MINIREVIEW—COMPUTATIONAL ANALYSIS OF GPCRS

Understanding Peptide Binding in Class A G Protein-Coupled Receptors

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ABSTRACT

Many physiologic processes are controlled through the activation of G protein-coupled receptors (GPCRs) by regulatory peptides, making peptide GPCRs particularly useful targets for major human diseases such as diabetes and cancer. Peptide GPCRs are also being evaluated as next-generation targets for the development of novel antiparasite agents and insecticides in veterinary medicine and agriculture. Resolution of crystal structures for several peptide GPCRs has advanced our understanding of peptide-receptor interactions and fueled interest in correlating peptide heterogeneity with receptor-binding properties. In this

review, the knowledge of recently crystalized peptide-GPCR complexes, previously accumulated peptide structure-activity relationship studies, receptor mutagenesis, and sequence alignment are integrated to better understand peptide binding to the transmembrane cavity of class A GPCRs. Using SAR data, we show that peptide class A GPCRs can be divided into groups with distinct hydrophilic residues. These characteristic residues help explain the preference of a receptor to bind the C-terminal free carboxyl group, the C-terminal amidated group, or the N-terminal ammonium group of peptides.

Introduction

Over 50 peptides released by endocrine and neuronal tissues play roles as circulating hormones, neurotransmitters, local regulators, or all of these at once, controlling human development, reproduction, physiology, and behavior. This regulatory peptide system is a major target of therapeutic intervention in the control of body function. Targeting the regulatory peptide system has been critical in oncology, endocrinology, neuroscience, and many other areas (Lau and Dunn, 2018).

Biologically active peptides are produced from large precursor molecules via proteolytic cleavage and other post-translational modifications, such as amidation, acetylation, cyclization, sulfation, glycosylation, and phosphorylation. Although some peptides are characterized by a common motif (e.g., RFamide

peptides are characterized by a Arg-Phe-NH₂ sequence at their C-terminus), overall they have great diversity in size, chemistry, and structure.

Most regulatory peptides elicit their biologic responses by binding to over 100 G protein-coupled receptors (GPCRs), also known as seven transmembrane-spanning receptors. Among the six known classes of GPCRs, receptors from classes A and B bind regulatory peptides. In particular, a large number of regulatory peptides bind to class A GPCRs.

A “message-address” concept of peptide binding was introduced by Schwyzner in the 1970s following studies on adrenocorticotropin peptide truncation (Schwyzner, 1977). Thus, the adrenocorticotropin N-terminus—which is important for receptor activation acts as a ‘message’, while the peptide C-terminus enhancing the activity represents an ‘address’. This concept was further explored in structure-activity relationship (SAR) studies of opioid peptide dynorphin (Chavkin and Goldstein, 1981) and design of opioid agonists and antagonists (Portoghese et al., 1988, 1993). From these studies, it was

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ABBREVIATIONS: APLNR, apelin receptor; AT₁ and AT₂, angiotensin receptors; DAMGO, H-Tyr-D-Ala-Gly-N(Me)Phe-Gly-OH, (2S)-2-[[[2(R)-2-[[[(2S)-2-amino-3-(4-hydroxyphenyl)propanoyl]amino]propanoyl]amino]acetyl]-methylamino]-N-(2-hydroxyethyl)-3-phenylpropanamide; DIPP-NH₂, H-Dmt-Tic-Phe-Phe-NH₂; DOR, opioid receptor; EMPA, N-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-N-pyridin-3-ylmethylacetamide; ET_A, ET_B, endothelin receptors; GPCR, G protein-coupled receptor; KOR, opioid receptor; MOR, opioid receptor; MOP, opioid receptor; NK₁, NK₂, NK₃, neurokinin/tachykinin receptors; NMU1, NMU2, neuromedin-U receptors; NOP, opioid receptor; NPY₁, NPY₂, NPY₄, NPY₅, neuropeptide Y receptors; NTS1, NTS2, neurotensin receptors; OX₁, OX₂, orexin receptors; PAR1, PAR2, PAR3, PAR4, PAR5, proteinase-activated receptors; PMX53, (2S)-2-acetamido-N-[(3S, 9S, 12S, 15R, 18S)-15-(cyclohexylmethyl)-9-[3-(diaminomethylideneamino)propyl]-12-(1H-indol-3-ylmethyl)-2,8,11,14,17-penta-oxo-1,7,10,13,16-pentazabicyclo[16.3.0]henicosan-3-yl]-3-phenylpropanamide; SAR, structure-activity relationship; SB-674042, 5-(2-fluorophenyl)-2-methyl-4-thiazolyl[[2(S)-2-[(5-phenyl-1,3,4-oxadiazol-2-yl)methyl]-1-pyrrolidinyl]methanone.

Fig. 1. Receptor-peptide terminal group interactions in the X-ray peptide-GPCR complexes. The overview of the peptide binding is on the left side and the zoom view of the peptide terminal group interactions is on the right side. (A) Endothelin with the free C-terminal carboxylic group and ET_B; (B) neurotensin with the free C-terminal carboxylic group and NTS₁; (C) apelin with the free C-terminal carboxylic group and APLNR; (D) [Sar1, Ile8] angiotensin II with the free C-terminal carboxylic group and AT₂; (E) DAMGO peptide with the N-terminal ammonium and MOR; (F) CVX15 with the N-terminal ammonium and CXCR4. Peptide and receptor ribbons are in red and gray, respectively. Peptide carbon atoms are in green; only receptor residues forming interactions are shown. Salt bridges and hydrogen bonds are shown in pink and black dashed lines.

shown that there are separate recognition sites for the address and message moieties of a peptide in the receptor.

In recent years, remarkable progress had been made in the structural biology of GPCRs, mostly owing to the determination of crystallographic structures of several family members, including peptide GPCRs. Currently, 12 peptide class A and four peptide class B GPCRs have been crystallized in complex with either peptides or nonpeptide ligands. A comprehensive overview of the crystal structures of peptide GPCRs with regard to peptide and nonpeptide ligand binding has been recently reviewed by Wu et al. (2017). The newly available structural information, in combination with peptide structure-activity studies accumulated over years, not only describes atomic details of peptide-receptor interactions for the crystallized GPCRs but also helps in understanding how these structurally diverse molecules bind within the peptide GPCR subfamily. In this review, using available crystal structures of peptide GPCRs in conjunction with peptide SAR studies, receptor mutagenesis, and sequence analysis, we analyze binding of the C-terminus or N-terminus of regulatory peptides to the transmembrane cavity of the class A GPCRs. We show that peptide SAR data has effectively subdivided the class A GPCR binding-site residues into different groups having distinct hydrophilic residues. These characteristic hydrophilic residues are linked to the preference of the receptor to bind the C-terminal free carboxyl group, the C-terminal amidated group, or the N-terminal ammonium group of peptides.

X-Ray Structures of GPCRs Bound to Peptides

The crystal structures of the following class A peptide-GPCRs complexed with peptide agonists or antagonists are currently

available: apelin (APLNR), angiotensin (AT₁), neurotensin (NTS₁), endothelin (ET_B), opioid (MOR and DOR), chemokine (US28 and CXCR4), and component peptide (C5a₁) receptors (Wu et al., 2010; White et al., 2012; Burg et al., 2015; Fenalti et al., 2015; Qin et al., 2015; Shihoya et al., 2016; Ma et al., 2017; Asada et al., 2018; Koehl et al., 2018; Liu et al., 2018). Furthermore, subtypes of the protease-activated (PAR1 and PAR2), chemokine (CXCR4, CCR2, CCR5 and CCR9), opioid (MOR, KOR, DOR, and NOP), orexin (OX₁ and OX₂), angiotensin (AT₁ and AT₂), neuropeptide (NPY₁), C5a, and neurokinin (NK₁) receptors are crystallized in complex with nonpeptide orthosteric and/or allosteric antagonists (Wu et al., 2017; Liu et al., 2018; Robertson et al., 2018; Yang et al., 2018; Yin et al., 2018; Schoppe et al., 2019).

As expected, peptides tend to bind to GPCRs in many different conformations. However, in general, one part of the peptide is buried in the helical cavity, whereas another part forms interactions with the extracellular loops and the N-terminus of GPCRs. Peptides, including apelin, angiotensin, endothelin, neurotensin, and cyclic PMX53, point the C-terminus inside the helical bundle, whereas peptides binding to the opioid and chemokine receptors (Fig. 1) bind in the reverse orientation with the N-terminus pointing to the receptor helical side.

Polar interactions are the primary driving force for peptide recognition and binding. To identify the polar interactions, hydrogen atoms were added and optimized in the crystal structures; and hydrogen bonds and salt bridges between the peptide and the receptor were then calculated using the Maestro software (Maestro 9.9, 2014; Schrodinger, LLC, New York, NY) with default setting (Table 1). Given that the

TABLE 1

Hydrogen bond (H) and salt bridge (SB) interactions between a peptide and the helical cavity from the available crystal structures of class A GPCRs bound to a peptide

Peptide	Receptor	Overall Number of Contacts	Number of Contacts with Peptide Side Chains	Number of Contacts with Peptide Backbone	Number of Contacts with Peptide Terminal Group	Number of Peptide Residues within the Cavity	PDB Code
Apelin	APLNR	8	2H+1SB ^a	2H	2H+1SB	6	5VBL
Neurotensin	NTS ₁	6	1H	1H	3H+1SB	4	4GRV
Endothelin	ET _B	16	4H+1SB	6H	3H+2SB	16	5GLH
[Sar1, Ile8] AngII	AT ₂	12	3H+1SB	6H	1H+1SB	6	5XJM
DIPP-NH2	DOR	4	1H		2H+1SB	3	4RWD
DAMGO	MOR	4	1H		2H+1SB	5	6DDF
CX3CL1	US28	5	1H	3H	1H	7	4XT3
CX3CL1/Nb7	US28	7	2H	4H	1H	11	4XT1
vMIP-II	CXCR4	13	6H+2SB	2H	2H+1SB	9	4RWS
CVX15	CXCR4	10	6H+3SB		1H	6	3OE0
cyclicPMX53	C5a ₁	12	6H+1SB	5H		3	6C1R

^aHydrogen bond criteria: 3 Å for the maximum distance between donor and acceptor atoms, 90° and 60° for donor and acceptor minimum angles, respectively. Salt bridge criteria: the maximum distance between atoms is 5 Å.

extracellular loops and the N-terminus vary in GPCRs and the helical bundle is conserved, we analyzed interactions of the peptides with the helical bundle to compare the binding of the peptides. The peptides form multiple hydrogen bonds and salt bridges with the residues of the transmembrane helices via side chains, backbone, and terminal groups (Table 1). Interestingly, the terminal charged group of peptides located within the helical bundle is engaged in several polar interactions (Fig. 1). Thus, the C-terminal carboxyl group of angiotensin, apelin, endothelin, and neurotensin forms salt bridges and hydrogen bonds with positively charged lysine and arginine residues. The N-terminal ammonium group of the peptides in the opioid and chemokine receptors is engaged in an ionic interaction with an aspartate residue (Fig. 1).

Earlier SAR studies utilizing fragments and amidated or esterified analogs of angiotensin, endothelin, and neurotensin have shown that the C-terminus together with the free carboxyl group is required for the activity of these peptides (Rioux et al., 1975; Rovero et al., 1990; Labbe-Jullie et al., 1998). Indeed, the ionic interaction patterns observed in the crystal structures confirm the SAR studies.

The correlation between direct and indirect structural information of peptide binding suggests that SAR data of peptide terminal ends could be further linked with available structural information to gain insight into interactions of peptides with not yet crystalized GPCRs. A question then arises as to whether the large heterogeneity of peptide ligands and ability to activate different GPCRs can be linked or classified somewhat using binding characteristics of the peptide C- or N-terminal groups interacting with the transmembrane helices. In an attempt to address this question, we propose to integrate the knowledge of binding-site residues gained from the crystal structures with peptide SAR studies and sequence analysis of the class A GPCRs.

Class A Receptor Grouping with Peptide SAR and Hydrophilic Binding Site Residues as a Basis

We collected SAR data from the literature for 47 peptides binding to class A GPCRs to compare peptide binding properties. Table 2 shows a sequence of peptides or short, active peptide fragments, derived from peptide truncation studies

and peptide end-terminal functional groups important for activity and obtained from analog studies. The peptides are grouped on the basis of the importance of the peptide terminal end to activity. The C-terminus of the peptide contains a free carboxyl or amide group. For the first 11 peptides in Table 2, the free carboxyl group is important for peptide activity, whereas for a large number of peptides (19 in the table) amidation of the carboxyl group is favorable for activity. Amidation is the most common post-translational modification of peptides. For many peptides, this modification not only improves peptide stability and delivery but is also required for activity, as determined in the SAR studies (Table 2). The table also includes a number of regulatory peptides for which the N-terminus or the cyclic portion of the peptide structure is important for activity. Furthermore, we review available receptor mutagenesis and receptor-peptide modeling studies and show in Table 2 the peptide terminal end that is predicted to bind inside the helical bundle. From available published data, it appears that the terminal end of peptides, which is critical for activity ("message" moiety), is pointed to the helical bundle.

Initially, 35 residues lining the helical cavity of the peptide class A GPCRs were selected using the "ligand binding pocket for class A" option in the GPCRDB server (www.gpcrdb.org) to relate the binding properties of the peptide GPCRs with the collected peptide SAR data. In addition to the human receptors, several other orthologs (bovine, chimpanzee, guinea pig, mouse, rat, and rabbit) were considered in the amino acid residue alignment. The Ballesteros-Weinstein index (Ballesteros and Weinstein, 1995) was used for the residue selection and comparison among GPCRs. Next, taking into consideration the polar interactions in the crystalized peptide-GPCR complexes, the number of residues for the analysis was reduced to 25 by focusing only on residue positions with a high occurrence of hydrophilic residues. The alignment of 25 binding site residues was split into five groups on the basis of the binding of the peptide terminal end that is important for activity (Fig. 2). Figure 2 shows the alignment of binding site residues for the human receptors and the conservation score for hydrophilic residues in each residue position from the analysis of receptor orthologs. In the next sections, we will assess each of the receptor groups and highlight patterns of residues anchoring the "message" peptide end.

TABLE 2
Peptides binding to class A GPCRs with key regions important for activity

Peptide	Receptor	Sequence of a Peptide or Short Active Peptide Fragment (Peptide Region Important for Activity is Highlighted in Grey, Cysteine Involved in the Disulfide Bridges Are in Bold.)	End Group Important for Activity	Peptide Terminal End Inside the Helical Bundle	Reference
Angiotensin	AT ₁ , AT ₂	H2N-DRVYIHPF-COOH	-COOH	C-end	Rioux et al., 1975; Karnik 2000; Asada et al., 2018
Apelin	APLNR	H2N-LVQPRGPRSGPWQGGRRKFRQRPRLSHKGPMPF-COOH	-COOH	C-end	Murza et al., 2012; Ma et al., 2017
Bradykinin	B ₁ , B ₂	H2N-RPPGFSPFR-COOH	-COOH	C-end	Rhaleb et al., 1990; Jarnagin et al., 1996; Ha et al., 2006
Complement Peptides	C3a	H2N-SVQLTEKRM ^{DK} VGKYPKELRKCCEDGMRENPMRFS ^Q RRTRFISLGEAC	-COOH	C-end	Mollison et al., 1989;
C5a	C5a ₁ , C5a ₂	KKVFLDCCNYITELRRQHARASHLGLAR-COOH H2N-TLQKKIEIAAKYKHSVVKCCYDGACVNNDETCEQRAARIS- LGPRCIKAFTECCVVASQLRANISHKMDQLGR-COOH	-COOH	C-end	Higginbottom et al., 2005; Klos et al., 2013; Liu et al., 2018
Endothelin	ET _A ET _B	H2N-CSSSLMDKECVYFCHLDIIW-COOH	-COOH	C-end	Rovero et al., 1990; Shihoya et al., 2016
Neurotensin	NTS ₁ , NTS ₂	H2N-LYENKPRRPYIL-COOH	-COOH	C-end	Labbe-Julie et al., 1998; White et al., 2012; Kleczkowska and Ljpkowski, 2013)
H2 relaxin	RXFP1	Chain A: H2N-QLYSALANKCCHVGCTKRS ^L AREFC-COOH Chain B: H2N-DSWMEEVKLCGR ^L ELVRAQIAICGM ^T WS-COOH		C-end of Chain B	Bathgate et al., 2013; Hu et al., 2017; Patil et al., 2017; Wong et al., 2018
Insulin-like peptide 3 (INSL3)	RXFP2	Chain A: H2N-AAATNPARYCCLSGCTQ ^Q DLTLCPY-COOH Chain B: H2N-PTPEMREKLCGHF ^R VALRVCGGPR ^W STEA-COOH		C-end of Chain B	
H3 relaxin	RXFP3	Chain A: H2N-DVL ^{AG} SSSCCKWGC ^S SKSEISSLC-COOH Chain B: H2N-RAAPYGV ^R LCGR ^E FI ^R AV ^I FTCGGSRW-COOH		C-end of Chain B	
INSL5	RXFP4	Chain A: H2N-QDLQTLCTDGC ^S MTDLSALC-COOH Chain B: H2N-KESVRLCGL ^E YIRTV ^I YICASSRW-COOH		C-end	
Cholecystokinin gastrin	CKK1 CKK2	H2N-RDY-SO3H-TGWLDF-CONH2 H2N-LEEEEEAYGWMDFNH2	-CONH2	C-end	Morley et al., 1965; Jensen et al., 1982; Black and Kalindjian, 2002; Archer-Lahlou et al., 2006
Orexin	OX ₁ , OX ₂	Orexin A: (pyroglutamoyl)Pyr- PLPDCCCRQKTCSCRLYELLHGAGNHAAGILTL-CONH2 Orexin B: H2N-RSGPPGLQGLR ^L QLRLQASGNHAAGILTM-CONH2	-CONH2	C-end	Darker et al., 2001; Ammoun et al., 2003; Lang et al., 2004; Heifetz et al., 2013
Neuropeptide Y	NPY1, 2, 4, and 5	H2N-YPSKPDNPGE^DAPAE^DLARYSALRHYINILTRQRY-CONH2	-CONH2	C-end	Boublik et al., 1989; Xu et al., 2013, 2018; Yang et al., 2018
Prolactin-releasing peptide	PrRP	H2N-SRTHRHSMEIRTPDINPAWYASGRIPVGRF-CONH2	-CONH2	C-end	Boyle et al., 2005; Findeisen et al., 2011; Rathmann et al., 2012
Pyroglutamylated RFamide peptide	QRFP	H2N-TSGPLGNLAEELNGYSRKKGGSFRF-CONH2	-CONH2	C-end	Findeisen et al., 2011
Neuropeptide FF	NPF1-2	H2N-SQAFLFQPRF-CONH2	-CONH2	C-end	Findeisen et al., 2011
Metastin/kisspeptin	KISS1R	H2N-YNWNSFGLRF-CONH2	-CONH2	C-end	Kofani et al., 2001;
Vasopressin	V _{1A} , V _{1B} , V ₂	H2N-CYFQNCPRG-CONH2	-CONH2	Cyclic end	Findeisen et al., 2011
Oxytocin	OT	H2N-CYIQNCPLG-CONH2	-CONH2	Cyclic end	Jard and Bockaert, 1975; Mouillac et al., 1995; Chini and Fanelli, 2000
			-CONH2	Cyclic end	Jard and Bockaert, 1975; Chini and Fanelli, 2000; Gimpl and Fahrenholz, 2001

(continued)

TABLE 2—Continued

Peptide	Receptor	Sequence of a Peptide or Short Active Peptide Fragment (Peptide Region Important for Activity is Highlighted in Grey, Cysteine Involved in the Disulfide Bridges Are in Bold.)	End Group Important for Activity	Peptide Terminal End Inside the Helical Bundle	Reference
Neuromedin B	BB ₁	pEQLGNQWAVGHLM-CONH ₂	-CONH ₂	C-end	Mervic et al., 1991; Lin et al., 1995; Jensen et al., 2008
Gastrin-releasing peptide	BB ₂	MYPRGNHWAVGHLM-CONH ₂			
Bombesin	BB ₃	GNLWATGHFM-CONH ₂			
Neuromedin-U	NMU1-2	H2N-FRVDEEFQSPFASQSRGYFLFRPRN-CONH ₂	-CONH ₂	C-end	Brighton et al., 2004; Kawai et al., 2014
Substance-P/K, neurokinin	NK ₁ , NK ₂	H2N-RPKPQQFFGLM-CONH ₂	-CONH ₂	C-end	Couture et al., 1979; Ganjiwale et al., 2011
Neurokinin A	NK ₃	H2N-HKTDSEFVGLM-CONH ₂			
Neurokinin B		H2N-DMHDFVGLM-CONH ₂			
Thyrotropin-releasing hormone	TRH1	pEHP-CONH ₂	-CONH ₂	All	Chang et al., 1971; Engel and Gershengorn, 2007
Opioid peptides endorphins	DOR, KOR, MOR, NOP	H2N-YGGFL-COOH H2N-GGFM-COOH	H2N-	N-end	Morley 1983; Koehl et al., 2018
Neuropeptides B/W	NPBW1-2	H2N-WYKPAAGHSSYSVGRAAGLLSGL-COOH H2N-WYKHWASPRYHTVGRAAGLLMGL-COOH		N-end	Kanesaka et al., 2007
Neuropeptide S	NPSR1	H2N-SFRNGVGTGMKTSFQRAKS-COOH	O=HC-NH-	N-end	Roth et al., 2006
N-formyl peptide	FPR1-3	O=HC-NH-MLFK-COOH		N-end	Prossnitz and Ye, 1997
Adrenocorticotrophic hormone	MC1-5	O=HC-NH-SYSMEHFRWGKPV-CONH ₂	O=HC-NH-	FRW	Schwyzler, 1977; Matsunaga et al., 1989; Schioth et al., 1997; Audinot et al., 2001; Haskell-Luevano et al., 2001
Melanocortins (α , β , and γ)	α	O=HC-NH-SYSMEHFRWGKPV-CONH ₂			
Motilin	MLNR	H2N-FVPIFTYGELOQMKEKERNKGQ-COOH		N-end	Poitras et al., 1992; Xu et al., 2005
Ghrelin	GHSR	H2N-GSS(octanov)FLSPQHQRVQQRKESKPPAKLQPR-COOH		N-end	Charron et al., 2017
Gonadotropin-releasing hormone	GNRHR	CONHCHO-EHWSYGLRPG-CONH ₂	CONHCHO-	N-end	Hoffmann et al., 2000; Padula, 2005; Barran et al., 2005
Galanin	GAL1-3	H2N-GWTLNSAGYLLGPHAVGNHRSFSDKNGLTSCOOH	H2N-	N-end	Church et al., 2002; Lang et al., 2015
Urotensin	UT	H2N-ETPDCFWKVCY-COOH		Cyclic portion	Labarrere et al., 2003; Merlino et al., 2013
Melanin-concentrating hormone	MCH1-2	H2N-DFDMLRCMLGRVYRPCWQV-COOH		Cyclic portion	Matsunaga et al., 1989; Schioth et al., 1997; Audinot et al., 2001
Somatostatin	SST1-5	H2N-AGCKNFFWKFTTSC-COOH		Cyclic portion	Vale et al., 1978; Martin-Gago et al., 2013
Fragments of a tethered ligand	PAR1-4	H2N-SFLLRN-COOH H2N-TFLLRN-COOH			Gerszten et al., 1994

BB₁, BB₂, BB₃, bombesin receptors; C3a, C5a₁, C5a₂, complement peptide receptors; CCK1, CCK2, cholecystokinin receptors; ET_A, ET_B, endothelin receptors; FPR1, FPR2, FPR3, N-formyl peptide receptors; GAL1, GAL2, GAL3, galanin receptors; GHSR, ghrelin receptor; GNRHR, gonadotropin-releasing hormone receptor; MC5, melanocyte-stimulating hormone receptor; MCH1, MCH2, melanin-concentrating hormone receptors; MLNR, motilin receptor; NPBW1, NPBW2, neuropeptide B/W receptors; NPFF1, NPFF2, neuropeptide FF receptor; NPSR1, neuropeptide S receptor; PrRP, prolactin-releasing peptide receptor; QRFP, pyroglutaminylated RFamide peptide receptor; SST1, SST2, SST3, SST4, SST5, somatostatin receptors; TRH1, thyrotropin-releasing hormone receptor; UT, urotensin receptor; V1A, V1B, V2, vasopressin receptors.

Fig. 2. Alignment of binding site residues for human class A peptide GPCRs. (A) GPCRs binding peptides with the C-terminal free carboxyl group pointing to the helical cavity. (B) GPCRs binding peptides with the C-terminal amidated group pointing to the helical cavity. (C) GPCRs binding peptides with the N-terminus pointing to the helical cavity. (D) GPCRs binding the cyclic peptides. (E) PAR receptors. Positively charged, negatively charged, and amide-containing residues are in cyan, purple, and yellow, respectively. Receptors co-crystallized with a peptide and synthetic ligands are in gray. Residues forming direct interactions with peptides are in bold. APLNR, apelin receptor; AT₁, AT₂, angiotensin receptors; B₁, B₂, bradykinin receptors; BB₁, BB₂, BB₃, bombesin receptors; C3a, C5a₁, C5a₂, complement peptide receptors; CCK1, CCK2, cholecystokinin receptors; DOR, KOR, MOR, NOP, opioid receptors; ET_A, ET_B, endothelin receptors; FPR1, FPR2, FPR3, N-formyl peptide receptors; GAL1, GAL2, GAL3, galanin receptors; GHSR, ghrelin receptor; GNRHR, gonadotropin-releasing hormone receptor; KISS1R, kisspeptin receptor; MC1, MC2, MC3, MC4, MC5, melanocyte-stimulating

GPCRs Binding Peptides with the C-Terminal Free Carboxyl Group Interacting with the Helical Cavity

The receptors binding angiotensin, apelin, complement C fragment, endothelin, neurotensin, bradykinin, and relaxin peptides are grouped together (Fig. 2A). From the alignment of the peptide binding site residues, we highlight positively charged residues at positions 4.64 (or placed within the second extracellular loop 2), 5.42, and 6.55 with a degree of conservation (67%, 46%, and 41%, respectively).

AT₂, APLNR, ET_B, NTS₁, and C5a₁ have been crystallized in complex with a peptide, providing direct information about peptide-receptor interactions (White et al., 2012; Shihoya et al., 2016; Ma et al., 2017; Asada et al., 2018; Liu et al., 2018). Arginine at position 4.64 forms polar interactions with the backbone of angiotensin, apelin, and PMX53 in AT₂, APLNR, and C5a₁, respectively (Fig. 1). Lysine at position 5.42 forms a salt bridge with the free carboxyl group of angiotensin in AT₂. Arginine at this position in C5a₁ is 4.3 Å away from the backbone of PMX53. Lysine or arginine at position 6.55 forms a salt bridge with the C-terminal carboxyl group of apelin and endothelin in APLNR and ET_B, respectively (Fig. 1). In the case of NTS₁, residue 6.55 is at a distance of 3.8 Å from the C-terminal carboxyl group of neurotensin and, instead, arginine 6.54 forms a salt bridge with the terminal group of the peptide. Mutation of the charged residues in these three conserved positions reduces the peptide activity at AT₂, APLNR, ET_B, NTS₁, and C5a₁ (Labbe-Jullie et al., 1998; Higginbottom et al., 2005; Shihoya et al., 2016; Ma et al., 2017; Asada et al., 2018).

The two relaxin family peptide receptors, namely relaxin family peptides 3 and 4 (RXFP3 and RXFP4), have a positively charged residue in position 5.42, whereas the other two receptors, RFXP1 and RFXP2, in position 6.55. Mutation of the positively charged residue in these positions in RXFP3 and RXFP1 decreases peptide activity (Hu et al., 2016; Wong et al., 2018). Unlike other peptides of this group, the relaxin peptides are composed of two chains A and B linked with three disulphide bonds (Table 2). The C-terminus of chain B of H3 relaxin carrying the peptide activity (Patil et al., 2017) is predicted to interact with the positively charged residue at position 5.42 in RXFP3 (Wong et al., 2018). In the bradykinin receptors (B₁ and B₂), arginine at position 4.64 is conserved; however, no information on its importance in peptide binding is available in the literature.

GPCRs Binding Peptides with the C-Terminal Amidated Carboxyl Group Interacting with the Helical Cavity

Amidation of the C-terminal carboxyl group in a large number of regulatory peptides is important for biologic

Fig. 3. Ligand binding sites of peptide GPCRs. Nonpeptide antagonist interactions with residues at positions 3.32, 4.60, and/or 6.55 in the crystal structures of NPY₁, OX₁, OX₂, and NK₁. Validated homology model of the CCK2 receptor complexed with the CCK4 peptide (Langer et al., 2005). The antagonists, the peptide CCK4, and the receptors are labeled and only the amide-containing residue is shown. Hydrogen bonding is in black-dashed lines.

activity (Eipper et al., 1992). This group of peptides includes cholecystokinin, gastrin, orexin, neuropeptide Y, prolactin-releasing peptide, pyroglutamylated RFamide peptide, neuropeptide FF, kisspeptin, vasopressin, oxytocin, bombesin, gastrin-releasing peptide, neuromedin B, neuromedin U, substance P, neurokinin, and thyrotropin-releasing hormone. The receptors binding these peptides are grouped together and the alignment of binding-site residues highlights three notably conserved asparagine or glutamine at positions 3.32, 4.60, and 6.55, with the conservation score of 66%, 44%, and 52%, respectively (Fig. 2B).

Currently, there is no crystal structure of a GPCR bound to an amidated peptide demonstrating direct interactions between the peptide C-terminal amidated group and the receptor. However, the crystal structures of the neuropeptide Y₁ (NPY₁), orexin (OX₁ and OX₂) and neurokinin (NK₁) receptors are available in complex with orthosteric nonpeptide ligands (Fig. 3), guiding thoughts on hydrophilic interactions between a peptide and the receptor helical bundle.

The NPY₁ crystal structure is obtained in complex with two synthetic antagonists, BMS-19885 and UR-MK299 (Yang et al., 2018). The carbonyl of BMS-19885 ester group forms H-bonding with glutamine and asparagine at positions 3.32 and 6.55 (Fig. 3). The peptide bond-like moiety of UR-MK299 is engaged in two acceptor- and donor- hydrogen bonds with residue

hormone receptors; MCH1, MCH2, melanin-concentrating hormone; MLNR, motilin receptor; NK1, NK2, NK3, neurokinin/tachykinin receptors; NMU1, NMU2, neuromedin-U receptors; NPBW1, NPBW2, neuropeptide B/W receptors; NPFF1, NPFF2, neuropeptide FF receptor; NPSR1, neuropeptide S receptor; NPY₁, NPY₂, NPY₄, NPY₅, neuropeptide Y receptors; NTS1, NTS2, neurotensin receptors; OX₁, OX₂, orexin receptors; OT, oxytocin receptor; PAR1, PAR2, PAR3, PAR4, PAR5, proteinase-activated receptors; PrRP, prolactin-releasing peptide receptor; QRFP, pyroglutamylated RFamide peptide receptor; RXFP1, RXFP2, RXFP3, RXFP4, relaxin receptors; SST1, SST2, SST3, SST4, SST5, somatostatin receptors; TRH1, thyrotropin-releasing hormone receptor; V_{1A}, V_{1B}, V₂, vasopressin receptors; UT, urotensin receptor. Conservation score (%) for each residue position calculated from the analysis of receptor orthologs (bovine, chimpanzee, guinea pig, human, mouse, rabbit, and rat) is shown at the bottom of each binding site residue alignment. The conservation score and the corresponded bar are shown for the most conserved positively charged (cyan), negatively charged (purple) or amide-containing residues (yellow).

6.55 (Fig. 3). In mutagenesis studies, residues at positions 3.32 and 6.55 are predicted to be in contact with the peptide C-terminus and important for receptor activation (Sautel et al., 1996; Kaiser et al., 2015). Structurally, UR-MK299 imitates the C-terminal Arg and Tyr residues of the natural peptide, and its binding helps to model the binding mode of the peptide in NPY₁ (Yang et al., 2018). Furthermore, a very recent study employing mutagenesis and synthesis of peptide analogs with the modified C-terminal amide group has suggested that the C-terminal amide group forms interactions with asparagine 6.55 (Xu et al., 2018).

The crystal structures of the orexin receptors are also available in complex with three synthetic antagonists, suvorexant, SB-674042, and EMPA (Yin et al., 2015, 2016; Suno et al., 2018). The tertiary amide carbonyl group of suvorexant and SB-674042 forms a hydrogen bond with asparagine at position 6.55 in OX₁ and OX₂ (Fig. 3, shown for Suvorexant only). This functional group of the antagonists mimics the peptide amide group, suggesting also a potential interaction with this residue either through the backbone or the amidated peptide C-terminus. Suvorexant and SB-674042 are in very close proximity to glutamine at position 3.32 (3.3 Å), whereas EMPA forms a water-mediated contact with this residue (Suno et al., 2018) (Fig. 3). Although the role of asparagine 6.55 has not yet been validated in mutagenesis, mutation of glutamine at position 3.32 reduces the functional activity of the orexin peptides (Malherbe et al., 2010).

Residues 3.32 and 6.55 are also important in vasopressin binding to the vasopressin (V_{1A}) receptor (Mouillac et al., 1995; Chini and Fanelli, 2000). In the case of the cholecystokinin receptors (CCK1 and CCK2), receptor mutagenesis and structure-affinity studies of modified CCK analogs suggest that asparagine 6.55 forms hydrogen bonds with the amidated carboxyl group of the peptide C-terminus (Fig. 3) (Gigoux et al., 1999; Galés et al., 2003; Langer et al., 2005).

The receptors binding the RF-amide peptides, including neuropeptide Y, prolactin-releasing peptide, pyroglutamylated peptide, neuropeptide FF, and kisspeptin, have conserved glutamine 3.32. However, apart from the NPY receptors, the role of this residue in the other RF-amide receptors has not been established yet by mutagenesis. Another conserved residue in the RF-amide receptors is aspartate 6.59; mutation of this residue significantly attenuates peptide activity (Findeisen et al., 2011). From functional and modeling studies, it has been suggested that aspartate 6.59 interacts with the arginine of the peptide RF-amide motif (Rathmann et al., 2012).

Mutagenesis studies of the bombesin receptors (BB₂ and BB₃) indicate the importance of asparagine or arginine 3.32 for bombesin binding (Akeson et al., 1997; Nakamura et al., 2016). Mutation of tyrosine at position 6.55 in BB₂ is critical for the activity of the peptide (Lin et al., 2000). Within the bombesin family, an additional three polar residues, at positions 2.61, 6.51, and 7.39, are conserved and critical for peptide binding (Donohue et al., 1999; Lin et al., 2000).

Glutamine 3.32 has not been mutated yet in the thyrotropin-releasing hormone receptor (TRH1). Mutations of other polar residues at positions 3.37, 6.52, and 7.39 significantly reduce the binding of thyrotropin-releasing hormone, suggesting direct interactions with the backbone of the peptide (Engel and Gershengorn, 2007).

In the neuromedin U receptors (NMU1 and NMU2), mutation of arginine 6.55 reduces peptide binding (Kawai

et al., 2014). In addition, the importance of glutamates at positions 2.61 and 3.33 for the interaction with the peptide C-terminus has been shown in mutagenesis (Kawai et al., 2014).

The neurokinin receptors (NK₁, NK₂, and NK₃) do not have polar residues at positions 3.32 and 6.55. However, the amide-containing residue at position 4.60, which is conserved within the subfamily, forms a hydrogen bond with the antagonists aprepitant (Fig. 2), CP-99,994, and netupitant in the NK₁ crystal structure complexes (Yin et al., 2018; Schoppe et al., 2019) and has been predicted to bind to the neurokinin C-terminus according to mutagenesis and modeling studies (Lundstrom et al., 1997).

GPCRs Binding Peptides with the N-Terminal End Pointing to the Helical Bundle

Peptides having an N-terminal end important for activity include opioid peptides, neuropeptides B/W, neuropeptide S, N-formyl peptide, adrenocorticotropin, melanocortins, motilin, ghrelin, gonadotropin-releasing hormone, and galanin (Table 2). The sequence alignment of the binding site residues (Fig. 2C) identifies negatively charged residues in helix 2 at position 2.61 or in helix 3 either at positions 3.29, 3.32, or 3.33 in a large number of the receptors binding these peptides, with conservation scores of 43%, 30%, 30%, and 19%, respectively.

The crystal structures of the DOR and MOR receptors bound to a bifunctional peptide, DIPP-NH₂, and the peptide agonist DAMGO, respectively, have been determined (Fenalti et al., 2015; Koehl et al., 2018). The N-terminus of both the peptides binds in a similar way, with the ammonium group forming a salt bridge with the aspartate at position 3.32 (Fig. 1). This interaction is conserved with opioid-like compounds as observed in their crystal complexes with the opioid receptors (Wu et al., 2017). Mutation of this residue is critical for the activity of endorphins and opioid-like compounds (Surratt et al., 1994; Li et al., 1999).

The importance of a negatively charged residue at position 3.32 for the receptors binding neuropeptides B/W (NPBW1 and NPBW2) is unknown. Mutagenesis of aspartate or glutamate at position 3.33 in the FPR, motilin receptor (MLNR), and gonadotropin-releasing hormone receptors shows the significance of this residue for the binding of the N-formyl peptide, motilin, and ghrelin, respectively (Feighner et al., 1998; Mills et al., 2000; Xu et al., 2005). Binding studies of various analogs of the N-formyl peptide and motilin suggest that the N-terminal formyl-amide or the ammonium group interact with negatively charged aspartate or glutamate (Mills et al., 2000; Xu et al., 2005).

The importance of the negative charge at position 3.29 in the melanocortin receptors (MC1 and MC4) for the binding and potency of the melanocortin peptides is demonstrated in mutagenesis studies (Yang et al., 1997; Haskell-Luevano et al., 2001). However, it is not clear whether the N-terminal group or arginine of the critical phenylalanine-arginine-tryptophan (FRW) motif bind to the residue. Within the five melanocortin receptor subtypes, there are two other negatively charged residues, at positions 2.61 and 4.64. While residue 4.64 does not substantially change the potency of the peptide, residue 2.61 has a profound effect on peptide binding (Yang et al., 1997; Haskell-Luevano et al., 2001).

The receptors binding gonadotropin-releasing hormone, galanin, and neuropeptide S do not have a negatively charged residue in helices 2 and 3. However, there are negatively charged residues in other helices that are known to be important for the peptide activity. Thus, aspartate at position 2.61 is critical for peptide binding and signaling in the gonadotropin-releasing hormone receptor (GNRHR) (Hoffmann et al., 2000; Flanagan et al., 2000). In the galanin receptor (GAL1), mutagenesis and galanin SAR studies suggest that the ligand ammonium group binds to glutamate 6.59 (Kask et al., 1996; Church et al., 2002). The role of other negatively charged residues in position 5.39 in GAL2 and GAL3 and 6.55 and 6.58 in the neuropeptide S receptor (NPSR1) is unknown.

Over 20 GPCRs are known to bind chemokines. Because chemokines are classified as small proteins (8–10 kDa), these receptors belong to the protein-bound GPCRs. As mentioned previously, several crystal structures of the chemokine receptors are available bound to short peptides whose N-terminus points inside the helical bundle. Following the analysis of the receptor binding-site residues performed here, the negatively charged residues at positions 2.63, 6.58, 7.32, and 7.39 have a level of conservation among the chemokine receptors. Direct interactions with some of these residues are observed in the crystal structures of the chemokine receptors in complex with peptide and nonpeptide ligands (Tan et al., 2013; Wu et al., 2017). Thus, in the crystal structure of the CXCR4 receptor bound with a viral chemokine antagonist, the peptide N-terminal ammonium group forms a salt bridge with residue 2.63 (Qin et al., 2015). For a large peptide, like a chemokine, it is especially true that many binding factors, including steric and electrostatic interactions of various peptide residues, contribute together to peptide activity, thus the binding of the terminal ammonium group to a counterpart residue in the binding site might not be crucial.

GPCRs Binding Cyclic Peptides with a Cyclic Part Important for Activity and the PAR Receptors

The receptors binding urotensin, melanin-concentrating hormone, and somatostatin form a group with a conserved negatively charged residue at position 3.32 and a conserved amide-based residue at position 6.55, with 100% conservation in orthologs (Fig. 2D). Residue 3.32 is known to be important for peptide binding (Strnad and Hadcock, 1995; Macdonald et al., 2000; Sainsily et al., 2013). From mutated peptide analog studies, it appears that the positively charged residue within the cyclic part of the peptide forms polar interactions with aspartate 3.32 (Audinot et al., 2001; Labarrere et al., 2003; Martin-Gago et al., 2013). The importance of residue 6.55 is demonstrated in the urotensin receptor (Holleran et al., 2009). Unlike other peptides with the C-terminal free carboxyl group, this group is not important for urotensin binding, as its amidation does not affect peptide activity (Labarrere et al., 2003; Merlino et al., 2013). In contrast, the cyclic part of these peptides conveys the activity (Audinot et al., 2001; Labarrere et al., 2003; Martin-Gago et al., 2013).

In the case of the PAR receptors, there are conserved charged residues at positions 2.61 and 4.64 and the asparagine at position 6.52 (Fig. 2E). Position 4.64 is similar to the first group. While the lysine at position 2.61 forms direct

interactions with the synthetic ligand AZ8838 in the crystal structure of PAR2 (Cheng et al., 2017), positions 4.64 and 6.52 have not been yet explored. The importance of the extracellular loops in peptide binding has been examined in mutagenesis studies (Gerszten et al., 1994); however, the role of residues in the helical cavity needs further investigation.

Concluding Remarks

In this review, using earlier peptide SAR studies combined with alignment of binding site residues derived from the recent GPCR-peptide complex crystal structures, we divided the peptide GPCRs of class A into three major groups. In particular, we used the 25 residues of the GPCR helical bundle to characterize the hydrophilic properties of the receptor binding cavity.

The first group consists of the receptors that bind the free C-terminal carboxyl group of peptides inside the helical bundle. These receptors have several relatively conserved, positively charged residues at positions 4.64, 5.42, and 6.55. From the available crystal structures, these residues form polar interactions either with the C-terminal free carboxyl group or the peptide backbone. A recognition residue in a receptor for the peptide C-terminal carboxylate is not fully preserved, and a positively charged residue at other nonconserved positions could contribute to peptide binding. Thus, the interaction with the terminal carboxylate involves arginine at position 6.54 in NTS₁ and lysine at positions 3.33 and 5.38 in ET_B.

The second large group involves the receptors binding amidated peptides. Interestingly, these receptors have relatively conserved asparagine or glutamine at positions 3.32, 4.60, and 6.55. The analysis of the peptide SAR studies combined with receptor mutagenesis and the available crystal structures of the receptors in complex with synthetic antagonists suggests that the peptide terminal amide forms interactions with a counterpart amide group of glutamine or asparagine at one of these positions. In contrast to the group of the receptors binding peptides with the terminal free carboxyl group, this group of the receptors does not have conserved polar residues at positions 4.64 and 5.42 but has conserved asparagine or glutamine at position 6.55, instead of arginine or lysine. Residue 6.55 appears to be critical in peptide binding and could contribute to the differentiation of the C-terminus property for several receptors of these two groups.

The third group includes the receptors binding peptides with the N-terminus interacting with the helical bundle. Our analysis identifies a regular presence of a negatively charged residue at positions 2.61, 3.29, 3.32, and 3.33. The crystal structures of the opioid receptors bound to peptides show that the ionic interaction between the peptide N-terminal ammonium group and residue 3.32. Analysis of available SAR and mutagenesis data for several receptors of this group suggests the presence of ionic interaction between the peptide terminal ammonium group and a negatively charged residue of the receptor. In comparison with the first two groups, these receptors have a few positively charged and amide-containing residues in the binding cavity. Unlike the receptors binding amidated peptides, several receptors have aspartate at position 3.32 instead of glutamine.

The receptors binding the cyclic peptides with the cyclic part being important for activity have conserved aspartate and

glutamine/asparagine at positions 3.32 and 6.55, respectively. The importance of these residues is shown in mutagenesis for several receptors of this group. The PAR receptors form a separate group with conserved polar residues at positions 2.61, 4.64, and 6.52.

Comparison of the conservation score for the hydrophilic residues in the alignment of the binding site residues in the three major receptor groups shows a clear pattern in the conserved residue preference in the receptor group. In particular, the highest conservation score in the first group is for the positively charged residues, in the second group, for the amide-containing residues, and in the third group is for the negatively charged residues. Thus, the proposed grouping of the receptors on the basis of the peptide SAR data are justified from the sequence analyses of receptor orthologs.

The recent reporting of crystal structures of GPCRs bound to peptides allows assessment of the structural diversity of peptide binding for the first time. Furthermore, the structural data facilitates interpretation of peptide SAR studies and allows extrapolation of findings to related GPCRs. Thus, our polar residue analysis allows a grouping of the peptide receptors that highlights conserved residues important for peptide binding. Despite a large heterogeneity in possible binding modes of peptides within GPCRs, some level of generalization of peptide binding can be established through the analysis of the peptide terminal-end binding to the transmembrane helical cavity of GPCRs.

Further understanding of peptide-GPCR recognition has important implications for the design of peptides and peptide-like molecules as new pharmacological tools and medicines. From the conservation of a specific type of hydrophilic residue in a receptor, the binding orientation of peptides in the helical bundle could be identified, and therefore the important part of peptides for activity could be determined. This, in turn, provides a rationale for peptide modification. This knowledge can also facilitate peptide receptor deorphanization. Although 47 mammalian GPCRs were only analyzed here, studies could be expanded to neuropeptide GPCRs of parasitic nematodes and insects to facilitate a better understanding of peptide binding sites and improve design of GPCR-targeting agrochemicals.

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Participated in research design: Tikhonova, Fourmy.

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Wrote or contributed to the writing of the manuscript: Tikhonova.

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