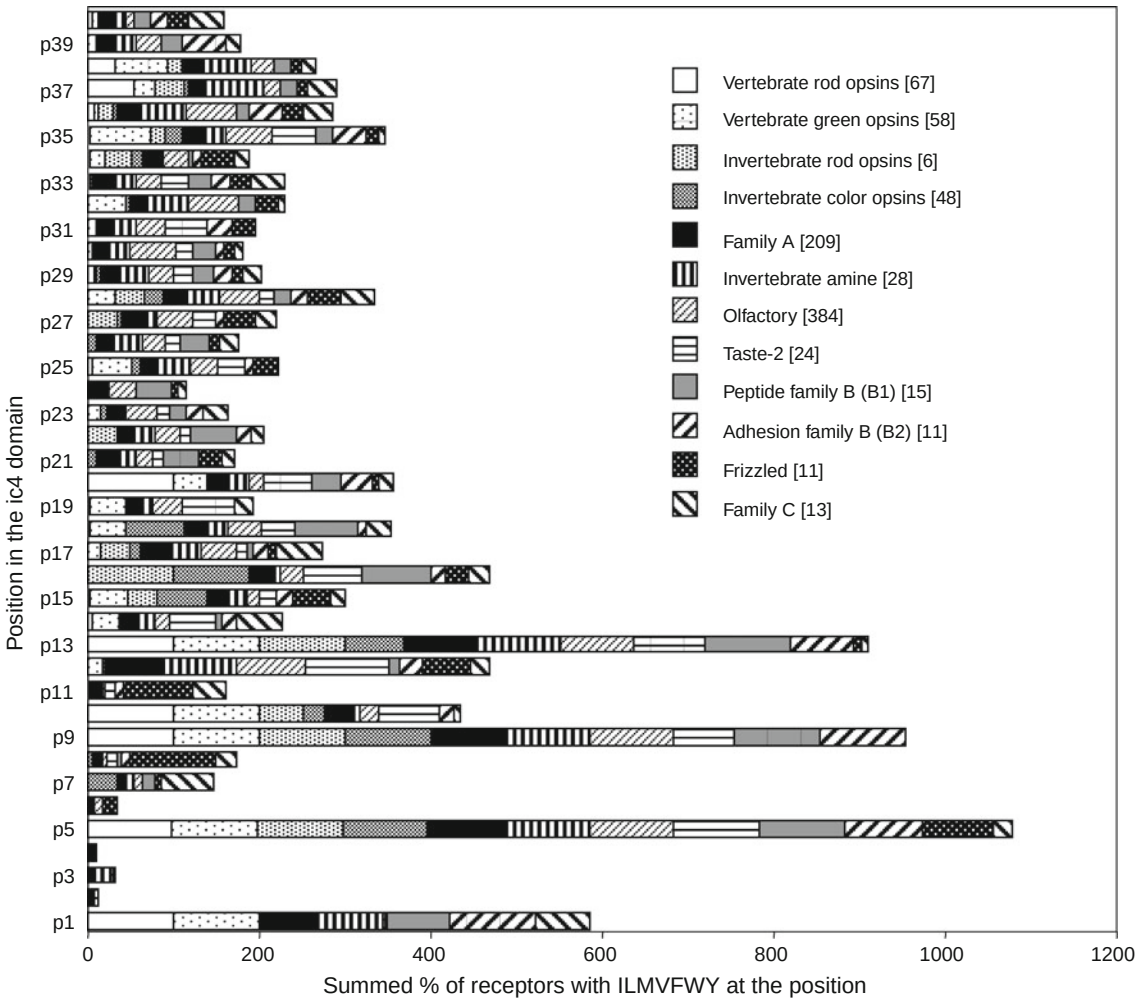

Fig. 2 Abundance of large hydrophobic sidechains (ILMVFWY) over the first 40 positions in the fourth intracellular domain of human heptahelical GPCRs. The number of receptors examined in each group is shown in *brackets* after the group name (the maximal cumulative percentage is 1,200)

The N-terminal juxtamembrane helix of the fourth intracellular domain in GPCRs

The fourth intracellular (ic4) domain has consistently high presence of large hydrophobic residues at positions 5, 9 and 13 in most GPCRs (Fig. 2). These residues should support strong $n/(n + 4)$ α-helical hydrogen bonding at the level of peptide bonds (Pauling et al. 1951; also see Steiner and Koellner 2001; Doig 2002). This pattern is ubiquitous in ciliary opsins (Table S3.2) and in A-GPCRs with high similarity to bRho (Fig. 3a), and is less frequent in A-GPCRs without proline at tm5.14, including most of *mas*, prostaglandin and purinergic A-GPCRs (Fig. 3a).

It should be recalled that positions 5, 9 and 13 are strongly conserved relative to bovine rhodopsin (as well as internally) in A-GPCRs and Taste-2 receptors (Parker and Parker 2010). This assures helix stability regardless of degree of association with the bilayer, and should be important for prolonged contact with G-protein heterotrimers. Throughout GPCR groups, large hydrophobic residues are much less abundant past Helix 8 (Fig. 2), and this section also has much lower helical predictions (Fig. 3c).

Use of the N-terminal tract of the fourth intracellular (ic4) domain in opsins and A-GPCRs for association with G-proteins/signal transduction was shown in a large number of studies (see "Discussion"), and this area is also known to be critical in surface expression of the receptors (Lin et al. 1998; Yasuda et al. 2009). Opsins and canonical GPCRs have at least 13 residues in the fourth intracellular domain, and most have more than 24 (Parker and Parker 2010). Residues 1–2 (as aligned with bRho) form a random-coil hinge preceding Helix 8 in bovine rhodopsin (Palczewski et al. 2000 and PDB files 1F88, 1HZX, 3DQB, 3C9L, 1U19), β_1-adrenergic receptor (PDB 2VT4), β_2-adrenergic receptor (PDB 3D4S), and adenosine A_{2A} receptor (PDB 3EML). In Y receptors and most other A-GPCRs, as well as

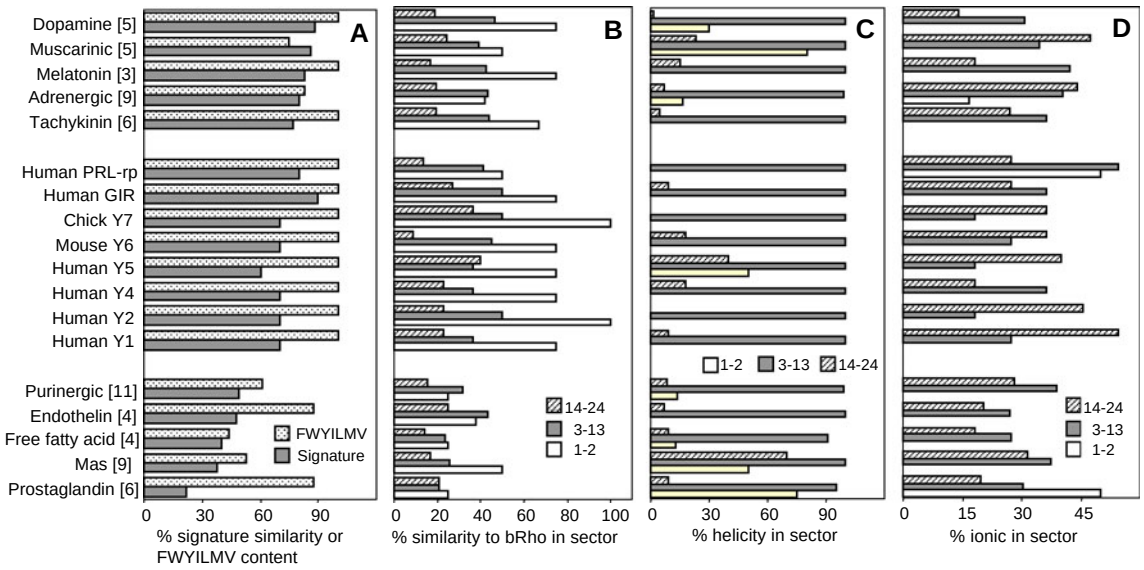

Fig. 3 A comparison of constituents in the N-terminal 1-24 section of the fourth intracellular domain of Y receptors and other A-GPCRs as grouped by agonist type. The receptors are human unless otherwise noted. All data are averages of the respective parameters. The receptors groups included represent the five highest (*top*) and lowest (*bottom*) scores for similarity to bRho at the juxtamembrane positions 3-13 (Helix 8) from 30 receptor groups responding to similar agonists (see the Supplementary Table S3). Numbers of receptors in the respective groups are shown in *brackets* after the group labels. For other details see "Methods" and Table S3. **a** Signature similarities to bRho at positions 2, 5, 6, 9, 13 and content of large non-ionic sidechains (Phe, Trp, Tyr, Ileu Leu, Met and Val) at positions 1, 5, 9 and 13 (see text). **b** Similarity to bovine rhodopsin at positions 1-2, 3-13 (Helix 8) and 14-24. **c** Predicted helicity for positions 1-2, 3-13 and 1-24. **d** Ionic residues (His, Lys, Arg, Asp and Glu) at positions 1-2, 3-13 and 14-24

in opsins, the ic4.1-2 residues are also predicted as unstructured by the *porter* program (Fig. 3c; Table S3). Residues ic4.3-13 are helical (Helix 8) in the above crystallographic structures, and also as forecast for all Y receptors and most A-GPCRs (Fig. 3c; Table S3). The ic4.14-24 residues show much lower structuring in the above PDB examples, as well as in program predictions (Fig. 3c; Table S3).

The ic4.1-13 sector of Y receptors exceeds the conservation observed in A-GPCRs, including the 2-5-6-9-13 signature similarity to bRho (Fig. 3a; for the 2-5-6-13 pattern see Parker and Parker 2010), and also general similarity to bRho and internal consensus (Fig. 3b). The Y receptors also share the predicted helicity profiles (Fig. 3c). The basic and ionic residues in ic4.3-13 (Helix 8) of Y receptors are however less abundant than A-GPCR averages for this sector (Fig. 3d; Table S3.2). Ionic content much above the random is, however, found in the ic4.14-24 tract of Y receptors, except the Y4 (Fig. 3d).

The electrostatic balance in the C-terminal 20-residue segment of the common G-protein α subunits is quite acidic in pertussis-toxin sensitive G-protein α subunits, but neutral or somewhat basic in Gs and Gq-type subunits (Table S5). This could favor interactions of PTX-sensitive subunits with basic motifs in ic3 and ic4 helices of the receptors.

Discussion

The C terminus of the ic3 domain (ic3-Ct) and Helix 8 are found to interact with G-proteins much more frequently than other intracellular tracts (see Okamoto et al. 1991; Ikezu et al. 1992; Liu et al. 1995, 1996; Wade et al. 1999; Huang et al. 2001; Chee et al. 2008 for the ic3-Ct, and Marin et al. 2000; Mukhopadhyay et al. 2000; Palczewski et al. 2000; Shi et al. 2001; Miura et al. 2003; Swift et al. 2006; Lehmann et al. 2007; Conner et al. 2008; Scheerer et al. 2008, 2009 for H8). In opsins and A-GPCRs both segments could generally be helical in association with the bilayer, and are helical upon co-crystallization with phospholipids. Both helices are preceded by less structured hinges, which should help a broadside positioning that can maximize contacts with transducers.

From PDB files 1F88, 1HZX, 3DQB, 3C9L, 1U19 for bovine rhodopsin and 3D4S, 2VT4, 3EML and 2JX4 for A-GPCRs, as well as from program predictions, the C-terminal helix of the ic3 domain could generally be contiguous with the tm6 helix, as part of a transductional engine (Bhattacharya et al. 2008; Scheerer et al. 2008; Holst et al. 2009). The ic3 portion, exposed to cytosol and rich in ionic residues, could be important in activation of transducing GTPases, possibly as related with transducer docking to Helix 8. Unlike Helix 8, the C-terminal helical

part of the ic3 domain does not have a $n/(n + 4)$ pattern of helix-stabilizing large hydrophobic residues, and could engage transducer motifs more dynamically.

There is considerable evidence for large and even critical involvement of Helix 8 in GPCR function and upkeep (Heding et al. 1998; Lin et al. 1998; Yasuda et al. 2009) and especially in interaction of GPCRs with transducers (Marin et al. 2000; Mukhopadhyay et al. 2000; Palczewski et al. 2000; Shi et al. 2001; Miura et al. 2003; Swift et al. 2006; Lehmann et al. 2007; Conner et al. 2008; Scheerer et al. 2008, 2009). This interaction frequently involves GPCR heteropentamers (Baneres and Parello 2003; Nobles et al. 2005), and is apparently stable in the resting state (Nobles et al. 2005; Estes et al. 2008), and important for surface expression of the receptors (Salahpour et al. 2004; Ulloa-Aguirre et al. 2007; Decaillot et al. 2008). Removal of Helix 8 results in loss of surface expression of several dimerizing GPCRs (Yasuda et al. 2009), and mutation of H8 basic residues can result in non-functional expressions unable to attach G-trimers (Ulloa-Aguirre et al. 2007). The gonadotropin releasing hormone receptor (the only A-GPCR that lacks H8) shows a high attrition at the level of ER, which is overcome by fusing a Helix 8-containing C terminus from an isoform receptor (Heding et al. 1998; Lin et al. 1998). As in Yasuda et al. (2009), this points to the role of H8 in evading the ER quality control system, via association with G-proteins. The apparent absence of H8 in C-GPCRs could be compensated by covalent dimerization of extracellular domains at the level of ER (see Jiang et al. 2004), and through stabilization of large ic4 domains of these receptors by satellite proteins (Shin et al. 2003).

The electrostatic interaction potential is strong in both ic4.3-13 (H8) and the less structured ic4.14-24 part (Fig. 3d; Table S3). However, the hydrophobic/van der Waals potential is much larger in H8, especially in Y receptors (Fig. 3a). The quadruple $n/(n + 4)$ repeat of large hydrophobic sidechains at ic4.1-13 (present invariantly in Y receptors and ciliary opsins; Fig. 3a; Table S3.2) should be important for sustained contacts in stable interactions of GPCRs and G-protein heterotrimers in epithelial-type cell lines (Nobles et al. 2005; Parker et al. 2007, 2008b) as well as in kidney tubular epithelia (Estes et al. 2008). Helix 8 may act as an ionic/hydrophobic zipper (Marti and Bosshard 2003), and has some similarity with zippers in *c-jun* and *c-fos* transcription factors (Chinenov and Kerppola 2001). The less structured remainder of the ic4 domain is known to be involved in contacts with receptor-processing proteins, including arrestins, kinases and phosphatases. Contact of H8 with the PTX-sensitive Gt_1 α subunit (transducin-α) in bovine rhodopsin involves primarily the C-terminal α_5 helix of the subunit (Scheerer et al. 2008, 2009), and this should also apply to the quite similar C termini of Gi α subunits (see Table S5) in partnering with A-GPCRs, including Y receptors.

Acknowledgments This work was partly supported by the US National Institutes of Health Grant R01 HD-13703.

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