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A Database of Mutants and Effects of Site-Directed Mutagenesis Experiments on G Protein-Coupled Receptors

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ABSTRACT **A database system and computer programs for storage and retrieval of information about guanine nucleotide-binding protein (G protein) -coupled receptor mutants and associated biological effects have been developed. Mutation data on the receptors were collected from the literature and a database of mutants and effects of mutations was developed. The G protein-coupled receptor, family A, point mutation database (GRAP) provides detailed information on ligand-binding and signal transduction properties of more than 2130 receptor mutants. The amino acid sequences of receptors for which mutation experiments have been reported were aligned, and from this alignment mutation data may be retrieved. Alternatively, a search form allowing detailed specification of which mutants to retrieve may be used, for example, to search for specific amino acid substitutions, substitutions in specific protein domains or reported biological effects. Furthermore, ligand and bibliographic oriented queries may be performed. GRAP is available on the Internet (URL: http://www-grap.fagmed.uit.no/GRAP/homepage.html) using the World-Wide Web system.**

Key words: Site-directed mutagenesis, G protein-coupled receptors, database, information storage and retrieval, software, World-Wide Web, Internet, ligand-receptor interactions, signal transduction, sequence alignment

INTRODUCTION

Performing substitution of single or multiple amino acids in proteins, and production of chimeric proteins are common techniques in modern molecular biology and protein engineering. These techniques have had a large impact on the knowledge of the structure and function of proteins, also for the large majority for which no experimentally determined three-dimensional structure has been reported. For several years, our research group has been involved in construction of three-dimensional models of guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs),[1,2] and the data obtained from site-directed mutagenesis studies have been useful in this work.[3] An iterative procedure involving molecular modeling, site-directed mutagenesis, and ligand-binding experiments has been suggested as a way to gradually improve the accuracy of such receptor models.[3] However, with the continuous and rapid increase in the number of reported experiments, it is difficult to keep track of all published mutation data in order to apply these in structure and function studies. By analysis of amino acid sequences, and by analysis of three-dimensional models of G protein-coupled receptors, various hypotheses for the structural and functional roles of individual amino acids have been proposed and examined by site-directed mutagenesis experiments (Table I). Information obtained from mutant receptors has also been utilized to construct three-dimensional GPCR models.[3,25–30]

The superfamily of GPCRs[30–33] are transmembrane receptors that transduce signals to effector proteins inside cells via coupling to G proteins.[34] Physiological actions of various endogenous compounds, including many hormones, autocrine and paracrine factors, and neurotransmitters, are mediated by GPCRs. This superfamily also includes receptors for sensory stimuli, for example, odorant compounds (odorant receptors) and photons (visual pigments or opsins). Structural data on GPCRs, based on biochemical, immunological, and biophysical approaches, have validated a consensus architecture of GPCRs with an extracellular N terminus, a cytoplasmic C-terminus, and seven transmembrane helices (TMHs) connected by loops.[35]

The GPCR superfamily has been divided into five different families.[36] A GPCR family is a collection of receptors whose protein sequences have more than or equal to 20% sequence identity in the predicted TMHs.[36] Currently, more than 200 unique receptor subtypes that are members of family A have been

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Table I. Hypotheses on the Structural and Functional Roles of Individual Amino Acids in GPCRs (Family A) That Have Been Tested by Using Site-Directed Mutagenesis Techniques*

Targets for site-directed mutagenesis
Consensus sites for glycosylation in extracellular domains (putative sites for N-linked glycosylation)[4]
Consensus sites for phosphorylation by different protein kinases in intracellular domains (putative sites for desensitization,[5] phosphorylation,[5] downregulation[6])
Residues in intracellular domains (putative sites for receptor sequestration)[7]
Residues in intracellular domains (putative sites for receptor downregulation)[8]
Cysteine residues in intracellular domains (putative sites for receptor palmitoylation)[9]
Cysteine residues in extracellular domains (putative sites for formation of disulfide bonds)[10]
Residues that take part directly in receptor-G protein coupling[11]
Residues that occur as point mutations† in abnormal functional genes[12] or as polymorphic variations in a population[13]
Consensus site for cleavage by thrombin in extracellular N terminus (putative role in signal transduction)[14]
Chromophore attachment site, Lys296 in TMH7, in rhodopsin[15]
Hydrogen-bonding or charged residues[16]
Residues that are highly conserved among family A receptors,[16] within a class of receptors,[16] within a subclass of receptors[17]
Residues that differ between receptor subtypes that bind members of the same class of ligands with different affinities[18]
Residues that differ between opsins[19]
Residues that differ between species homologues of a particular receptor subtype[20]
Residues that are unique for a particular receptor isoform (e.g., residues in the long isoform of the D_2 dopamine receptor that are absent in the short isoform of the D_2 dopamine receptor)[21]
Residues that are predicted to take part in ligand binding in three-dimensional models of ligand-receptor complexes[22–24]

*Only a few publications for each target are included.
†Mutations in human rhodopsin causing autosomal dominant retinitis pigmentosa.

cloned and sequenced.[36] GPCR family A includes opsins, odorant receptors, and receptors for various endogenous ligands, including biogenic amines, neuropeptides, glycoprotein hormones, platelet-activating factor, thrombin, adenosine, nucleotides, and eicosanoids. These receptors are characterized by amino acid sequences that have seven hydrophobic segments containing very distinctive sequence patterns.[37–39] These common patterns imply that all these receptors share the same basic three-dimensional structure, at least in the transmembrane domains.

Recently, molecular genetics has demonstrated that certain human diseases are caused by mutations in genes coding for specific GPCRs, including missense mutations, frame-shift mutations, and mutations that change any codon to a terminator codon.[40] The structural and functional effects of such mutations have been investigated by site-directed mutagenesis experiments.[12]

The number of single- and multiple-point mutants in GPCRs produced by site-directed mutagenesis have increased substantially over the last few years, and continues to increase. The amount of data from site-directed mutagenesis experiments, and the usefulness of the data from such experiments, have prompted us to produce a database containing results from site-directed mutagenesis studies and to develop software to access such a database. The software was used to construct a database of mutants and biological effects in GPCRs, but has been designed such that it also may be used for other protein classes.

Information obtained from experiments with single- and multiple-point mutants of GPCRs from family A have been assembled and organized into a searchable database (GRAP). GRAP includes data for mutants involving single and multiple amino acid substitutions in family A receptors[36] published in the English language before December 1995. Among the ~200 unique members of family A receptors (excluding species homologues) which have been cloned,[36] point mutations in 62 members of the family have been reported up to now and are included in the current version of GRAP (Table II).

MATERIALS AND METHODS

The GRAP database and software was implemented on a Sun SparcStation 10/51 using unix (SunOS 4.1.3) shell scripts, the AWK programming language,[41] and the World-Wide Web (WWW) system.[42–44]

Database Usage and Maintainance Software Package (PMDbW)

Every substitution stored in the database has one associated data file and a unique identification number. The searchable data on receptor class, subclass, species, secondary structure domain, substitution, and characterized properties, for example, agonist

Table II. Receptor Subtypes Registered in GRAP

Class	Subclass	Receptor subtype GRAP name*		
Adenosine		A_1AdenR	A_{2A}AdenR	A_3AdenR
Biogenic amine	Adrenergic	α_{1A}AdrenR	α_{1B}AdrenR	α_{2A}AdrenR
		β_2AdrenR		
	Dopaminergic	D_1DR	D_{2L}DR	D_{2S}DR
	Histaminergic	H_1HisR	H_2HisR	
	Muscarinic acetylcholine	M_1MAR	M_2MAR	M_3MAR
		M_5MAR		
	Serotoninergic (5-hydroxytryptamine)	$5HT_{1A}$R	$5HT_{1D\alpha}$R	$5HT_{1D\beta}$R†
		$5HT_{1E}$R	$5HT_{1F}$R	$5HT_{2A}$R
		$5HT_{2C}$R		
Eicosanoid	Thromboxane	TPR		
	Prostaglandin	$EP_{3\alpha}$R‡	EP_{3D}R‡	
Glycoprotein hormone	Follicle-stimulating hormone	FSHR		
	Luteinizing hormone/ choriogonadotropic hormone	LH/CGR		
	Thyroid-stimulating hormone	TSHR		
Neuropeptide	α-Chemokine	IL_{8a}R		
	Angiotensin	AT_{1A}AngR	AT_{1B}AngR	
	Bombesin/neuromedin	BB_1R	BB_2R	
	Bradykinin	B_2BKR		
	Complement 5a	C_{5a}R		
	Cholecystokinin	CCK_BR		
	Endothelin	ET_AR	ET_BR	
	Gonadotropin-releasing hormone	GNRHR		
	Melanocortin	MC_1R	MC_2R	
	Neuropeptide Y	Y_1NPYR		
	Neurotensin	NTR		
	Opioid	δOR	κOR	μOR
	Somastostatin	$SRIF_{1A}$R	$SRIF_{1B}$R	$SRIF_{1C}$R
		$SRIF_{2A}$R		
	Tachykinin (neurokinin)	NK_1R	NK_2R	NK_3R
	Thyrotropin-releasing factor	TRFR		
	Vasopressin	V_{1A}AVPR	V_2AVPR	
	fMLP related	fMLPR		
Nucleotide (purinergic)		P_{2U}R		
Opsin	Green	Opsin-green		
	Red	Opsin-red		
	Rod	Opsin-rod		
Platelet-activating factor		PAFR		
Thrombin		ThR		

*A unique GRAP name is defined as an abbreviation of an IUPHAR (International Union of Pharmacology) name for each receptor subtype, and for each isoform of a particular receptor subtype. Abbreviations: D_{2L}DR, D_2 long dopamine receptor (one of two isoforms); D_{2S}DR, D_2 short dopamine receptor (one of two isoforms); IL_{8a}R, interleukin$_{8A}$ receptor; $SRIF_{1A}$R, somatotropin release inhibiting factor$_{1A}$ receptor; V_{1A}AVPR, V_{1A} arginine vasopressin receptor; fMLP, *N*-formyl-met-leu-phe.

†The 5-HT_{1B}R is the rodent homologue of the human 5-$HT_{1D\beta}$R.

‡$EP_{3\alpha}$R and EP_{3D}R are isoforms of the prostaglandin EP_3 receptor subtype.

binding, were included in the data file names as short strings, and the components of each file name was separated with a separator character. Thus, the standard unix *find* program could be used as a search engine. When searching for bibliographic information, the standard unix *grep* program is used.

Every bibliographic record has its identification number for association between mutation data and literature references, and the reverse association (from bibliographic data to mutant data) is performed by a simple indexing system by collecting and storing the associated substitution identifica-

tion numbers. A similar association mechanism was used for the association between ligand and mutation data, while special characters were used in the mutation data files to identify a ligand name. Data for all the individual substitutions in one multimutant are collected in one file with a name determined by the first amino acid substitution entered for the multimutant. The remaining substitutions in the multimutant are stored as symbolic links that refer to this mutant file. The data files are stored in a directory hierarchy. Each directory contains one particular kind of information, for example, mutation data, bibliographic data, and ligand data.

To be able to use PMDbW for other protein classes, information dependent on protein class is kept in separate configuration files. The information kept in these configuration files define, for example, the protein domains and biological effect types, and will thus define the characteristics of the database. A number of other files are dependent on the actual information stored in the database, for example, subtypes and amino acid sequences. When one or more of these files are updated, database management programs need to be run to update files in the database system that are dependent on them, for example, the WWW-documents, which will enable searches using fill-in forms and menus.

GRAP Mutation Database

Literature searches for molecular biological data on GPCRs were performed in MEDLINE EXPRESS 1982–1994, REFERENCE UPDATE 1994–1995 (December) and CURRENT CONTENTS LIFE SCIENCES 1994–1995 (December). Data for single- and multiple-point mutants of members of the family A of GPCRs,[36] described in journal articles in English, were included in GRAP. Mutation data and bibliographic data were collected in GRAP, and GRAP has been continuously extended.

Database statistics for the current version of GRAP is shown in Table III. Each substitution was assigned to one or two of the following 15 receptor secondary structure domains: extracellular amino terminus, intracellular carboxyl terminus, extracellular loop 1–3, intracellular loop 1–3, or transmembrane helix (TMH) 1–7. Information regarding the effects (or lack of effects) by the substitution(s) on receptor properties were included for each of the mutants. Each mutant also has a hypertext link to either a Swiss-Prot[45] or a G protein-coupled receptor database (GCRDb)[36] entry so that further information on the amino acid sequence can easily be found.

For each ligand that is registered in the database, either an abbreviated or a full ligand name has been used to define a unique ligand identifier. For most of the ligands that are registered in the database various alternative names (full ligand names, synonyms, systematic names) and the Chemical Abstracts Registry number are available. Amino acid sequences of short peptide ligands are also available. Where the enantiomeric forms of ligands are given, GRAP uses the (D)/(L), (S)/(R) or (+)/(−) conventions from the original papers describing the mutants. Isotope-labels in radioligands are indicated by [^{125}I] or [^{3}H] prefixes preceding the ligand identifiers.

Table III. Statistics for the Current Version of the GRAP Database

	Number of entries in GRAP
Receptor classes	9
Receptor subclasses	30
Receptor subtypes	94*
Mammalian species	8
Mutants	2136†
Amino acid substitutions	3551‡
Positions substituted	1732§
Ligands	413
Papers referenced	332

*Isoforms and species homologues are included.
†The total number of all single and multiple mutants. The same receptor mutant may have been investigated in several studies.
‡The total number of amino acid substitutions in all single and multiple mutants.
§Number of positions in the alignment that have been linked to mutants.

The GRAP database includes multiple-point mutants where a low number of amino acids (up to 12) has been substituted, including some chimeric receptors where small segments were exchanged without any deletions or insertions of amino acids. Since the exact start- and endpoints of each TMH in GPCRs are not known, the assignments of point mutations to protein domains are not obvious for mutations in a region joining two domains. The domain assignments from the papers describing the mutants were used in GRAP. Thus, the domain assignment will depend on the actual defined endpoints of the TMHs. Some point mutations were assigned to two domains, that is, they were supposed to be near the interface between the two domains.

Detailed information regarding quantitative (Table IV) and qualitative (Table V) effects of mutations on agonist binding (chromophore binding in visual pigments) and antagonist binding properties were included for each mutant. Ligands (agonist or antagonist) were classified based on observed effects or lack of effects of mutations on receptor binding parameters. If the mutant showed at least a threefold increase or decrease in ligand-binding affinity compared to the wild-type receptor, or if the ligand-binding capacity (B_{max}) or the sensitivity of the receptor-binding affinity to either monovalent cations, guanine nucleotides, cholera toxin, or pertussis toxin, were markedly affected, then the receptor-binding properties for this particular ligand in

Table IV. Quantitative Information Provided for Mutants in the GRAP Database

Property	Parameter	Registered effects in GRAP
Binding of radioligands	Dissociation constant for radioligand-receptor complex (K_d)	$K_{d,mutant}/K_{d,wild\text{-}type}$ ratio*
		$K_{d,wild\ type}/K_{d,mutant}$ ratio†
	Ligand-binding capacity (B_{max})	$B_{max,mutant}/B_{max,wild\text{-}type}$ ratio
Binding of competitive ligands (unlabeled)	Inhibition constant for competitive ligand (K_i)	$K_{i,mutant}/K_{i,wild\text{-}type}$ ratio*
		$K_{i,wild\text{-}type}/K_{i,mutant}$ ratio†
	Concentration of competitive ligand that displaces 50% of radioligand (IC_{50})	$IC_{50,mutant}/IC_{50,wild\text{-}type}$ ratio‡
		$IC_{50,wild\text{-}type}/IC_{50,mutant}$ ratio§
Binding of allosteric ligands (unlabeled)	Dissociation constant for binding of the allosteric ligand A to the allosteric site in the absence of any ligand binding at the primary binding site (K_{dA})	$K_{dA,mutant}/K_{dA,wild\text{-}type}$ ratio*
		$K_{dA,wild\text{-}type}/K_{dA,mutant}$ ratio†
	Cooperative factor between an allosteric ligand and a radioligand that binds to another site on the receptor (α)	$\alpha_{mutant}/\alpha_{wild\text{-}type}$ ratio¶
		$\alpha_{wild\text{-}type}/\alpha_{mutant}$ ratio‖
	Concentration of allosteric ligand required to slow the dissociation of a radioligand by 50% (IC_{50A})	$IC_{50A,mutant}/IC_{50A,wild\text{-}type}$ ratio‡
		$IC_{50A,wild\text{-}type}/IC_{50A,mutant}$ ratio§
Chromophore binding	Wavelength for the maximal absorbance of chromophore (λ_{max})	$\lambda_{max,mutant}$

*If $K_{mutant} > K_{wild\text{-}type}$.
†If $K_{mutant} < K_{wild\text{-}type}$.
‡If $IC_{50,mutant} > IC_{50,wild\text{-}type}$.
§If $IC_{50,mutant} < IC_{50,wild\text{-}type}$.
¶If $\alpha_{mutant} > \alpha_{wild\text{-}type}$.
‖If $\alpha_{mutant} < \alpha_{wild\text{-}type}$.

GRAP were defined as to be affected. The reported increase or decrease in receptor-binding affinity were associated with the ligand as K_i or IC_{50} values for competitive ligands, and K_d values for radioligands. Some of the registered mutants in GRAP contain quantitative effects of mutations on the binding of agonists to low-affinity states (G protein uncoupled receptor) and high-affinity states (G protein-coupled receptor). Quantitative effects of mutations on the cooperative factor (α), which describes the interactions between an allosteric ligand and a radioligand that binds to another site on the receptor, were included. The chromophore-binding properties of visual pigments (opsins) were characterized by λ_{max} values obtained from absorption spectroscopy experiments. If a mutation caused a large shift (no exact limit was defined) in the λ_{max} value, then chromophore binding was defined to be affected.

Detailed information regarding qualitative effects on binding of monovalent cations, receptor-G protein coupling and signal transduction properties were included for each mutant (Table V). Effects of guanine nucleotides, cholera toxin, or pertussis toxin on ligand-binding affinities, the binding of guanine nucleotides to G protein, and effects of cholera toxin or pertussis toxin on signal transduction, have been examined in mutant and wild-type receptors to investigate the effects of mutations on receptor-G protein coupling. Signal transduction properties were classified based on observed effect or lack of effect on agonist-induced signal transduction, and also on effect or lack of effect on basal (agonist-independent) activity of signal transduction.

GRAP Sequence Alignment

The majority of the receptor amino acid sequences with registered mutants were retrieved from the Swiss-Prot protein database.[45] The amino acid sequence of the short form of the rat dopamine D_2 receptor (415 amino acids) was obtained from GCRDb,[36] and the sequence of the long form (444 amino acids) was obtained from Swiss-Prot. Since multiple receptor forms of a single receptor subtype are not included in Swiss-Prot, the GCRDb[36] was used in order to obtain receptor isoforms not included in Swiss-Prot. The amino acid sequences were aligned using the Pileup program (gap weight = 3.0, length weight = 0.1) in the GCG program package.[46] Other alignment programs may be used to align the receptor sequences in GRAP. We may provide a better alignment of GPCRs in a future version of GRAP; however, high-quality alignments may be difficult to obtain. The alignment and its associated hypertext links can be updated by using software in the PMDbW package whenever new receptor subtypes and mutants are added to the database. In some cases where the residue number or type of a substituted amino acid did not agree with

TABLE V. Qualitative Information Provided for Mutants in the GRAP Database

Property	Effects of mutations on
Ligand binding	Ligand-receptor dissociation rate; receptor binding affinity and ligand binding capacity; proportion of receptors at the cell surface
	High-affinity state, low-affinity state, proportion of sites in high-affinity state*
Regulation of ligand binding	Receptor binding affinity or ligand-receptor dissociation rate: sensitivity to monovalent cations (e.g., Na^+), guanine nucleotides (e.g., GTPγS, Gpp(NH)p, GTP), pertussis toxin, cholera toxin, and $pH^\dagger$
Guanine nucleotide binding to G proteins	Light-induced or agonist-stimulated GTPγ^{35}S
	Basal GTPγ^{35}S binding to G proteins
GTP-GDP‡ exchange of G proteins	Light-induced GTP-GDP exchange activity of transducin
GTPase activity of G proteins	Light-induced or agonist-stimulated GTPase activity of G proteins
	Basal GTPase activity of G proteins
Inactivation of G proteins by venom toxin treatment	Signal transduction: sensitivity to pertussis toxin treatment and cholera toxin treatment
Adenylate cyclase (I-VIII) activity	Agonist-stimulated cAMP accumulation§
	Basal cAMP
	Basal cAMP-dependent transcription
	Antagonist-stimulated decrease in basal level of cAMP
	Agonist-inhibition of cAMP accumulation§
	Basal inhibition of cAMP accumulation
Phospholipase C-β activity	Agonist-stimulated PI hydrolysis§
	Basal inositol phosphates
	Antagonist-stimulated decrease in basal level of inositol phosphates
Activity of K^+ channels	Agonist-stimulated inwardly rectifying K^+ currents
Activity of Ca^{2+} channels	Agonist inhibition of Ca^{2+} currents
Activity of intracellular Ca^{2+} channels (IP_3R)¶	Agonist-stimulated Ca^{2+} sensitive Cl^- currents
	Agonist-stimulated increase in cytoplasmic $[Ca^{2+}]$
	Agonist-stimulated $^{45}Ca^{2+}$ release from *Xenopus* oocytes
MAP kinase kinase activity	Agonist-stimulated mitogen-activated protein (MAP) kinase kinase activation
Phospholipase A_2 (cytosolic) activity	Agonist-stimulated arachidonic acid release
Neoplastic transformation	Agonist-stimulated focus formation (morphology)
	Focus formation in unstimulated cells (morphology)
Mitogenesis	Agonist-stimulated mitogenesis (morphology)
Release of serotonin	Agonist-stimulated [^{3}H]serotonin release from intracellular granules
Secretion of β-hexosaminidase	Agonist-stimulated secretion of β-hexosamidase
Actin-polymerization	Agonist-stimulated actin polymerization

*Mutations may change the relative proportions of agonist-occupied receptors in high- and low-affinity states either by affecting receptor-G protein contacts or by affecting the interconversion between distinct conformational states of the receptors.
†Monovalent cations may bind to certain GPCRs and modulate receptor binding affinities for other ligands allosterically. Binding of guanosine 5′-*O*-3-thiotriphosphate (GTPγS), guanyl-5′-(β,γ-imido)diphosphate (Gpp(NH)p) or guanosine triphosphate (GTP) to G proteins uncouples receptors from G proteins. Pertussis toxin treatment causes selective inactivation of $G_{i/o}$ proteins. Cholera toxin treatment causes selective inactivation of G_s proteins.
‡GTP-GDP, guanosine triphosphate-guanosine diphosphate.
§Potencies, efficacies and coupling efficacies. cAMP, cyclic adenosine monophosphate. PI, phosphatidylinositol.
¶IP_3R, inositol 1,4,5-trisphosphate receptor.

the amino acid sequences obtained from protein databases, the assignments of amino acid sequence number or type were adjusted, and comments were added to the associated data file.

RESULTS AND DISCUSSION

Protein-Oriented Databases for GPCRs

WWW[42–44] is a system for managing and retrieving hyperlinked data across the Internet,[47] and has reached an enormous popularity in a short period of time. User-friendly software has been developed to display WWW documents and to follow the embedded links from server to server.[47] Information related to GPCRs is available from the Swiss-Prot protein database[45] at the ExPASy server,[48] the GCRDb,[36] and the Online Mendelian Inheritance in Man (OMIM) database,[49] using WWW. Further-

Point Mutations Database

Go to: Homepage, Search Form, Full Alignment, View Parts of Alignment.

Mutation search specification

❑ Single point ❑ Multiple points

Group: Adenosine
Biogenic amine
____Adrenergic
____Dopaminergic
____Histaminergic
____Muscarinic
____Serotoninergic(5-HT)
Eicosanoid
____Thromboxane
Glycoprotein Hormone

Species: Any

Domain: TM Segment 3 OR

Mutation: Asp ==>> Ala OR Asn OR Ser

Registered effect on: ❑ Signal Transduction ❑ Agonist Binding ❑ Antagonist Binding

Output: ❑ Group ❑ Subgroup ☒ Subtype ☒ Specie ☒ Domain ☒ Effect

Start the mutation search | Clear mutation search specification

Probe search specification

Enter a full or partial probe name or related information: bosentan

Start the probe search | Clear probe search specification

Literature search specification

Enter a full or partial author name, title string or journal reference in MEDLINE format:

strader

Start the literature search | Clear literature search specification

Fig. 1. The GRAP mutant search form. In the mutation search specification (upper part), the user may select one or several receptor groups, one or two receptor domains, and specify particular amino acid substitutions. Clicking on the species, domain, and mutation boxes brings up menus to select from. The receptor group list is a scrollable list. By setting toggles, one may narrow the search to only single-point mutants, only multiple-point mutants, only mutants with characterized signal transduction, or agonist-binding or antagonist-binding properties. Similarly, the amount of information to print in the list of matching mutations returned from a search may be specified. In the probe search specification (middle part), the user may enter a partial or a full ligand name or a Chemical Abstracts Service (CAS) registry number. Alternatively, one may click on the underlined word *probe*, and view all registered ligand names in GRAP. In the literature search specification (lower part), the user may enter bibliographic information to search for. An example GRAP reference may be seen by clicking on the underlined word *format*. In each of the sections, the search is initialized by clicking on the start button, and a clear button may be used to clear all user-defined settings. One can move to other parts in the database by clicking on the underlined phrases at the top of the figure.

Point Mutations Database

Go to: Homepage, Search Form, Full Alignment, View Parts of Alignment.

Domains:TM=Transmembrane segment; XC=Extracellular; IC=Intracellular

21 substitutions were found:

1. 5HT1AR Human in TM 3 Asp(116)->Ala Agonist Binding Antagonist Binding effect(s)
2. 5HT2AR Rat in TM 3 Asp(155)->Asn Signal Transduction Agonist Binding Antagonist Binding effect(s)
3. {ALPHA}2AAdrenR Human in TM 3 Asp(113)->Asn Signal Transduction Antagonist Binding effect(s)
4. {ALPHA}2AAdrenR Human in TM 3 Asp(130)->Asn Signal Transduction Agonist Binding Antagonist Binding effect(s)
5. {ALPHA}2AAdrenR Pig in TM 3 Asp(130)->Asn Agonist Binding Antagonist Binding effect(s)
6. {BETA}2AdrenR Human in TM 3 Asp(130)->Asn Signal Transduction Agonist Binding Antagonist Binding effect(s)
7. {BETA}2AdrenR Hamster in TM 3 Asp(113)->Asn Antagonist Binding effect(s)
8. {BETA}2AdrenR Hamster in TM 3 Asp(113)->Asn Signal Transduction Antagonist Binding effect(s)
9. {BETA}2AdrenR Hamster in TM 3 Asp(113)->Asn Signal Transduction Antagonist Binding effect(s)
10. {BETA}2AdrenR Hamster in TM 3 Asp(113)->Ser Signal Transduction Antagonist Binding effect(s)
11. D2SDR Human in TM 3 Asp(114)->Asn Agonist Binding Antagonist Binding effect(s)
12. H1HisR Human in TM 3 Asp(107)->Ala Signal Transduction Antagonist Binding effect(s)
13. H1HisR Human in TM 3 Asp(107)->Asn Signal Transduction Agonist Binding Antagonist Binding effect(s)
14. H2HisR Dog in TM 3 Asp(98)->Asn Signal Transduction Antagonist Binding effect(s)
15. M1MAR Rat in IC Loop 2/TM 3 Asp(122)->Asn Signal Transduction Agonist Binding Antagonist Binding effect(s)
16. M1MAR Rat in TM 3 Asp(105)->Asn Antagonist Binding effect(s)
17. M1MAR Rat in TM 3 Asp(122)->Asn Antagonist Binding effect(s)
18. M1MAR Rat in XC Loop 1/TM 3 Asp(99)->Asn Antagonist Binding effect(s)
19. M1MAR Rat in XC Loop 1/TM 3 Asp(99)->Asn Signal Transduction Agonist Binding Antagonist Binding effect(s)
20. M2MAR Human in TM 3 Asp(97)->Asn Antagonist Binding effect(s)
21. M2MAR Human in TM 3 Asp(97)->Asn Antagonist Binding effect(s) *

*This is a multi point mutant.

Fig. 2. List of mutants produced by a search for all Asp→Ala, Asp→Asn, and Asp→Ser mutations in the transmembrane helix 3 within the class of biogenic amine receptors (see Fig. 1). By clicking on one particular entry in this list, information for the mutant is displayed. Underlined phrases indicate hypertext links to other data in GRAP.

more, a large number of other databases related to biology and various other research tools are available, using WWW. We have therefore chosen to use the WWW technology for the purpose of constructing a user interface to the present database of point mutants and associated data.

In the implementation of GRAP, standard unix shell tools and text-processing tools were used. This enabled the generation of computer programs that easily can be used on a number of unix variants. The PMDbW software package has been tested on the Solaris 1.1 and IRIX 4.0.5 H operating systems. In contrast to C-programs, shellscripts do not require compiler software, and in many cases compiler software is not supplied as standard by computer vendors. Due to the fact that shell programs are interpreted and not compiled, they will run slowly. However, the present database search programs have been optimized for speed, by precreating routines that are dependent on database configuration, by algorithm optimization, and by using the text processing utility *awk*. In the current version of the software a response time of 5 seconds or less was observed for most database searches. For remote us-

Point Mutations Database

Go to: Homepage, Search Form, Full Alignment, View Parts of Alignment.

Asp (107) was changed to **Asn** in TM Segment 3 .

Amino acid sequence: HH1R_HUMAN

Signal Transduction effect:

Histamine-stimulated PI hydrolysis (Very weak)

Agonist Binding effect:

Histamine (No specific binding)

Antagonist Binding effect:

[3H]Mepyramine (126-)

Literature reference

Fig. 3. GRAP mutant entry example (entry number 13 in Fig. 2).[52] The underlined word *HH1R_HUMAN* is a hypertext link to the protein sequence entry in the Swiss-Prot database. One may view the associated literature reference by clicking on the underlined words *Literature reference.* One may view ligand data by clicking on the underlined words *Histamine* or *Mepyramine.* The text (126-) means that this mutation reduced the receptor binding affinity (K_d) for [3H]mepyramine by 126-fold.

ers the main obstacle for quick response will be data transfer over the network.

To the best knowledge of the authors, GRAP is the first effort toward a systematic organization of point mutant data for family A of GPCRs[26] into a searchable database allowing different user-defined queries to be performed. The GRAP database may be used for retrieval of relevant mutant data on specific proteins, and this may facilitate the use of mutant data in molecular modeling projects and in the planning of molecular biological projects. Thus, data from GRAP may be used to assist the construction of more accurate three-dimensional models of GPCRs and their complexes with ligands and G proteins. Such data may also stimulate the proposal of novel hypotheses for receptor activation, that is, the actual molecular events that transmit ligand binding into activation of G proteins, which remain to be elucidated.[50] In addition to GRAP, detailed data for a few hundred mutants in GPCRs have been assembled and made available on the Internet by the European Molecular Biology Laboratory (EMBL)[51] and by the National Institutes of Health (NIH). In contrast to the GRAP database, the source of mutation data available from EMBL[51] is a text file containing only a portion (around 300 mutants) of the mutations found in the GRAP database, and there has been no report of any program for searching in this data file. Compared to GRAP, which allow various user-defined queries to be performed, the WWW mutation database at NIH is currently restricted to mutation searches, which list a few registered mutations in a particular receptor domain.

In GRAP, receptor subtypes have been classified according to the structure of their endogenous ligands. A class was defined as a collection of receptors for one category of endogenous ligands, for example, neuropeptides, biogenic amines, and a subclass as a collection of receptors that share the same endogenous ligand(s). One class for all opsins and subclasses for individual opsin proteins that have been registered in the database were also defined. Sequence analysis of 59 biogenic amine receptors suggests that ligand-based classification schemes may neglect sequence similarities.[27] It has been suggested that serotonin (5-HT_{1A}, 5-$HT_{1D\alpha}$), dopamine (D_2, D_3, D_4), adrenergic (α_{2A}, α_{2B}, α_{2C}), and octapamine receptors that inhibit adenylate cyclase activity may form a subclass of receptors according to sequence similarities in their transmembrane segments.[27] However, the ligand-based classification was chosen because this is a well-established strategy for receptor classification, unlike sequence-based classifications.

Point Mutations Database

Go to: Homepage, Search Form, Full Alignment, View Parts of Alignment.

Bosentan

Synonym(s):

Ro470203

Systematic ligand name:

4-(1,1-dimethylethyl)-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxyphenoxy)[2,2'-bipyrimidin]-4-yl]-Benzenesulfonamide

CAS registry number:

147536-97-8

Bosentan has been used to test the following mutants:

1. ETAR Human in TM 2 Asp(133)->Val Agonist Binding Antagonist Binding effect(s) *
2. ETAR Human in TM 2 Ile(132)->Ala Agonist Binding Antagonist Binding effect(s) *
3. ETAR Human in TM 2 Ile(136)->Phe Agonist Binding Antagonist Binding effect(s) *
4. ETAR Human in TM 2 Leu(134)->Val Agonist Binding Antagonist Binding effect(s) *
5. ETAR Human in XC Loop 1 Asp(149)->Gly Agonist Binding Antagonist Binding effect(s) *
6. ETAR Human in XC Loop 1 His(150)->Asn Agonist Binding Antagonist Binding effect(s) *
7. ETAR Human in XC Loop 1 Phe(148)->Phe Agonist Binding Antagonist Binding effect(s) *
8. ETAR Human in XC Loop 1 Pro(147)->Thr Agonist Binding Antagonist Binding effect(s) *

*This is a multi point mutant.

Fig. 4. Ligand information and list of mutations produced by a search for the ligand name bosentan (see Fig. 1). CAS, Chemical Abstracts Service.

Database Usage

In the mutation search form (Fig. 1), the user selects from several options for receptor classes (Table II), receptor species, amino acids in mutations, and domains. Various wild-card searches (e.g., Asp → any in any domain in any of the registered receptors) as well as more specific searches may be performed. The possibility to specify either one or two domains is useful to retrieve mutations that are located in a segment joining two domains. The user is allowed to select multiple classes/sublcasses in each search. This means that complex queries may be specified where the user marks each class/subclass that should be included in the search, for example, serotonergic, dopaminergic, histaminergic, and adrenergic. By setting toggles, one may narrow the search to only single-point mutants, only multiple-point mutants, only mutants with characterized signal transduction, agonist-binding properties, or antagonist-binding properties. One may also specify the amount of information to print in the list of matching mutations returned from a search. The mutation query in Figure 1 specifies a search for all Asp→Ala, Asp→Asn and Asp→Val mutations in the TMH3 within the class of biogenic amine receptors, and Figure 2 shows the list of mutants returned by this query. By clicking on a particular entry in this list, information for the mutant is displayed (Fig. 3). From this view, it is possible to access either a Swiss-Prot[45] or a GCRDb[36] amino acid sequence entry for this particular receptor subtype, details about the ligand(s) used for testing the mutant, and the literature reference. When displaying the literature reference in MEDLINE format, a list of all substitutions characterized in the paper is given.

One may search for a full or a partial ligand name (Fig. 1) or a Chemical Abstracts Registry number. This will retrieve a list of matching ligands or mutations where the ligand has been used to test the biological activity of the mutant (Fig. 4). Bibliographic data may be searched by specifying a full or partial author name (Fig. 1), title string, or journal

Point Mutations Database

Go to: Homepage, Search Form, Full Alignment, View Parts of Alignment.

Found 19 matching references.

1. Fong TM et al. J Biol Chem 1994;269:14957-14961
2. Cheung AH et al. Mol Pharmacol 1992;41:1061-1065
3. Strader CD et al. J Biol Chem 1989;264:16470-16477
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8. Fong TM et al. Nature 1993;362:350-353
9. Strader CD et al. Proc Natl Acad Sci USA 1987;84:4384-4388
10. Strader CD et al. J Biol Chem 1988;263:10267-10271
11. Fong TM et al. Biochem 1992;31:11806-11811
12. Fong TM et al. J Biol Chem 1992;267:25668-25671
13. Fong TM et al. J Biol Chem 1992;267:25664-25667
14. Fong TM et al. Regulatory Peptides 1993;46:43-48
15. Huang R-RC et al. Biochem 1994;33:3007-3013
16. Cascieri MA et al. J Biol Chem 1994;269:6587-6591
17. Strader CD et al. J Biol Chem 1989;264:13572-13578
18. Huang R-RC et al. Mol Pharmacol 1994;45:690-695
19. Fong TM et al. J Biol Chem 1994;269:2728-2732

Fig. 5. List of literature references produced by a search for the author name Strader (see Fig. 1). By clicking on one literature reference in such list, the MEDLINE formatted literature reference (excluding abstracts) and the list of mutants described in the paper are displayed.

reference, and this query produces a list of literature references in a short format. By clicking on one reference in such list, the MEDLINE formatted reference (excluding abstracts) and the list of mutants described in this paper are displayed (Fig. 5). Search queries may be constructed using phrases in title strings or in journal references (abbreviated journal names). All mutants may be accessed from an alignment of all the sequences registered in the database. One may view a full or a partial alignment and access the mutant data from the alignment via hypertext links (Fig. 6).

The amino acid sequence alignment and the associated hypertext links to mutant data are important components of GRAP. The ligand-based classification of receptors (Table II) allows the user to search for mutations in specific classes of these receptors and to view parts of the alignment. Although the sequence alignment is not a very good one, the main issue, when including the alignment, was to allow access to substitution data directly from the amino acid sequences, for example, to extract data for mutations (in different GPCRs) that have been done at a particular position. By using this feature, one can compare novel results obtained in site-directed mutagenesis experiments against the data in GRAP. Furthermore, GRAP may be used in comparative analysis of GPCRs, revealing similarities and differences among family A receptors, or between subclasses and subtypes within the family. Due to the presence of gaps in transmembrane segments in the present alignment, equivalent amino acid residues in different receptors are not always aligned at equivalent positions in the alignment (same "column"). If one assumes regular α helices for all the transmembrane segments in GPCRs, no gaps should be allowed in these segments. However, current alignment methods fail to produce good sequence alignments if the amino acid similarities are low between some of the aligned sequences. The current GRAP alignment should be used with caution, particularly when distantly related receptor sequences are compared. However, each of the TMHs in the GRAP alignment include a few residues that either are conserved among most or among all of the aligned sequences (e.g., Asn in TMH1, Asp in TMH2, Arg in TMH3/second intracellular loop, Trp in TMH4, and Pro in TMHs5–7), and the position of such residues may be used to identify equivalent amino acid positions in diverse receptors, either by using a previously proposed common numbering scheme for family A GPCRs,[35] or by inspection of the GRAP alignment.

Possible Extensions of the Database

Additional biochemical, pharmacological, and molecular biological data for the mutants may be added

```
GRHR_RAT E.....FLCK VLSYLKLFSM YAPAFMMVVI SLDRSLAVTQ
V2R_BOVIN D.....ALCR AVKYLQMVGM YASSYMILAM TLDRHRAICR
V2R_PIG D.....ALCR AVKYLQMVGM YASSYMILAM TLDRHRAICR
V2R_HUMAN D.....ALCR AVKYLQMVGM YASSYMILAM TLDRHRAICR
V1AR_RAT D.....WLCR VVKHLQVFAM FASAYMLVVM TADRYIAVCH
GRPR_MOUSE R.....IGCK LIPFIQLTSV GVSVFTLTAL SADRYKAIVR
NMBR_RAT K.....LGCK LIPAIQLTSV GVSVFTLTAL SADRYRAIVN
ETBR_HUMAN AE.....MCK LVPFIQKASV GITVLSLCAL SIDRYRAVAS
ETBR_RAT AE.....MCK LVPFIQKASV GITVLSLCAL SIDRYRAVAS
ET1R_HUMAN HNDFGVFLCK LFPFLQKSSV GITVLNLCAL SVDRYRAVAS
OPRK_MOUSE D.....VLCK IVISIDYYNM FTSIFTLTMM SVDRYIAVCH
OPRK_RAT D.....VLCK IVISIDYYNM FTSIFTLTMM SVDRYIAVCH
OPRD_MOUSE E.....LLCK AVLSIDYYNM FTSIFTLTMM SVDRYIAVCH
OPRD_RAT E.....LLCK AVLSIDYYNM FTSIFTLTMM SVDRYIAVCH
OPRM_RAT T.....ILCK IVISIDYYNM FTSIFTLCTM SVDRYIAVCH
SSR2_RAT K.....AICR VVMTVDGINQ FTSIFCLTVM SIDRYLAVVH
SSRA_MOUSE K.....AICR VVMTVDGINQ FTSIFCLTVM SIDRYLAVVH
SSR3_RAT S.....LMCR LVMAVDGINQ FTSIFCLTVM SVDRYLAVVH
SSR5_RAT S.....FLCR LVMTLDGINQ FTSIFCLMVM SVDRYLAVVH
SSR1_HUMAN A.....LLCR LVLSVDAVNM FTSIYCLTVL SVDRYVAVVH
C5AR_HUMAN G.....AACS ILPSLILLNM YASILLLATI SADRFLLVFK
FMLR_HUMAN W.....FLCK FLFTIVDINL FGSVFLIALI ALDRCVCVLH
AG2R_HUMAN N.....YLCK IASASVSFNL YASVFLLTCL SIDRYLAIVH
AG2R_RAT N.....HLCK IASASVSFNL YASVFLLTCL SIDRYLAIVH
AG2S_RAT N.....HLCK IASASVSFNL YASVFLLTCL SIDRYLAIVH
IL8A_HUMAN T.....FLCK VVSLLKEVNF YSGILLLACI SVDRYLAIVH
BRB2_RAT E.....VLCR VVNTMIYMNL YSSICFLMLV SIDRYLALVK
PAFR_CAVPO K.....FLCN LAGCLFFINT YCSVAFLGVI TYNRFQAVKY
PAFR_HUMAN K.....FLCN VAGCLFFINT YCSVAFLGVI TYNRFQAVTR
P2UR_MOUSE T.....VLCK LVRFLFYTNL YCSILFLTCI SVHRCLGVLR
THRR_HUMAN S.....ELCR FVTAAFYCNM YASILLMTVI SIDRFLAVVY
GASR_HUMAN T.....VICK AVSYLMGVSV SVSTLSLVAI ALERYSAICR
GASR_RAT T.....VICK AISYLMGVSV SVSTLNLVAI ALERYSAICR
GASR_CANFA T.....VVCK AVSYLMGVSV SVSTLSLVAI ALERYSAICR
TRFR_MOUSE Y.....VGCL CITYLQYLGI NASSCSITAF TIERYIAICH
OPSG_HUMAN H.....PMCV LEGYTVSLCG ITGLWSLAII SWERWMVVCK
OPSR_HUMAN H.....PMCV LEGYTVSLCG ITGLWSLAII SWERWLVVCK
OPSD_BOVIN P.....TGCN LEGFFATLGG EIALWSLVVL AIERYVVVCK
OPSD_HUMAN P.....TGCN LEGFFATLGG EIALWSLVVL AIERYVVVCK
NK1R_HUMAN L.....FYCK FHNFFPIAAV FASIYSMTAV AFDRYMAIIH
NK1R_RAT L.....FYCK FHNFFPIAAL FASIYSMTAV AFDRYMAIIH
NK3R_RAT A.....NYCR FQNFFPITAV FASIYSMTAI AVDRYMAIID
NK2R_HUMAN R.....AFCY FQNLFPITAM FVSIYSMTAI AADRYMAIVH
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Fig. 6. Part of the GRAP amino acid sequence alignment (TMH3). The left column contains hypertext links (underlined words) to the protein entries in the Swiss-Prot database or in the G-protein coupled receptor database (GCRDb). The underlined amino acids in the alignment have been linked to mutant data by hypertext links.

to the database. The results obtained from site-directed mutagenesis experiments depend on expression of other endogenous and exogenous proteins (e.g., G proteins, effectors, kinases) in the cell type chosen for study and on the density of cell surface receptors. Therefore, future versions of GRAP may be extended to contain information about the cell type used for receptor expression. Other extensions may be structure implications suggested from the mutations, including the identification of specific ligand-receptor interactions and intramolecular interactions in the receptor, which could be used to assist the modeling of receptors[25,26,53] and ligand-receptor interactions.[27–30] Sequence-derived information may also be added to the mutations, including consensus sites for different protein kinases, consensus sites for N-linked glycosylation, cysteine residues at putative disulfide bridging sites, and cysteine residues at putative palmitoylation sites. The GRAP database contains many mutant records

corresponding to gene defects leading to human diseases: for example, autosomal dominant retinitis pigmentosa, congenital night blindness (rhodopsin), and X-linked nephrogenic diabetes insipidus (V_2 arginine vasopressin receptor). In future versions of GRAP, links to single and multiple amino acid substitutions corresponding to gene defects described in OMIM[49] may be added. This way, one can use the search facilities in GRAP to access information about the genetic causes and clinical consequences of mutations in specific domains in GPCRs.

Use of Mutation Data in GPCR Modeling

At present, the amino acid sequences for more than 250 subtypes of GPCRs are known,[36] whereas no detailed three-dimensional structure of any GPCR has yet been reported. The mutant data in GRAP indirectly provide structural information for GPCRs. Such information may be used to assist the construction of more accurate three-dimensional models of GPCRs and their ligand complexes. By using the ligand search option in GRAP, it is possible to obtain data about the quantitative and qualitative effects of mutations on the binding of specific ligands to specific receptors. This information may be used to assist the docking of specific ligands into receptor models either by using interactive computer graphics or by using distance restraints to restrain specific ligand-receptor interactions during energy calculations.

The overall three-dimensional arrangement of the seven TMHs of GPCRs are likely to be similar for all members of this family. When a detailed three-dimensional structure of a GPCR becomes available, for example, for rhodopsin,[54,55] information contained in GRAP may be useful in homology modeling of other receptors in family A.

CONCLUSION

GRAP may be regarded as a prototype for protein-oriented mutation databases for the WWW. The present database is a powerful tool for structure-activity studies, including molecular modeling studies on GPCRs and their interactions with ligands. Due to extensive use of hypertext links in GRAP, easy access to all relevant data is provided whether a substitution-oriented, ligand-oriented or bibliographic-oriented approach is used. The computer software developed for this purpose enables the construction of databases of mutant data for other classes of proteins.

GRAP is currently available on the Internet, and the PMDbW software can be obtained from the authors.

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