Molecular Pharmacology Fast Forward. Published on December 27, 2006 as doi:10.1124/mol.106.029470
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MOL #29470

The Highly Conserved DRY Motif of Class A GPCRs: Beyond the Ground State

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Molecular Pharmacology Fast Forward. Published on December 27, 2006 as DOI: 10.1124/mol.106.029470
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Running title:
Alternative roles for the highly conserved E/DRY motif

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Abbreviations:
GPCR, heptahelical G protein-coupled receptors; CAM, constitutively active mutant; CA, constitutive activity; CIM, constitutively inactive mutant; ETC, extended ternary complex model.

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Abstract

Despite extensive study of heptahelical G protein-coupled receptors (GPCRs), the precise mechanism of G protein activation is unknown. The role of one highly conserved stretch of residues, the amino acids Glu/Asp–Arg–Tyr, (i.e. the E/DRY motif) has received considerable attention with respect to regulating GPCR conformational states. In the consensus view, Glu/Asp maintains the receptor in its ground state, since mutations frequently induce constitutive activity (CA). This hypothesis has been confirmed by the rhodopsin ground-state crystal structure and by computational modeling approaches. However, some class A GPCRs are resistant to CA, suggesting alternative roles for the Glu/Asp residue and the E/DRY motif. Here we propose two different subgroups of receptors within class A GPCRs that make different use of the E/DRY motif, independent of the G protein type (G_s, G_i or G_q) to which the receptor couples. In P1-type receptors, non-conservative mutations of the Glu/Asp-Arg residues, besides inducing CA, increase affinity for agonist binding, retain G protein coupling, and retain an agonist-induced response. In contrast, in P2-type receptors, the E/DRY motif is more directly involved in governing receptor conformation and G protein coupling/recognition. Hence, mutations of the Glu/Asp residues do not induce CA. Conversely, non-conservative mutations of the Arg of the E/DRY motif always impair agonist-induced receptor responses and, generally, reduce agonist binding affinity. Thus, it is essential to look beyond the rhodopsin ground state model of conformational activation to clarify the role of this highly conserved triplet in GPCR activation and function.

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The completion of the human genome project in 2003 (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml) identified approximately 720 genes that encode for the heptahelical G protein-coupled receptors (GPCRs) (Wise et al., 2004), which are the largest family of cell surface receptors (Fredriksson et al., 2003; Maudsley et al., 2005) and constitute the most diverse form of transmembrane signaling protein (Lefkowitz, 2000; Pierce et al., 2002). Of these genes, 282 belong to the Class A or Rhodopsin family (http://www.iuphar-db.org/list/index.htm). Members of this family respond to ligands that are extremely different in terms of chemical structure and size (small organic molecules, lipids, ions, hormones, short and large polypeptides, glycoproteins, and even photons of light), exert a wide range of physiological functions (neurotransmission, hormone response, inflammation, etc), mediate communication with the outside environment (taste, odor, vision), and contribute to diffusion and progression of infectious diseases. Importantly, more than 30% of the clinically marketed drugs target GPCR function, representing approximately 9% of global pharmaceutical sales (Brink et al., 2004; Drews, 2000). The broad range of biological functions together with the potential for pharmacological interventions has generated considerable interest in the mechanisms by which GPCRs mediate their effects.

Does a common structure predict a common behavior?

Although these putative GPCRs have no overall sequence homology, their primary structure is characterized by a common structural motif of seven transmembrane-spanning regions (7TM) (Bockaert and Pin, 1999). While the extracellular receptor surface is known to be critically involved in ligand binding (Schwartz, 1994; Strader et al., 1994), the intracellular receptor surface is known to be important for recognition and activation of heterotrimeric GTP-binding proteins (G

proteins) (Dohlman et al., 1991), the primary, but not sole, signal transducing system (Hall et al., 1999; Marinissen and Gutkind, 2001) for GPCRs.

It has not, however, been possible to define a consensus sequence of the binding interface(s) between receptor and G proteins (Bourne, 1997; Wess, 1998). Thus, there has been a sustained effort to elucidate the functional mechanisms of GPCRs, including their ability to undergo conformational changes and activate G proteins (Schwartz et al., 2006). Such efforts have focused on highly conserved aminoacid sequence motifs including one highly conserved stretch of residues, the triplet of amino acids Glu/Asp–Arg–Tyr. This E/DRY or DRY motif is located at the boundary between transmembrane domain (TM) III and intracellular loop (ICL) 2 of class A GPCRs (rhodopsin family). It plays a pivotal role in regulating GPCR conformational states (see Table 1).

The consensus picture

Indeed, the consensus picture derived in part from the rhodopsin structure is that the basic Arg (denoted residue 3.50) forms stabilizing intramolecular interactions, notably with the neighboring Asp or Glu (3.49) (Ballesteros et al., 1998; Ballesteros et al., 2001; Li et al., 2001), and/or with another charged residue (6.30) on helix 6 (Angelova et al., 2002; Ballesteros et al., 2001; Greasley et al., 2002; Shapiro et al., 2002; Zhang et al., 2005), thereby constraining GPCRs in the inactive (R) conformation. The crystal structure of the ground state of rhodopsin indicates that the Arg is engaged in a double salt bridge with the adjacent Glu (3.49) as well as with Glu (6.30) on helix 6 (Palczewski et al., 2000; Teller et al., 2001), suggesting that disruption of these salt bridges may be a key step in receptor activation (Angelova et al., 2002; Cohen et al., 1993; Greasley et al., 2001). Mutation of the Glu/Asp of the E/DRY motif has been proposed to induce a conformational change that repositions

the Arg from its polar pocket, resulting in the ability of some GPCRs to adopt an active (R*) conformation (Cotecchia et al., 2002; Scheer et al., 1996; Scheer et al., 1997). Thus, this **first phenotype** (P1-type) is characterized by an increase of agonist-independent basal receptor activity (constitutive activity, CA) upon mutation of Glu/Asp 3.49 (constitutive active mutant, CAM), which occurs, for example, in (rhod)opsin (Acharya and Karnik, 1996; Cohen et al., 1993; Franke et al., 1992), α_{1B} adrenergic receptors (α_{1B}-AR) (Scheer et al., 1996; Scheer et al., 1997), vasopressin type II receptors (V_2R) (Morin et al., 1998), β_2-AR (Ballesteros et al., 2001; Rasmussen et al., 1999), histamine H_2 receptors (H_2R) (Alewijnse et al., 2000), μ opioid receptors (μO-R) (Li et al., 2001), α_{2B}-AR (Ge et al., 2003), and oxytocin receptors (OT-R) (Favre et al., 2005) (Table 1).

The consensus picture does not apply to all GPCRs.

By analyzing the available literature concerning mutations at the E/DRY motif of class A GPCRs to find a common pattern to predict its function, we were able to discriminate at least one other phenotype. This **second phenotype** (P2-type) does not exhibit increased CA upon mutation of Glu/Asp 3.49 (constitutive inactive mutant, CIM), and is observed for muscarinic M1 and M5 (M1 and M5 AchRs) (Burstein et al., 1998; Lu et al., 1997), gonadotropin-releasing hormone (GnRH) (Arora et al., 1997; Ballesteros et al., 1998), cannabinoid 2 (CB_2R) (Feng and Song, 2003; Rhee et al., 2000), α_{2A}-AR (Chung et al., 2002; Wang et al., 1991), TP (TP-R) (Capra et al., 2004), V_{1A} ($V_{1A}R$) (Hawtin, 2005), and chemokine 5 (CCR5) (Lagane et al., 2005) receptors. Nonetheless, for receptors of the P2-type, mutations can still affect receptor function as Glu/Asp non-conservative (i.e. charge-neutralizing or hydropathy-reversing) mutations have a number of effects that support an important role in stabilizing receptor conformation (see Table 1). For example, in the TP-R, the E129V

mutant displayed a 2-6 fold increase in agonist affinity, a 10-fold decrease in EC_{50} and an approximately 2-fold increase in E_{max} for agonists compared to wild type receptor (Capra et al., 2004). This phenomenon, also observed for other GPCRs such as M1 AchR (Lu et al., 1997), GnRH (Arora et al., 1997; Ballesteros et al., 1998) and α_{2A}-AR (Chung et al., 2002), has been interpreted as a mutation-specific conformational change toward an active-like conformation in accordance to the extended ternary complex (ETC) model (Samama et al., 1993) however, this is not accompanied by detectable constitutive activity. For some receptors assignment to a defined phenotype was difficult due to differences in the methodologies used by various laboratories and/or lack of complete data. These receptors are listed as Undefined in Table 1.

Does Arg mutations add complexity or fit into these defined phenotypes?

In contrast to the enhancement of basal activity observed for Glu/Asp mutations, non-conservative mutations of Arg 3.50 show variable effects on function of the P1-type receptors but invariably exert strongly disruptive effects on P2-type receptor activity (Table 1). This correlation between effects of E/D and R mutations within the P1 and P2 groups of receptors is a key aspect of this phenotypic division. Furthermore, naturally occurring mutations in P2-type receptors have been identified that result in receptor dysfunction and are responsible for certain diseases e.g., nephrogenic diabetes insipidus (NDI) (Birnbaumer, 1995; Innamorati et al., 1997; Rosenthal et al., 1993) and hypogonadotropic hypogonadism (Costa et al., 2001). Interestingly, Arg 3.50 mutations also show two patterns of effects on agonist binding. The first (generally in P1-type receptors) preserves high affinity agonist binding and G protein coupling (rhod, α_{1B}-AR, V_2R, β2-AR, H_2R, μO-R, α_{2B}-AR and OT-R) whereas the second (in P2-type) disrupts high affinity agonist binding and,

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conceivably, G protein coupling (M1 AchR and possibly M5 AchR, GnRH, CB_2R α_{2A}-AR, TP−R, $V_{1a}R$ and CCR5).

The effect of non-conservative Arg 3.50 mutations in P2-type GPCRs to disrupt receptor function concomitant with decreased agonist affinity is consistent with loss of G protein coupling in agreement with the ETC model (Table 1). Acharya and Karnik have suggested that Arg 3.50 interacts directly with the G protein to catalyze GDP release (Acharya and Karnik, 1996); however direct evidence in support of this conclusion is not available. The relation between binding and response for some P1-type receptors is harder to reconcile. There is an apparent paradox between the increased or unchanged agonist affinity and loss of function. There are two possible explanations for this. Arg 3.50 may serve as an effector for G protein activation as suggested by Acharya and Karnik (Acharya and Karnik, 1996) and Chung et al (Chung et al., 2002). Alternatively, mutations in Arg 3.50 of the V_2R may produce a "constitutively desensitized" phenotype, reported as a loss-of-function mutant due to decreased expression at the plasma membrane (Barak et al., 2001). This latter observation has been extended to other GPCRs, suggesting that this emerging paradigm of constitutive receptor desensitization might represent a general mechanism of hormonal resistance (Wilbanks et al., 2002).

Other considerations

The Tyr residue is the least conserved and studied among the triad sequence, with cysteinyl, histidyl, and serine residues occurring in some GPCRs, such as OT-R, V_2R, and GnRH. Tyr residue mutation often does not (Arora et al., 1997; Gaborik et al., 2003; Lu et al., 1997; Ohyama et al., 2002) or only marginally (Auger et al., 2002; Hawtin, 2005; Rhee et al., 2000; Zhu et al., 1994) affect receptor function.

GTPγS effects on agonist binding (i.e. GTP-induced affinity shift) has been seldom examined and, thus, the results are difficult to interpret (Table 1). Most CAMs become resistant to GTPγS effects, while the only CIM studied maintains the GTP shift for Asp 3.49 mutations, while having their affinity lowered for Arg 3.50, as one would expect. More variable are the effects for the receptors listed as Undefined.

Despite our efforts to find a common pattern within each class of receptors, there does not appear to be a specific aminoacid sequence or polarity profile in the ICL2 loop that accounts for the different functional properties of P1- and P2-type receptors (data not shown), as might be expected given that very closely related receptor subtypes (e.g. α_{2a} and α_{2b} AR) fall in different groups. Thus, given the present understanding of the mechanisms underlying receptor activation, it is not possible at present to predict the likely phenotype for a receptor that has not yet been mutated in this region.

Conclusions

The classification described above is certainly an over-simplification. First, some receptors did not fall into the two categories outlined. Also, an individual receptor might have constitutive activity or might be inactive depending on the particular signal output, but behave differently for another output. For example, the triple mutant DRY/AAY of the AT_1R, while being unable to induce inositol 1,4,5-trisphosphate (IP3) accumulation and to couple to G proteins (Shibata et al., 1996) (Gaborik et al., 2003), results in activation of the MAPK cascade, which is functionally Gq-independent but β-arrestin-dependent (Wei et al., 2003). Recently, Favre et al. demonstrated that the mutation D136N of the OT-R, enhances signaling through G_q proteins while disrupting interactions with G_i proteins (Favre et al., 2005). Furthermore, all the studies reviewed here are, of course, performed in recombinant

systems where only homodimerization is present or prevalent. In native systems the presence of heterodimerization may add complexity to some of the features here highlighted.

The two subgroups (P1- and P2-type) of class A GPCRs use the E/DRY motif in different ways (Figure 1). This is apparently independent of the class of G protein (G_s, G_i or G_q) to which the receptor is preferentially coupled (Burstein et al., 1998; Chung et al., 2002). In the P1-type group, E/DRY is involved in constraining the receptor in the ground state. In fact, activating mutations tend to weaken the ground state interactions of the central Arg and increase the solvent accessibility of selected amino acids at the cytosolic extensions of TM3 and TM6. Accordingly, all non-conservative mutations of the Glu/Asp or Arg residues increase or induce CA of the receptors, increase (or not affect) affinity for agonist binding, and retain G protein coupling. An agonist-induced response that is sometimes evident may also be masked by an increase in receptor internalization (constitutively desensitized receptor, apparent loss-of-function phenotype). While high affinity agonist binding is usually interpreted to reflect G protein coupling, it is possible that mutations may induce a high affinity (R*) state even in the absence of G protein coupling. Thus, the main role of Arg 3.50 in this group might be to maintain the inactive state of the receptor (Flanagan, 2005). In contrast, in the P2-type group, the E/DRY motif is more directly involved in governing G protein coupling/recognition. Hence, mutations of the 3.49 Glu/Asp residue do not induce CA, whereas agonist-induced responses are altered in a mutation-specific manner. Indeed, some non-conservative mutants yield receptors with more efficient signaling properties (increase in agonist potency and/or efficacy), an observation that suggests a conformational change in the ground state toward an active-like conformation, which, despite the absence of CA as generally intended (i.e.

increase in basal receptor signaling), might be viewed as a form of constitutive "activatability". Conversely, the central Arg of the DRY motif seems to be more directly involved in receptor–G protein coupling/recognition. Non-conservative mutations of this residue invariably impair agonist-induced receptor responses and also reduce affinity for agonist binding.

Measuring receptor cell surface expression, and especially their coupling efficiency to alternative signaling pathways should be considered in analysis of such mutants in the future. In this respect, ligand-induced regulation of [^{35}S]GTPγS binding can provide an excellent measures of the basic pharmacological characteristics and the relative efficacy of different mutants (Milligan, 2003), and should be, despite its technical difficulties, the primary choice in this type of studies.

We would also like to stress the importance, besides the charge, of the hydropathic characteristic of the residues involved in G protein–receptor binding (Capra et al., 2004; Greasley et al., 2001; Janz and Farrens, 2004; Moro et al., 1993; Wess, 1998). In fact, when mutagenesis was performed mutating the D142 of the α_{1B}-AR to all possible natural amino acid, a clear relationship was found between the empirically deduced hydrophathy index of the substituted residues and the extent of CA (Scheer et al., 1997). Thus, not only charge-neutralizing, but also hydropathy-reversing substitutions should be considered non-conservative and have been demonstrated to affect receptor functionality (Capra et al., 2004).

While other subclasses of class A GPCRs may exist with yet a different function of the conserved E/DRY motif, there are striking parallels between the functional behavior of the Glu/Asp and Arg mutations in the P1- and P2-type receptors. Extension of this concept to other Class A GPCRs and elucidation of the molecular basis for these distinct functional behaviors would be of significant interest

and should help clarify the role of this highly conserved triplet in GPCRs activation and function.

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Molecular Pharmacology Fast Forward. Published on December 27, 2006 as DOI: 10.1124/mol.106.029470
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Footnotes

This work was supported in part by grants from the Italian Ministry of University and Research, MIUR –PRIN 2004 (prot**.** 2004052155) to G.E. Rovati and V. Capra.

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Molecular Pharmacology Fast Forward. Published on December 27, 2006 as DOI: 10.1124/mol.106.029470
This article has not been copyedited and formatted. The final version may differ from this version.

Legends to the Figures

Figure 1. Effect of the mutation and proposed role for the DRY motif of class A GPCRs.

Table 1. Comparative study of E/DRY mutations in human GPCRs of family A.

Receptor*	Asp/Glu mutant	Basal activity	Agonist affinity	Agonist induced activity	Loss of GTPγS Shift	Reference	Arg mutant	Basal activity	Agonist affinity	Agonist induced activity	Loss of GTPγS Shift	Reference
CAM												
α_{1B}-AR	R, I	⇑	⇑			(Scheer et al., 1996; Scheer et al., 1997)	K	⇑		⇑		(Scheer et al., 2000)
							A, N, E, H, D	=/⇑		⇓ (CD)		"
α_{2B}-AR	A	⇑	⇑	⇑		(Ge et al., 2003)	NA					
β_2-AR	N, A	⇑	⇑	⇓/⇑	_	(Ballesteros et al., 2001; Fraser et al., 1988; Rasmussen et al., 1999)	NA					
H_2R	N, A	⇑	⇑	⇑	+	(Alewijnse et al., 2000)	N, A	⇓		⇓		(Alewijnse et al., 2000)
μ-OR	H, Q, Y, M	⇑	⇑	⇓	+	(Li et al., 2001)	NA					
	E	⇓	⇓	=		"						
OT-R	N	⇑	⇓/=	⇑	+	(Favre et al., 2005)	A	⇑		=	+	(Fanelli et al., 1999)
rhodopsin	Q, S, I	⇑		⇑		(Acharya and Karnik, 1996; Cohen et al., 1993; Franke et al., 1992)	Q-G	⇓/=		⇓		(Acharya and Karnik, 1996; Franke et al., 1992)
	D, L, F	⇓		⇓		"						
							Double mutants R-A, E, Q D A, R, Q	⇓/=		⇓		"
V_2R	A	⇑	=	⇑		(Morin et al., 1998)	H		=	⇓ (CD)		(Barak et al., 2001)

CIM											
α_{2A}-AR	I, N	=	⇑	=		(Wang et al., 1991) (Chung et al., 2002)	Q	⇓	⇓	⇓		(Chung et al., 2002)
CB_2R	A	⇓		⇓		(Feng and Song, 2003; Rhee et al., 2000)	A	⇓	=	=/⇓		(Feng and Song, 2003; Rhee et al., 2000)
CCR5	V	⇓	=	=		(Lagane et al., 2005)	N	⇓	⇓	⇓		(Lagane et al., 2005)
GnRH	N, E	=	=	=/⇑	_	(Arora et al., 1997; Ballesteros et al., 1998)	Q, A, S	=	=/⇓	⇓	+	(Arora et al., 1997; Ballesteros et al., 1998)
M1 AchR	E, N	=	=/⇑	=		(Lu et al., 1997)	N, A, L, Q, E		⇓	⇓		(Jones et al., 1995; Zhu et al., 1994)
M5 AchR	All	No ⇑ found				(Burstein et al., 1998)	All	No ⇑ found				(Burstein et al., 1998)
TP-R	V	=	⇑	⇑		(Capra et al., 2004)	V	=	⇓	⇓		(Capra et al., 2004)
$V_{1A}R$	E	=	⇓	⇓		(Hawtin, 2005)	A, H	=	⇓	⇓		(Hawtin, 2005)

Undefined												
A_3-R	N, K, R	=	=	=		(Chen et al., 2001)	A, K	⇓/⇑		⇑		(Chen et al., 2001)
							Double mutants D-K; R-K	=		⇓		"
AT_1-R	A, G	=/⇑	=	=/⇓	+	Ohyama, 2002 #778; Gaborik, 2003 #862]	A, G			=/⇓	_	(Gaborik et al., 2003; Ohyama et al., 2002)
							H			=/⇓ (CD)		(Wilbanks et al., 2002)
							Double/Triple mutants D-A, G; R-A, G; Y-A			⇓	+	(Gaborik et al., 2003; Ohyama et al., 2002; Shibata et al., 1996)
AT_2-R	A		=/⇓		+	(Moore et al., 2002)	A				_	(Moore et al., 2002)
							Triple mutants D-A; R-A; Y-A			=/⇓	+	"
CCR3	A, N			⇓		(Auger et al., 2002)	L			⇓		(Auger et al., 2002)
CXCR2	V	⇑				(Burger et al., 1999)	NA					
CXCR3	NA						N			⇓		(Haskell et al., 1999)
							Double mutants D-N; R-N			⇓		"
FP-R	NA						G, A		=/⇓	⇓	+	(Bennett et al., 2000; Miettinen et al., 1999; Prossnitz et al., 1995)
5-HT2AR	NA						E	⇓	=	⇓		(Shapiro et al., 2002)

CAM, constitutively active mutants; CIM, constitutively inactive mutants; CD, constitutively desensitized
* Receptor nomenclature follows the Official IUPHAR Nomenclature, available at http://www.iuphar-db.org/list/index.htm