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Review

7TM domain structures of adhesion GPCRs: what's new and what's missing?

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Adhesion-type G protein-coupled receptors (aGPCRs) have long resisted approaches to resolve the structural details of their heptahelical transmembrane (7TM) domains. Single-particle cryogenic electron microscopy (cryo-EM) has recently produced aGPCR 7TM domain structures for ADGRD1, ADGRG1, ADGRG2, ADGRG3, ADGRG4, ADGRG5, ADGRF1, and ADGRL3. We review the unique properties, including the position and conformation of their activating tethered agonist (TA) and signaling motifs within the 7TM bundle, that the novel structures have helped to identify. We also discuss questions that the kaleidoscope of novel aGPCR 7TM domain structures have left unanswered. These concern the relative positions, orientations, and interactions of the 7TM and GPCR autoproteolysis-inducing (GAIN) domains with one another. Clarifying their interplay remains an important goal of future structural studies on aGPCRs.

Highlights

Heptahelical transmembrane (7TM) domains of adhesion-type G protein-coupled receptors (aGPCRs) have proved to be recalcitrant to structural biological interrogation for a long time and their unusual mode of activation has underlined their peculiarity within the superfamily of GPCRs.

Nearly 30 structures of 7TM domains of human and mouse aGPCRs have now been released within a short period of time that cover a quarter of all mammalian aGPCRs and support the fundamental principles of aGPCR signaling.

Nonetheless, several key questions on adhesion GPCR activation, signaling, and physiology remain open.

Adhesion GPCRs have exceptional structural and functional profiles

Adhesion-type/class B2 GPCRs (**aGPCRs**; see Glossary) execute crucial tasks during development and in the operation of all major organ systems [1], and genetic lesions of aGPCR loci are associated with human disorders including many cancers [2]. In addition to a **7TM domain**, aGPCRs display exceptional structural features including large extracellular regions (ECRs) for adhesive interactions [3–5] and a **GAIN domain** [6]. The GAIN domain permits self-cleavage of nascent aGPCR proteins and sustained non-covalent attachment of the resultant receptor fragments to one another to generate the characteristic two-subunit layout of this large GPCR family (Box 1).

The GAIN domain of aGPCRs executes another pivotal function through a **TA/*Stachel*** element contained within, which activates receptor signaling [7,8] akin to protease-activated GPCRs [9] and glycoprotein hormone receptors [10–12]. The GAIN domain consists of two subdomains denoted A and B, of which the former is composed of two to six α-helices, and the latter of 13 β-strands in a β-sandwich [5,13]. Irrespective of whether the GAIN fold undergoes autocatalytic cleavage at the GPCR proteolysis site (GPS) or not, the TA component corresponds to the most C-terminal β-strand of the fold, which was shown in previous structural investigations of isolated GAIN domains [6] or GAIN domains embedded in the context of the wider ECR of the receptors [13,14]. In these structures, the TA is concealed within the autoproteolyzed GAIN domain including a hydrophobic network between the TA β-strand and its immediate environment. In this configuration, the TA – and therefore also the GAIN domain – acts as a junction between the N-terminal fragment (NTF) and the C-terminal fragment (CTF) of the receptor protein. Also in non-proteolyzable aGPCRs this TA position in the GAIN fold is indistinguishable from that of self-cleaved aGPCRs [6].

Studies on the structural flexibility of the GAIN domain suggest that it allows TA–7TM contact to regulate receptor activity [15–19], likely through partial exposure of the TA to a so far undefined

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Box 1. Nomenclature convention for adhesion GPCR structural domains

At the identification of the first human aGPCR, ADGRE5/CD97, only few distinct topological features of aGPCRs were deciphered, including a 7TM domain, an extracellular region (ECR) with adhesion motifs [84], and a juxtamembrane mucin-like stalk and cysteine box [85]. This set of tell-tale structural aGPCR characteristics remained unchanged for a long time. ADGRE5, and shortly thereafter ADGRL1, were found to be cleaved into two fragments before exiting from the endoplasmic reticulum (ER) that remain non-covalently associated [58,60]. The extracellular N-terminal fragment and the C-terminal membrane-spanning fragment were named NTF and CTF, or α and β subunits, respectively (not to be confused with Gα and Gβ subunits of G proteins) [22]. The cleavage point at which NTF and CTF are severed through an autocatalytic reaction [59] is located ~20–30 residues N-terminal to the start of the 7TM domain, and was termed the GPCR proteolysis site (GPS) [86]. All members of the aGPCR family except ADGRA1 contain a GPS, and most have a consensus sequence of $H^{-2}/R^{-2}L^{-1}|S^{+1}/T^{+1}$ (numbering with reference to the GPS) [87].

The GPS was first localized in an ill-defined juxtamembrane cysteine/tryptophan-rich receptor segment that was necessary but not sufficient for self-cleavage [58–60], and was later resolved as part of the GPCR autoproteolysis-inducing (GAIN) domain [6]. The GAIN domain consists of two subdomains and is necessary and sufficient for the autocatalytic cleavage of aGPCRs. Subdomain A is composed of up to six α-helices, while subdomain B adopts a twisted sandwich structure made up of 13 β-strands and two α-helices in which the five most C-terminal β-strands constitute the GPS motif. The N terminus of the CTF represents the tethered agonist (TA)/*Stachel* sequence which activates the aGPCR [7,8]. Despite the high variability in extracellular adhesion motifs across different aGPCR homologs, the GAIN domain is surprisingly well conserved in 32 of 33 mammalian aGPCR members (ADGRA1 has no GAIN domain) and similar conformations are observed in solved structures [6,14]. Note that humans only have a total of 32 aGPCRs because ADGRE4P is pseudogenized [88].

Several authors use the term 'stalk' or 'stalk region' synonymously for TA/*Stachel*. This is misleading and we believe that it should be avoided at all cost because 'stalk' and 'TA/*Stachel*' are non-identical aGPCR elements: they differ structurally and functionally.

With the advent of aGPCR 7TM structures, it is of note that the generic residue numbering scheme employed throughout primary literature reviewed herein is the sequence conservation-based Wootten scheme [35]. This numbering scheme has therefore been employed here as well. However, a structure-based generic numbering scheme is available and should ideally be used in future publications on aGPCR 7TM structures [89,90]. This scheme corrects for structural gaps in individual 7TM domains, and extends to helix 8 and other structurally conserved segments of the first two extracellular and intracellular loops, respectively [91]. Beyond the added segments, this scheme prevents offsets in the numbering relative to canonical α-helices such that numbers remain generic and equivalent across different structures.

interface in the 7TM domain [20]. In consequence, the NTF–CTF receptor heterodimer remains intact under these conditions, but the receptor can now transduce TA-dependent signals (**non-dissociation/displacement/tunable model**); in the alternative **dissociation/handgrenade/one-and-done model**, NTF shedding from self-cleaved aGPCRs [21] causes extraction of the TA from the GAIN domain, which results in irreversible collapse of the fold [6–8,20,22,23]. Under which circumstances aGPCRs switch from non-dissociative to dissociative TA-dependent signaling, which is likely a multistep process rather than a single event, is currently under intense investigation [24]. Open questions concern the dynamics of this process – how stimuli including those transmitted through ligand engagement and mechanical forces impact on the GAIN domain structure (Box 2), how many contacts need to be rearranged to gradually liberate the TA from its GAIN domain encasement, and what structural changes in the TA take place during this process.

Although class A, B1, and C GPCRs have become structurally well-defined over the past 15 years [25], high-resolution structures for the 7TM region of aGPCRs were not available for a long time. The past 2 years have seen the release of a flurry of single-particle cryo-EM structures of 7TM domains of several aGPCRs [26–33]. The overarching key observation in these reports is that the TA is bound to the orthosteric binding pocket in each receptor. This has now opened the opportunity to examine and compare aGPCR 7TM domains with those of other GPCR families to understand their structure–function dualism in greater detail. Undoubtedly, the new stock of high-resolution structural data will also assist the design of compounds that interfere with aGPCR signals.

Glossary

Adhesion GPCRs (aGPCRs): also known as family B2 GPCRs, these constitute one of the five main branches of the GPCR superfamily. They contain GAIN–7TM domain pairs, and many aGPCRs additionally harbor elaborate extracellular adhesive domains for interactions with matricellular and membrane-linked ligands.
Dissociation/handgrenade/one-and-done model: posits that the NTF–CTF heterodimer is physically separated to initiate and sustain receptor activity.
GPCR autoproteolysis-inducing (GAIN) domain: an extracellular hallmark domain of all aGPCRs (except ADGRA1) that is positioned immediately adjacent to the 7TM domain. Most aGPCRs are self-cleaved within the GAIN domain at a GPCR proteolysis site (GPS) which generates N- and C-terminal receptor fragments (NTF and CTF, respectively). The GAIN domain therefore contributes to both the NTF and the CTF. The NTF and the CTF remain non-covalently bound to one another after cleavage.
Heptahelical transmembrane (7TM) domain: the hallmark domain of all GPCRs. Structural changes in the 7TM domain upon ligand binding or stimulus encounter pass the extracellular signal across the membrane to intracellular messengers such as G proteins.
Mechanosensitive receptors: molecules that perceive and transduce force stimuli into intracellular responses. Can be located in membrane systems (e.g., aGPCRs and ion channels) or intracellularly (e.g., cytoskeletal components).
Non-dissociation/displacement/tunable model: suggests that the NTF and the CTF cooperate during receptor activation without physical disruption of the NTF–CTF heterodimer.
Tethered agonist (TA)/*Stachel*: the most C-terminal β-strand of the intact GAIN domain that is positioned C-terminal to the GPS. It remains connected to the CTF after receptor cleavage, and is necessary and sufficient for aGPCR activation.
Wootten numbering scheme: uses the X.YY format to denote the transmembrane helix number (X) and residue position (YY) relative to the most conserved residue in the helix (e.g., X.50). The numbering is used to refer to structurally equivalent residue positions in different class B GPCRs.

Box 2. Mechanobiological roles of adhesion GPCRs

GPCRs are commonly activated by soluble endogenous ligands of diverse origins and composition (e.g., monoamines, peptides, unmodified and modified proteins, and fatty or bile acids) [92]. aGPCRs also bind to a scope of proteinaceous ligands via their ECRs. In contrast to other GPCR families, those ligands are often part of the extracellular matrix or are transmembrane proteins fixed to the surface of cells [5,93,94]. This choice of interactors makes sense in light of the exceptional characteristic of aGPCRs as **mechanosensitive receptors** where receptor activation is triggered by direct force application or force-dependent interactions with their ligands. For example, ADGRG1/GPR56 regulates myotube hypertrophy upon resistance/loading-type exercise via activation of the $G\alpha_{12/13}$ pathway [95]. ADGRG1 is also required in platelets for sensing shear forces to regulate their shape in response to collagen binding [96]. In addition, ADGRL/LPHN/CIRL is necessary for the discrimination of mechanical stimuli such as sound, stretch, and touch [97], and ADGRG6/GPR126 is required for Schwann cell development in zebrafish by polymerization with laminin-211 in a force-dependent manner [42,98]. Furthermore, ADGRF5/GPR116 induces pulmonary surfactant secretion upon inflation of lung alveoli *in vivo* [99]. Downregulation of ADGRE5/CD97 in circulating leukocytes is triggered by shear stress transmitted through CD55 binding [100], and the positioning of dendritic cells in the spleen near blood-exposed regions requires the same interaction and the forces exerted by blood flow [101]. Dysfunctional processing of mechanical stimuli by an ADGRE2 mutant is also involved in the pathophysiology of the allergy-related condition vibratory urticaria [102]. Finally, ADGRD1/GPR133 can be activated by antibodies targeting the N terminus of the receptor [103], and ADGRG5/GPR114 can be activated by cell culture shaking [17], although the physiological relevance of these sensitivities to mechanical force application is not known.

How is the remarkable mechanosensitivity of aGPCRs reflected in their molecular structures? The diverse collection of adhesion motifs in aGPCR ECRs provides specific anchors for mechanical receptor fixation. Cleavage of the GAIN domain generates non-covalent NTF-CTF dimers in which the NTF can be shed by mechanical forces, thereby activating the receptor (dissociation model) [38,101]. However, there is also ample evidence for non-dissociative aGPCR signaling because GAIN domain cleavage is often not required for TA-dependent physiological or pharmacological receptor effects [16,18,19,68]. The novel 7TM structures of aGPCRs provide direct evidence that the TA is located in the 7TM domain binding pocket if the receptor lacks the NTF (as put forward by the dissociation scenario). Several structures also offer indirect evidence that, in NTF–CTF complexes of full-length receptors, the TA is missing from the pocket, and transitory structural intermediates may also be observable [28], supporting the non-dissociation and pre-bound/isomerization models [17,104]. Gradual TA exposure afforded by the structural flexibility of the GAIN domain and 7TM domain interaction constitutes a logical framework for how the scale of a mechanical stimulus can be decoded into relative levels of aGPCR activity [20]. It remains a daunting challenge to study how the structural dynamics of tethered agonism in aGPCRs depends on mechanical stimuli regimes, which are still as ill-defined as they are poorly applicable in standard pharmacological and structural biological experimentation.

Nonetheless, elementary questions about the process of aGPCR activation remain unresolved despite the overall similarity of the structural insights afforded by the new 7TM domain structures of aGPCRs. In particular, the dynamics of TA–7TM domain engagement and the role of glucocorticoid ligands for aGPCR 7TM domain activation are areas that require further active research. Moreover, all currently available 7TM domain structures are G protein complexes that represent active states of the 7TM domain, regardless of TA or ligand binding (Table 1). Accordingly, inactive states of the 7TM domain will need to be resolved to obtain insights into the mechanism of aGPCR activation and for structure-based design of antagonists. We highlight general and receptor-specific structural characteristics found in the new aGPCR 7TM domain datasets and discuss findings that are currently under dispute.

What's new

Although GAIN domain structures were solved in 2012 for the first time [6], atomic-level details of the 7TM domain of aGPCRs remained elusive until recently. In a series of recent publications, cryo-EM structures for eight of 33 mammalian (32 human) aGPCR homologs have been released (Table 1) [26–33]. These encompass four (D, F, G, and L) of the nine subfamilies of aGPCRs [1,34]. Several receptors are covered by multiple structures – ADGRD1 (2), ADGRG2 (6), ADGRG3 (2), ADGRF1 (8), ADGRL3 (5) – whereas for each of ADGRG1, ADGRG4, and ADGRG5 a single structure is now available (Table 1).

TA binding mode in active aGPCR 7TM domain conformations

As opposed to the β-strand conformation when positioned in the GAIN domain (Figure 1A–D), all TA-bound 7TM domain structures find the TA in a bent C-shaped partial α-helical conformation

Table 1. Cryo-EM structures of aGPCR 7TM domains[a,b]

Receptor	Protein	FL or CTF	Tethered agonist/*Stachel*	GAIN domain cleavage	PDB	Refs
Human ADGRD1	GPR133-CTF–G_s–Nb35	CTF	Included in protein	NA	7EPT	[27]
Human ADGRD1	ADGRD1-β–miniG_s–Nb35	CTF	Included in protein	NA	7WU2	[28]
Human ADGRG1	GPR56-CTF–miniG_{13}	CTF	Included in protein	NA	7SF8	[30]
Human ADGRG2	Apo-ADGRG2-FL–DNG_s–Nb35	FL	Included in protein	Suppressed	7YP7	[32]
Human ADGRG2	DHEA–ADGRG2-βT-120CT–DNG_s–Nb35	CTF	Not present	NA	7XKD	[32]
Human ADGRG2	DHEA–ADGRG2-FL-AA-120CT–miniG_s–Nb35	FL	Included in protein	Suppressed	7XKE	[32]
Human ADGRG2	DHEA–ADGRG2-βT-120CT–DNG_s–Nb35	CTF	Not present	NA	7XKF	[32]
Mouse ADGRG2	ADGRG2-β–G_s–Nb35	CTF	Included in protein	NA	7WUQ	[29]
Mouse ADGRG2	ADGRG2-FL–IP15–G_s–Nb35–scFv16	FL	Included in protein and as synthetic peptide	Suppressed	7WUI	[29]
Human ADGRG3	BCM–GPR97-FL-AA–Go	FL	Included in protein	Suppressed	7D76	[26]
Human ADGRG3	Cortisol–GPR97-FL-AA–Go–scFv16	FL	Included in protein	Suppressed	7D77	[26]
Human ADGRG4	ADGRG4-β–G_s–Nb35–scFv16	CTF	Included in protein	NA	7WUJ	[29]
Human ADGRG5	GPR114-CTF–G_s–scFv16	CTF	Included in protein	NA	7EQ1	[27]
Human ADGRF1	ADGRF1-FL–miniG_s–Nb35	FL	Included in protein	Yes	7WU3	[28]
Human ADGRF1	ADGRF1-FL–miniG_{i1}	FL	Included in protein	Yes	7WU4	[28]
Human ADGRF1	ADGRF1$^{H565A/T567A}$-FL–miniG_{i1}	FL	Included in protein	Suppressed	7WU5	[28]
Human ADGRF1	GPR110-CTF–G_q–Nb35	CTF	Included in protein	NA	7WXU	[31]
Human ADGRF1	GPR110-CTF–G_s–Nb35	CTF	Included in protein	NA	7WXW	[31]
Human ADGRF1	GPR110-CTF–G_i–scFv16	CTF	Included in protein	NA	7X2V	[31]
Human ADGRF1	GPR110-CTF–G_{12}–scFv16	CTF	Included in protein	NA	7WZ7	[31]
Human ADGRF1	GPR110-CTF–G_{13}–scFv16	CTF	Included in protein	NA	7WY0	[31]
Human ADGRL3	LPHN3-CTF–miniG_{13}	CTF	Included in protein	NA	7SF7	[30]
Mouse ADGRL3	ADGRL3-CTF–miniG_q–Nb35	CTF	Included in protein	NA	7WY5	[33]
Mouse ADGRL3	ADGRL3-CTF–miniG_s–Nb35	CTF	Included in protein	NA	7WY8	[33]
Mouse ADGRL3	ADGRL3-CTF–miniG_i–scFv16	CTF	Included in protein	NA	7WYB	[33]
Mouse ADGRL3	ADGRL3-CTF–miniG_{12}–scFv16	CTF	Included in protein	NA	7X10	[33]

[a]For future updates refer to https://gproteindb.org/structure/gprot_statistics.
[b]Abbreviations: ADGRG2-FL-AA-120CT, mutant version of ADGRG2 in which the autoproteolysis motif has been mutated and the receptor C-tail has been replaced with that of GPR120; ADGRG2-βT-120CT, mutant version of ADGRG2 in which the N-terminal region has been deleted and the receptor C-tail has been replaced with that of GPR120; BCM, beclomethasone; CTF, C-terminal fragment; DN_{Gs}, dominant negative G_s protein; FL, full length; GPR97-FL-AA, mutant version of GPR97 in which the autoproteolysis motif has been mutated; NA, not applicable; Nb35, nanobody 35; scFv16, single-chain antibody fragment variable of monoclonal antibody mAb16 that stabilizes GPCR–G protein complexes.

with remarkably high similarity (Figure 1A,G). In this general binding mode, the TA can be divided into a C-terminal and N-terminal section. The C-terminal part is positioned on top on the extracellular side of the 7TM fold. A hinge region bends the TA by 180°, and connects it to seven N-terminal residues containing the hydrophobic binding motif F/Y/L/VXφφφXφ, where φ is a hydrophobic amino acid. In some structures, a single helix winding with a hydrogen bond between the fifth and eighth TA residues is visible. This motif extends deeply into the putative hydrophobic binding pocket of the 7TM domain [27–30] (Figure 1B). The hydrophobic side chains of the F^{+3} residue (numbering with reference to the GPS), as well as the less conserved and φ^{+6} and φ^{+7} residues, point into the 7TM core like extended fingers [28,29] (Figure 1H). The residues interact with residue $W^{6.53}$ (residues are numbered according to the **Wootten numbering scheme** for class B GPCRs [35]) of the 7TM domain, a putative homolog to the class A GPCR W6.48 [30],

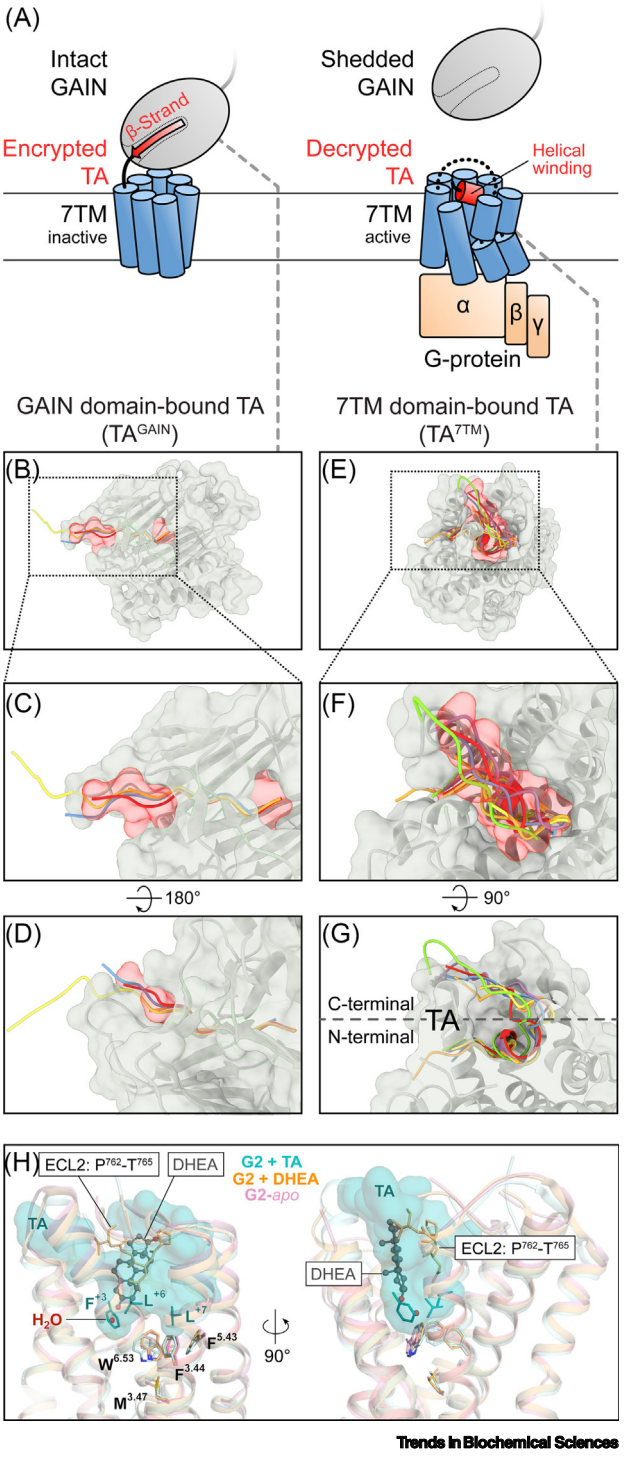

Figure 1. Different conformations of tethered agonist bound to GAIN or 7TM domains of aGPCRs. (A) (Left) In intact NTF–CTF heterodimeric aGPCRs, the TA is encased in the GAIN domain and adopts a β-strand conformation. (Right) When bound to the active 7TM domain complexed with a G protein, the TA displays a partly helical conformation, as observed in several cryo-EM structures. This scenario is thought to occur in the absence of the NTF. (B) The TA is deeply buried within the GAIN domain of ADGRG1 (red; PDB: 5KVM) [13]. This is very similar to the TA conformation in other receptors aligned and superimposed as a cartoon in different colors: ADGRB3 (blue; PDB: 4DLO), ADGRG6 (orange; PDB: 6V55), and ADGRL1 (yellow; PDB: 4DLQ). (C) The front view shows partial solvent accessibility of the GPS (red surface). (D) The rear view reveals TA encasement within the GAIN domain. (E) When bound to the 7TM domain, the TA adopts a C-shape. Top view of ADGRG1 TA (red; PDB: 7SF8) [30]. The 7TM-bound TAs of other receptors have been aligned and superimposed as a cartoon in the following colors: ADGRD1 (blue; PDB: 7WU2), ADGRF1 (purple; PDB: 7WU3), ADGRG2 (orange; PDB: 7WUQ), ADGRG4 (yellow; PDB: 7WUJ), and ADGRL3 (lime green; PDB: 7SF7). (F) The top detail illustrates the high solvent accessibility of the TAs. (G) The cut side-view shows the C-shape with the N-terminal TA part embedded within the putative 7TM domain binding pocket. (H) Structural comparison of DHEA-bound, TA-bound, and apo-ADGRG2 7TM domains. Structural alignment of the DHEA-bound (shades of orange; PDB: 7XKE) [32] and TA-bound 7TM domain of ADGRG2 (shades of blue; PDB: 7WUQ) [29] and apo-ADGRG2 (pink, PDB: 7YP7). The dark cyan surface corresponds to the TA bound in the 7TM domain, and selected hydrophobic side chains of deeply interacting F^{+3}, L^{+6}, and L^{+7} residues are shown in cyan. The upper quaternary core (UQC) residues (Box 3) in the structures, indicated as light blue, orange, and pink sticks, respectively, show highly similar arrangements. Overlay of TA surface with DHEA (gray ball-and-sticks) and the ECL2 fragment P^{762}–T^{765} (orange sticks) shows an almost complete overlap with the TA surface in the aligned structure together with a water molecule (red sphere) which is found in the binding pocket, matching the F^{+3} side-chain position.

Abbreviations: aGPCR, adhesion G protein-coupled receptor; cryo-EM, cryogenic electron microscopy; CTF, C-terminal fragment; DHEA, dehydroepiandrosterone; ECL2, extracellular loop 2; GAIN domain; GPCR autoproteolysis-inducing domain; GPS, GPCR proteolysis site; NTF, N-terminal fragment; TA, tethered agonist; 7TM domain, heptahelical transmembrane domain.

whose conformational change upon activation was initially termed 'toggle-switch', before high-resolution structures revealed that it is mainly a rotation of the backbone of transmembrane helix 6 (TM6) rather than a rotamer toggle [36]. Furthermore, $W^{6.53}$ is part of a putative 'upper quaternary core' motif (UQC; $F^{3.44}$, $M^{3.47}$, $F^{5.43}$/$F^{5.47}$, $W^{6.53}$) – as defined for ADGRG2 and ADGRG3 – and is implied to rope the TM helices together to promote receptor activation (Figure 2) [26–29,37]. Although three of the four residues are present in all available structures, the $F^{5.43}$/$F^{5.47}$ residue is not conserved and is absent in ADGRF1, ADGRL3, and ADGRG5. Crucially, the conformation of the UQC remains almost identical in ADGRG2 in the apo-, TA-bound, and dehydroepiandrosterone (DHEA)-bound structures, with only minimal displacement of $W^{6.53}$ in the TA-bound structures (Figure 1H) [29,32]. These binding modes match the experimental evidence that the 7 N-terminal TA positions are the most important interacting residues [7,38–41].

Small-molecule ligands mimic the TA binding mode in the 7TM domain of aGPCRs

Comparison of the ligand-binding pockets of the TA-bound structure of the ADGRG2 CTF, the uncleaved full-length and DHEA-bound ADGRG2 structure, and its apo-structure (PDB IDs:

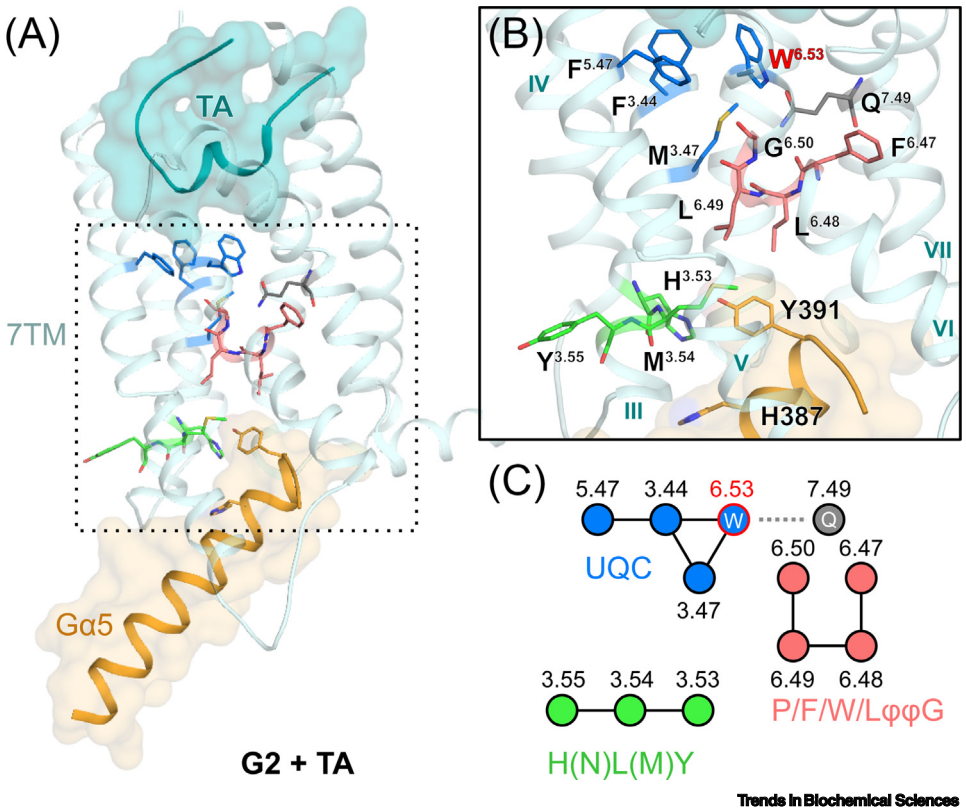

Figure 2. Putative activation motifs in adhesion GPCR 7TM domains. (A) Structure of the TA-bound ADGRG2 complex (PDB: 7WUQ) [24] with TA (dark cyan), the C-terminal α5-helix of Gα (orange), and the 7TM domain (light blue cartoon); core motifs are shown as sticks. Magnification of broken box shown in panel B. (B) Detail of the motifs. The upper quaternary core (UQC) residues (blue) tether the 7TM domain helix bundle (helix numbers indicated in roman numerals) to the conserved ligand-interacting $W^{6.53}$ (red) via a hydrogen bond with $Q^{7.49}$ (gray). $Q^{7.49}$ is closely packed against the PxxG or P/F/W/LφφG motif (salmon) mediating the TM6 kink. The H(N)L(M)Y motif (green) interacts with the Gα5 helix of the Gα (orange). (C) Residues of putative signaling motifs in aGPCR 7TM domains (Wootten numbering scheme). The hydrogen bond between $W^{6.53}$ and $Q^{7.49}$ is highlighted as a broken line. Abbreviations: φ, hydrophobic amino acid; aGPCR, adhesion GPCR; TA, tethered agonist; 7TM domain, heptahelical transmembrane domain; TM6, transmembrane helix 6.

7WUQ, 7XKE, 7YP7) [29,32] reveals a high structural similarity (Figure 1H). Interestingly, the volumetric overlay of DHEA with the TA binding mode shows that its conformation primarily matches F^{+3} and occupies the space of the S^{+2}, F^{+3}, and G^{+4} residues. However, the space of the F^{+3} aromatic side chain is substituted by a water molecule and the hydroxyl moiety of DHEA, resulting in a more polar environment. In the TA-bound structure, the L^{+7} residue directly interacts with $W^{6.53}$. In the DHEA-bound structures, in which the $W^{6.53}$ side chain is found in a similar orientation, the space of the missing L^{+7} residue is partially occupied by a four-residue stretch of extracellular loop 2 (ECL2) residues P^{762}–T^{765}, and all four residues directly interact with DHEA (Figure 1H). This stretch of residues is part of an unresolved section of ECL2 in the TA-bound structure (residues 758–767), indicating a high conformational flexibility [29].

The ligand-bound ADGRG3 structures (PDB IDs: 7D76 and 7D77) [26] show a ligand configuration similar to DHEA, with an identical orientation of the $W^{6.53}$ side chain. However, the absence of the TM6/TM7 kinks causes a shift in the helices compared to TA-bound structures and results in an intracellularly oriented void to which the palmitoylated cysteine of the G protein binds. By contrast, this void is absent in the TA-bound structures. The alternative conformation of active ADGRG3 without the notable TM7 kink has been suggested to reflect the partial agonistic property of beclomethasone (BCM) [26,30].

Mechanism of aGPCR–G protein coupling

Similarly to other GPCR classes, aGPCRs are capable of coupling to multiple Gα protein families. For instance, ADGRG6 was found to couple to both $G\alpha_{i/o}$ and $G\alpha_s$ with comparable fidelity [42], whereas others showed activation of $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ pathways [43]. Different aGPCRs such as ADGRL1, ADGRG3, and ADGRV1 show similar promiscuity in G protein choice [44]. The structural basis for the coupling specificity of GPCRs is generally still not well understood. However, several structural characteristics accounting for G protein selectivity in class A/rhodopsin-like GPCRs have been proposed, including the accessibility of the receptor intracellular cavity for G protein α5 helix binding, the length and a subset of residues of TM5 and TM6, and the peripheral domains of receptor–G protein interfaces [45–50].

The solved structures of aGPCR–G protein complexes suggest that the G protein coupling mechanism coaligns with that of class A GPCRs. In the available aGPCR 7TM–$G\alpha_s$ structures, intracellular loop 2 (ICL2) appears to be important for receptor interaction with α5 and αN helices (and/or neighboring regions) of $G\alpha_s$ [27–29,32]. This is also observed in all $G\alpha_s$-bound structures of class B1/secretin GPCRs [51] and class A GPCRs such as the β2-adrenoceptor (β2AR) [52]. In contrast to the 7TM–$G\alpha_{i1}$ binding mode of ADGRF1, the intracellular tip of TM6, which tilts out upon receptor activation, shows an additional outward tilt by 3 Å to accommodate the bulky residues from the α5 C terminus of $G\alpha_s$, consistent with a molecular dynamics (MD) study that TM6 of the receptor contributes to the extent of opening of the cytoplasmic cavity for accommodating different Gα C termini [53]. Moreover, ICL3 of ADGRF1 is more compact and bent upwards to accommodate the long αG–α4 loop of $G\alpha_s$, and the ICL3 was extended when bound to $G\alpha_{i1}$ [28]. The involvement of ICLs in G protein selectivity is also observed in structural data for other GPCR classes. For instance, a nuclear magnetic resonance (NMR) study showed that ICL2 of β2AR adopts different conformations when coupling to $G\alpha_s$ and $G\alpha_{i1}$ [54]. A recent Förster resonance energy transfer (FRET) study has highlighted the role of ICL3 in permitting only strong coupling between Gα subtypes and GPCRs [50]. Furthermore, $G\alpha_{i1}$ and $G\alpha_s$ distinctly interact with ICL3 and ICL2 of GCGR, respectively [55].

Elucidation of the structures of ADGRF1 bound to members of all four main G protein families, including $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$, provided comparisons on the engagement of Gα subunit

α5 helices on coupling with the receptor at the single-residue level [31]. Different attractions between the extreme C terminus of the α5 helices of Gα subunits and the cytoplasmic cavity of ADGRF1 have been observed, which is consistent with previous observations that the C terminus of Gα subunits mainly determines G protein coupling specificity [53,56]. Similar observations were made on ADGRL3 in complexes with $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, respectively [33]. However, as with class A GPCRs, the available structures of nucleotide-free receptor–G protein complexes from the aGPCRs do not show a more open intracellular cavity when coupled to $G\alpha_{i1}$, unlike the glucagon receptor (GCGR) which belongs to the phylogenetically related class B1 GPCRs [55,57].

What's missing

How are GAIN and 7TM domains positioned relative to one another in a full-length aGPCR?

An exceptional feature of aGPCRs is their bipartite molecular architecture (Box 1). Following GAIN domain-dependent self-cleavage, aGPCRs form stable NTF–CTF heterodimers at the cell surface [6,20,58–60]. Therefore, they adopt a similar spatial arrangement to uncleaved aGPCRs [6]. It has been a scientific challenge to grasp how NTF and CTF cooperate, and how this interaction impacts on receptor activation and signaling. Several isolated GAIN domain structures and structures of aGPCR 7TM domains are now available for this task [5,6,13,14,26–33].

To gain insights into the structure of the GAIN–7TM complex, it is important to distinguish cryo-EM datasets of full-length from CTF-only proteins, the latter of which represent the majority of the recently released structures (Table 1). Importantly, cleaved full-length receptor proteins with an intact GAIN domain may display NTF–CTF separation caused by protein preparation for cryo-EM imaging. In this case, only protein particles corresponding to the isolated CTF receptor portion would be generated. For example, in the cleavage-competent full-length ADGRG2 structure bound to a 15-mer TA-derived peptide agonist, no NTF density is visible in the cryo-EM snapshots (PDB ID: 7WUI) [29]. Therefore, GAIN domain-cleaved full-length and CTF-only receptors will not provide insights into the structure of the NTF–CTF complex. Nevertheless, all available structures unequivocally found that the TA is in a highly similar conformation when bound to the 7TM domain structure representing the active receptor conformation (Figure 1) [27–30].

Full-length 7TM domain structures have been obtained from protein samples in which suppression of GAIN domain self-cleavage artificially sustained the NTF–CTF complex (Table 1 and Figure 1). These structures provide potential hints about the interaction of the two receptor fragments, and thus between the GAIN and 7TM domains. Intriguingly, none of these structures delivered a high-resolution vantage point on the form, position, and interaction of the GAIN domain, let alone the entire ECR, in relation to the 7TM fold. This indicates that the GAIN domain adopts multiple orientations relative to the 7TM (or NTF relative to CTF), arguing for a more dynamic rather than a rigid interaction. In agreement with this notion, the density maps of the cleavage-resistant ADGRG3 complexes – in which the orthosteric binding site is occupied by a steroid ligand (cortisol or BCM) – reflect the intrinsic flexibility of the NTF, of which only a low density can be discerned that curtailed NTF structural assignments [26]. Similar results were obtained from two structures of cleavage-incompetent ADGRG2 mutant bound to DHEA (PDB ID: 7XKE) or in its apo-state (PDB ID: 7YP7). In cryo-EM snapshots of both datasets, NTF density can be discerned; however, the weak density in 2D class averages of the NTF prevents structural assignment in the proteins [32]. Notwithstanding this lack of insight with regard to the holo-receptor conformation, four structures derived from uncleaved full-length receptor protein constructs (PDB IDs: 7D76, 7D77, 7YP7, 7XKE) divulged an important structural aspect about aGPCR heterodimers: in no case was the TA positioned in or near the orthosteric binding site of the 7TM domain. This suggests that, provided that the NTF–CTF complex is intact, the TA cannot transition

from its GAIN domain encasement (TA^{GAIN}) to fully engage with the 7TM domain (TA^{7TM}), or at least that the equilibrium between these two states is strongly shifted towards TA^{GAIN} (Figure 1). At this point, it will be interesting to see whether structures of the full-length inactive receptor provide insights into the relative orientation of the NTF–CTF complex.

One structure derived from a cleavage-deficient full-length ADGRF1 construct (PDB ID: 7WU5) is unique in that it shows a TA-bound conformation [28]. This suggests that TA liberation from the GAIN domain and binding to the 7TM pocket is possible with the NTF still attached to the CTF. This prebound TA/isomerization scenario could thus potentially accommodate findings on the full TA response in aGPCRs that are naturally not cleaved [17] or were rendered uncleavable [18] (Box 2). However, the EM snapshots (2D class averages) of full-length ADGRF1 constructs lack any NTF density. This suggests that, alternatively, the receptor protein could have undergone NTF shedding after proteolytic cleavage, either through GAIN domain processing or other routes. It is well known that the ECRs of aGPCRs are subject to alternative cleavages. For example, ADGRB2, ADGRF5, and ADGRG6 have furin cleavage sites within their ECR, ADGRF1 and ADGRF5 have an autoproteolytically cleaving SEA (Sperm protein, Enterokinase, Agrin) domain, and for ADGRL1 and ADGRL2 a 'sheddase' cleaves the NTF close to the cell membrane (reviewed in [61,62]). It is tempting to speculate that the lack of NTF density arises from alternative cleavage. If this turns out to be true, this structure would be ultimately derived from a CTF-only receptor protein.

Do cryo-EM preparations reflect physiologically relevant receptor–G protein states?

All known receptor–G protein complexes, including some of the 7TM domain structures of aGPCRs (Table 1), are nucleotide-free complexes. Under cellular conditions, these complexes are presumably as short-lived as the intermediate GDP-bound complexes that precede them and the GTP-bound complexes that arise from them [63–65]. A major reason for the overrepresentation of these nucleotide-free complexes in the published structures is that this intermediate, unlike the intermediates preceding and following it, can be artificially enriched and structurally characterized by nucleotide-free preparation. All nucleotides are removed from the preparation by treatment with apyrase for several hours. During this period stable nucleotide-free complexes are formed, which are expected to be energetically at a very low minimum, whereas under physiological conditions GTP is immediately incorporated into the nucleotide-free Gα subunit owing to the high concentration of intracellular GTP [65].

For one of the best-studied systems, β2AR–$G\alpha_s$ protein coupling, time-resolved structural mass spectrometry analyses have shown that the C terminus of the α5 helix, which is crucial for specificity and signal transfer [53,56], remains flexible for a long time after the release of GDP and assumes the rigid and stable position of the nucleotide-free state only after hours [66]. Based on these studies, Kobilka and colleagues concluded that 'one would not expect to observe a complex represented by the crystal structure of the β2AR–G_s complex at physiological concentrations of GDP and GTP (36 μM and 305 μM in human, respectively)' [66]. The nucleotide-free structures that are formed in the cell, like the related GDP and GTP states, are therefore likely to be metastable and flexible in nature [67]. Considering the present nucleotide-free 7TM–G protein structures against this background, some questions arise.

Does the conserved TA binding mode – which is virtually identical in all 7TM structures despite the different orthosteric binding pockets (Figure 1) – reflect the physiologically relevant position, or does it arise from the long incubation time with nucleotide-free G proteins? Similarly, how can the high similarity of the binding pockets between ligand-free (apo) (PDB ID: 7YP7) [32] and agonist-bound ADGRG2 7TM domain structures be explained, which display remarkable steric

overlay of key signaling motifs such as the UQC and its conserved $W^{6.53}$ residue (PDB ID: 7XKE; Figure 1H and Box 3) [29,32]? The overall similarity of the orthosteric binding site suggests that intracellular G protein binding to the 7TM domain allosterically induces a conformation of the orthosteric binding pocket, which can then be selected by the TA without further structural changes. However, it remains unclear to what extent the preformed orthosteric binding pocket is due to a putatively high baseline activity, as suggested for ADGRG1 and ADGRB1 [68,69], or is merely an artifact from a long incubation of receptors with nucleotide-free G proteins [70].

In some structures, Gα subunits were replaced by highly modified, thermostable mini G proteins (Table 1) which can no longer exchange nucleotides but contain an intact α5 helix that allows binding to and stabilization of active receptors through this key structural motif. However, because they lack the lipidation motif, mini G proteins do not associate with detergent micelles and thus may allow binding to the intracellular cavity of any 7TM domain, provided that the α5 helix is adopted. Interestingly, in an elegant study, Jang *et al.* observed that the ability of the receptor to differentiate between cognate and non-cognate interactions is lost when G proteins lack GDP–GTP exchange activity, which poses additional doubts about how representative coupling specificity of a particular receptor can be inferred from the use of mini G proteins [70]. This analysis also provides additional evidence that G protein coupling specificity requires a functional GDP-bound G protein and is controlled at the level of one or several intermediate states preceding the nucleotide-free state of G proteins, as suggested by a recent analysis of the stepwise engagement of G_s protein by β2AR [66,67]. The use of mini G proteins as well as stabilization by single-chain fragment variable (scFv) antibodies or nanobodies, or the use of binary technology (LgBiT/SmBiT)-fused receptor/G protein constructs, may accordingly result in non-physiological receptor–G protein complexes.

Alongside the available structures, it is also worth noting that binding of the TA to the 7TM domain, receptor activation, and signal transfer to the intracellularly engaged G protein are dynamic

Box 3. Putative activation motifs in adhesion GPCR 7TM domains

The host of new aGPCR 7TM domain structures has led to the identification of potential signaling motifs inferred from sequence conservation and the impact of mutations on signaling. Notably, the lack of inactive aGPCR 7TM domain structures and information on aGPCR activation dynamics leave the importance of any motifs subject to further investigation. An overview of the most frequently described aGPCR signaling motifs observed in the current repertoire of structures is shown in Figure 2 in main text. The UQC motif – of which the helix 5 residue is not conserved – mediates agonist binding with the conserved $W^{6.53}$ [26] (see Figure 2 in main text) as a key interacting residue. The $W^{6.53}$ side chain forms a stabilizing hydrogen bond with $Q^{7.49}$ [27,30], which has further been described as part of a conserved $\varphi^{7.45}XXXQ^{7.49}G^{7.50}$-$X\varphi^{7.52}XXX\varphi^{7.56}$ motif that mediates the TM7 kink [32]. Another potential kink-mediating motif is found in TM6 as the F/W/L/$V^{6.47}\varphi\varphi G^{6.50}$ motif shared by 11 aGPCRs [32] or the P$\varphi\varphi$G motif in ADGRD1 and ADGRF1 [27,28] that contact the UQC. Zhu *et al.* proposed a central 'penta-core' motif containing $\varphi^{6.48}$ and $\varphi^{6.49}$ of the P$\varphi\varphi$G motif, $M^{3.47}$ of the UQC, and $I^{2.53}$, $I^{6.53}$, two so far unaddressed residues [31].

On the intracellular side, a H(N)L(M)Y motif was proposed – possibly analogous to the conserved class A ionic lock D(E)RY motif – that interacts with the α5 helix of the Gα subunit. Here, $H^{3.53}$ and $L^{3.54}$ coordinate the Gα5 helix and $Y^{3.55}$ points outwards from the 7TM bundle [26,27,32]. The motif was also identified as 'core 3' [28]. Furthermore, a lower triad core motif, consisting of $F^{3.54}$, $F^{5.54}$, and $L^{6.42}$ [26], was identified as the key motif interacting with the G protein palmitoyl moiety; however, this was not described in other aGPCR 7TM domains.

A central ionic lock between $H^{2.50}$ and $E^{3.50}$ – homologously conserved between class B1 and aGPCRs – was proposed to be relevant for receptor function. This motif is part of a larger HEXH polar motif that is analogous to HETX in class B1 receptors in which $H^{7.57}$ coordinates another histidine residue, $H^{2.50}$ [105]. The ionic lock, however, is not present in all aGPCRs; for example, ADGRF1 contains apolar residues at the respective positions [28].

The newly determined structures open up new avenues into structure-based drug design. Currently explored approaches include glucocorticoids [26], monobodies [13,106], and TA-derived peptides, possibly repeating the success story of the impact of class A GPCR structures on drug design [107].

processes that require further consideration and investigation. It will be exciting to see how future structural biology methods with higher time resolution or methods that explicitly map the dynamics of the systems – such as NMR, time-resolved cryo-EM, and MD simulations – will enrich and complete the present static picture of the receptor–G protein interaction [71]. It will be particularly crucial to obtain structural information for both GDP- and GTP-bound receptor–G protein complexes. The guanine nucleotide exchange factor activity of the receptor and thus its central physiological function requires elucidation of GDP-bound states in which the receptor lowers the energy barrier for nucleotide release – the rate-determining step in the process of G protein activation [65]. The nucleotide-free states – on which our knowledge of the structure of aGPCRs is currently exclusively based – merely reflect the outcome of this process that represents a state ready to take up GTP. In addition, aGPCRs are generally believed to be activated mechanically by cell–cell interaction or interactions with the extracellular matrix *in vivo* (Box 2). Hence, real-time activation of aGPCRs by force application directly coupled with sophisticated structural analytical tools would certainly contribute valuable details for how the receptor–G protein activation cascade is propagated from extrinsic mechanical forces onwards [72–74].

Finally, structural analysis of GPCR–G protein complexes has so far focused on combinations of receptors with Gα proteins from different families, but not with Gβγ. In the available aGPCR 7TM–G protein complex structures, $G\beta_1\gamma_2$, of 48 possible combinations of Gβγ formed by four Gβ isoforms and 12 Gγ isoforms, is used exclusively as the binding partner for Gα ($G\beta_5$ is excluded because it does not preferentially couple to Gγ *in vivo*). This limits the current structural understanding of the preference for particular Gβγ permutations by aGPCRs, as showcased for class A GPCRs [75]. Indeed, $G\gamma_3$ is required for somatostatin-induced activation of a voltage-sensitive calcium channel in GH3 cells, whereas such activation by a muscarinic receptor requires $G\gamma_4$ [76]. Interestingly, in the available aGPCR–G protein structures, ICL1 is involved in the interaction with Gβ subunit [26–28]. Future structural studies employing variations of Gβγ proteins may also evaluate the putative role of the flexible ICL1 in selective binding of Gβγ.

aGPCRs as membrane-bound steroid hormone receptors?

In addition to transmembrane and matricellular ligands, two aGPCRs of the G subfamily were found to be activated by steroid hormones. This was first shown through an *in vitro* ligand screen for ADGRG3 whose activity is stimulated by BCM and cortisol [77]. Both ligands were subsequently found to bind to the orthosteric binding pocket in structures of the G3 7TM domain [26]. An analogous observation was made for ADGRG2, which is activated by DHEA, DHEA sulfate, androstenedione, and 20α-hydroxycholesterol. The cryo-EM structure of the ADGRG2 7TM CTF (excluding the TA from the construct; Table 1) showed that DHEA in the binding pocket has a similar binding mode as BCM in ADGRG3 [32]. Comparison of BCM, cortisol, and DHEA in TA-bound complexes indicates that steroid ligands mimic the F^{+3} binding mode of the TA (Figure 1H) [29,32]. These similar binding modes, which are also reflected by the ability of these ligands to activate the receptor in a TA-independent manner [26,32], suggest that they directly compete with TA binding. Although these data provide valuable hints that subfamily aGPCRs may serve as steroid hormone receptors, there is so far no physiological evidence in support of this possibility. For ADGRG6, another subfamily G aGPCR, another set of small-molecule modulators were identified by an elegant *in vivo* assay, and yet none of which structurally resembles steroid hormones [78]. Similarly, *in vitro* screens for the subfamily G aGPCRs ADGRG1 and ADGRG5 found that the rotenoid derivative dihydromunduletone [79] is a small-molecule antagonist of both receptors, whereas 3α-acetoxydihydrodeoxygedunin is a partial agonist [80,81]. Future studies may reveal whether aGPCRs can also be activated *in vivo* by endogenously synthesized steroid hormones such as glucocorticoids and gonadosteroids.

Finally, lipids resolved in structures of aGPCR complexes provide initial hints that lipids may potentially modulate these receptors. In the structures of ADGRF1 lysophosphatidylcholine interacts with ICL2, H3, and H4. Synaptamide, which was first hypothesized to bind to the GAIN domain, retained its potency even in a GAIN-less construct, suggesting that this lipid acts on the receptor through another binding site. However, a mutation that abolishes lipid binding of ADGRF1 (G4.45Y) was found to reduce synaptamide potency [28]. Likewise, the GAIN domains of the ADGRG1 S4 isoform and ADGRB1 have been shown to bind phosphatidylserine (PS) [82]. However, the binding site was not localized in the case of ADGRB1 [83], leaving open the possibility that PS binds to the 7TM in a similar manner to lysophosphatidylcholine.

Concluding remarks

The recent advances in structural elucidations of aGPCRs, summarized here, support previous physiological, biophysical, and pharmacological observations highlighting the importance of tethered agonism for aGPCR activation. Delineation of TA–7TM and small ligand–7TM interaction interfaces in several aGPCR homologs paves the way for the development of pharmacological strategies, such as structure-based drug design, to interfere with aGPCR signaling. However, the physiological relevance of steroid hormone binding to the 7TM domain observed in two structures of aGPCR complexes demands additional support through *in vivo* experimental evidence. Finally, several unresolved issues remain and new questions arise that will require future research (see Outstanding questions). This includes the pressing need for structural assessments of full-length receptor complexes to determine the TA fold and position in intact aGPCR NTF–CTF heterodimers. The dynamic interplay of the ECR and 7TM domain, GAIN–7TM domain, and G protein–7TM domain will be of particular interest to understand the structure–function relationship of aGPCRs. Moreover, how ligands and mechanical forces – which activate aGPCRs physiologically – impact on the dynamics of TA release and the NTF–CTF complex (including conditions resulting in physiologically relevant NTF release [21]) will require further structural analyses that may not be directly amenable to cryo-EM and X-ray crystallographic experimentation. The importance of aGPCRs in the development and functioning of several major organ systems will drive the quest for answers to these questions, and will stimulate the identification of strategies to rectify disrupted aGPCR signals.

Outstanding questions

How is a full/complete GAIN–7TM domain pair structured?

Are there other GAIN–7TM–G protein states, and how does each of them contribute to signal transduction?

How do active and inactive 7TM states of individual aGPCRs compare to one another?

How do the dynamics of the NTF affect the signaling behavior of the CTF?

How are mechanical forces transmitted onto the NTF–CTF complex and transduced by the CTF?

What is the physiological relevance of steroid hormone agonists of some aGPCRs?

What does the structural dynamic continuum between encrypted versus decrypted TA/*Stachel* entail?

Which interactions (GAIN–7TM domain precoupling, G protein–7TM domain precoupling) account for the high basal activity of aGPCRs in pharmacological assays?

What are physiological conditions and effects of NTF shedding?

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Declaration of interests

The authors declare no conflicts of interest.

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