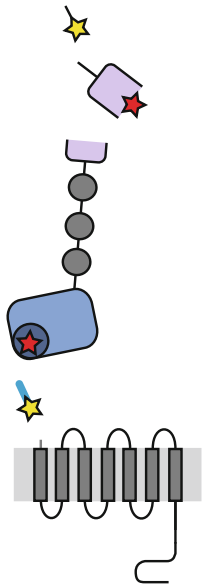
Control of Adhesion GPCR Function Through Proteolytic Processing

Matthias Nieberler, Robert J. Kittel, Alexander G. Petrenko, Hsi-Hsien Lin, and Tobias Langenhan

Graphical Abstract



Proteolytic processing events in adhesion GPCRs. aGPCRs can undergo multiple autoproteolytic (*red asterisks*) and proteolytic processing events by exogenous proteases (*yellow asterisks*) that may be involved in signaling events of the receptors.

Contents

M. Nieberler • R.J. Kittel • T. Langenhan (✉)
Department of Neurophysiology, Institute of Physiology, University of Würzburg, Röntgenring 9, Würzburg 97070, Germany
e-mail: tobias.langenhan@gmail.com

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Abstract

Proteolytic processing is an unusual property of adhesion family G protein-coupled receptors (aGPCRs) that was observed upon their cloning and biochemical characterization. Ever since, much effort has been dedicated to delineate the mechanisms and requirements for cleavage events in the control of aGPCR function. Most notably, all aGPCRs possess a juxtamembrane protein fold, the GPCR autoproteolysis-inducing (GAIN) domain, which operates as an autoprotease for many aGPCR homologs investigated thus far. Analysis of its autoproteolytic reaction, the consequences for receptor fate and function, and the allocation of physiological effects to this peculiar feature of aGPCRs has occupied the experimental agenda of the aGPCR field and shaped our current understanding of the signaling properties and cell biological effects of aGPCRs. Interestingly, individual aGPCRs may undergo additional proteolytic steps, one of them resulting in shedding of the entire ectodomain that is secreted and can function independently. Here, we summarize the current state of knowledge on GAIN domain-mediated and GAIN domain-independent aGPCR cleavage events and their significance for the pharmacological and cellular actions of aGPCRs. Further, we compare and contrast the proteolytic profile of aGPCRs with known signaling routes that are governed through proteolysis of surface molecules such as the Notch and ephrin pathways.

A.G. Petrenko
Laboratory of Receptor Cell Biology, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

H. Lin (✉)
Department of Microbiology and Immunology, College of Medicine, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-San, Tao-Yuan, Taiwan

Chang Gung Immunology Consortium and Department of Anatomic Pathology, Chang Gung Memorial Hospital-Linkou, Tao-Yuan 333, Taiwan
e-mail: hhlin@mail.cgu.edu.tw

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1 Forms of Proteolytic Processing Events in aGPCRs

1.1 GAIN-Mediated GPS Cleavage

One of the structural and functional hallmarks of aGPCRs is the juxtamembrane localization of a highly conserved GPCR proteolysis site (GPS) motif (Fig. 1) [1–3]. All aGPCRs, except GPR123/ADGRA1, contain the GPS motif [1]. Proteolytic modification of aGPCRs was first reported for CD97/ADGRE5 in 1996 by Kelly and colleagues [4]. They revealed a novel two-subunit structure of CD97, consisting of an extracellular fragment and a seven-transmembrane (7TM) fragment derived from a proprotein precursor. Petrenko et al. later identified the cleavage site and coined the term GPS to describe the proteolytic processing of CIRL/latrophilin/ADGRL1 [5, 6].

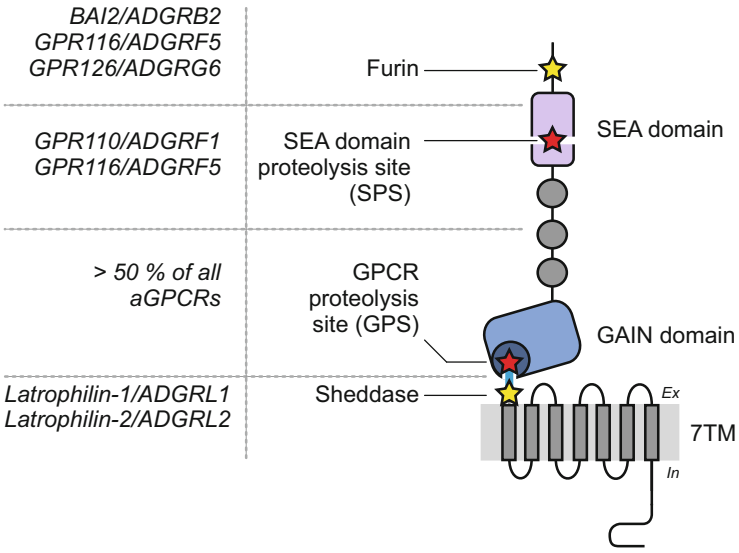

Fig. 1 Proteolytic processing events of adhesion GPCRs. aGPCRs can undergo multiple autoproteolytic (*red asterisks*) and proteolytic processing events by exogenous proteases (*yellow asterisks*) that may be involved in signaling events of the receptors. The most prevalent cleavage of aGPCR family occurs at the GPCR proteolytic site (GPS; *dark blue circle*) and is catalyzed by the GPCR autoproteolysis-inducing (GAIN) domain (*light blue*). Another type of autoproteolysis is governed by the SEA domain (*pink box*) and shares similarities with GAIN domain cleavages. aGPCRs can also be substrates for proteases and release parts of the ECD. Exemplary aGPCR homologs and associated proteolytic processing events are indicated on the *left*

The GPS motif of ~50 amino acids contains a highly conserved tripeptide cleavage sequence and several canonical cysteine and tryptophan residues [2]. Moreover, the 6–8 residues C-terminal to the cleavage site are usually small and hydrophobic [7]. The cleavage tripeptide almost always starts with His, followed by Leu/Ile and Ser/Thr, with proteolysis occurring between Leu/Ile and Ser/Thr (HL/I↓S/T) [2, 3]. Most interestingly, GPS proteolysis is not executed by any proteinases, but is brought about by an autocatalytic mechanism analogous to that of *hedgehog* morphogens [8, 9] and Ntn-hydrolases [10–14]. It is concluded that the GPS proteolytic reaction is most likely initiated by the deprotonation of the hydroxyl group of the P^{+1} residue (Ser/Thr) by the P^{-2} His residue. This is followed by a *cis*-nucleophilic attack on the α-carbonyl carbon of the P^{-1} Leu/Ile residue, producing a tetrahedral intermediate. An ester intermediate is subsequently generated via an N→O acyl shift. Finally, the attack by H_2O cleaves the ester bond splitting the receptor into two protein fragments (Fig. 2) [11]. The two fragments usually do not separate after proteolysis, but instead associate non-covalently to form a mature heterodimeric receptor complex on the cell surface [6, 15]. Interestingly, the GPS motif is absolutely necessary for proteolysis, but is insufficient to mediate the autoproteolytic reaction on its own [7].

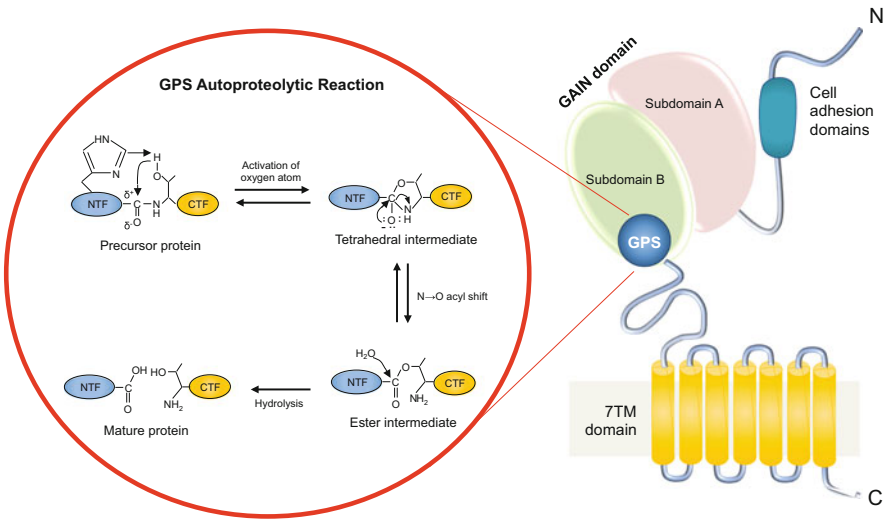

Fig. 2 The GAIN domain and GPS autoproteolysis of adhesion GPCRs. A schematic diagram of an aGPCR is shown. Located at the C-terminal half of the NTF, the GAIN domain is divided into the subdomain A (*pink*) and subdomain B (*light green*). The GPS motif (*blue*) is a part of subdomain B. The proposed mechanism of the GPS autoproteolytic reaction is shown inside the *red circle*. A His or another general base withdraws a proton from the hydroxyl group of a Ser or Thr at position +1. The resulting negatively charged oxygen makes a nucleophilic attack on the carbonyl group of the residue at position −1 (e.g., a Leu), yielding a tetrahedral intermediate and subsequently an ester intermediate. The resulting ester is then hydrolyzed to produce the NTF and CTF that form the mature protein

Interestingly, while it is well accepted now that the GPS proteolysis is a self-catalytic intramolecular reaction, the proteolytic efficiency is not always complete. Indeed, both processed and unprocessed GPR56/ADGRG1 and polycystin-1 receptors have been detected in vivo [16–18]. Moreover, crystals of two structurally similar GPS-containing fragments of CIRL/latrophilin and BAI3/ADGRB3 were described, the former in a completely cleaved and the latter in a non-cleaved conformation [19]. Hence, it is suggested that the GPS domain-containing receptors might adopt receptor folding conditions that either promote or demote GPS cleavage depending on cell types and cellular environments [20, 21].

One critical factor regulating the GPS proteolysis is found to be the first step of N-glycosylation during receptor biosynthesis in the ER [20, 21]. Other potential factors include the specific conditions of aGPCR expression, including the cell type and expression levels. While in the brain tissue only the cleaved form of CIRL/latrophilin is detected, its heterologous expression in transfected cells yields only a minor portion of the processed form that depends on the cell line used [5, 15]. Interestingly, the receptor cleavage yield in transfected cells can be regulated by pharmacological agents such as PMA and ionomycin, regulators of the protein kinase pathway, suggesting the existence of intracellular signaling mechanisms to fine-tune the autoproteolysis [22]. As with any chemical reaction, one can also anticipate a contribution of local pH and ionic changes, as well as a direct involvement of available nucleophilic molecules that serve as cofactors.

Pulse-chase experiments and use of various recombinant receptors and protein trafficking inhibitors have identified the ER lumen as the major subcellular localization of GPS proteolysis [2, 4, 11, 15]. However, due to the highly regulated nature of the GPS proteolytic reaction mentioned above, it is likely that GPS proteolysis could also occur at a later time point during protein maturation or perhaps even at the cell surface. Indeed, cell type-specific location of the GPS cleavage was reported for polycystin-1, whose proteolysis may occur within the ER or post-ER [21].

Recent structural analyses finally delineate the peculiar requirement and characteristics of GPS autoproteolysis. Crystallization of two aGPCRs, Latrophilin-1/ADGRL1 and BAI3, identifies a much larger extracellular GPCR autoproteolysis-inducing (GAIN) domain (~320 residues) that is sufficient and minimally required for GPS autoproteolytic reaction [19] (see also [23]). In fact, the GPS motif is an integral part of the GAIN domain. The crystal structure of the GAIN domain shows a subdomain A of 6 α-helices and a subdomain B consisting of a twisted β-sandwich of 13 β-strands and 2 small α-helices [19, 24]. The GPS motif is enclosed in the last five β-strands of subdomain B, and the cleavage takes place in a short kinked loop between the last two β-strands (Fig. 2) [19, 24]. The proper folding of the GAIN domain, hence the arrangement of the scissile bond in a unique configuration, provides an essential environment for the GPS autoproteolytic reaction. Due to the lack of the conformational constraint and chemical environment required for the proteolytic reaction, the GPS motif alone cannot mediate autoproteolysis. In addition, the cleaved last β-strand is tightly embedded within the rest of the GAIN domain, hence it is energetically unfavorable for the two fragments to dissociate [19, 24].

Interestingly, phylogenetic analysis shows that the GAIN domain is evolutionarily conserved from tetrahymena to mammals. In fact, it is believed that the GAIN domain is one of the most evolutionarily ancient and functional autoproteolytic protein folds identified to date [19, 24]. The close proximity to the TM region and the unique structural requirement for the GPS autoproteolytic reaction all suggest an important role for the GAIN domain in aGPCR function [25]. Furthermore, the GAIN domain is also present in all members of human polycystic kidney disease 1 (PKD1) protein family, suggesting a much wider usage of this novel domain in receptor biology [19, 24]. Indeed, sequence mutations in the GAIN domain have been linked to various human diseases, a clear indication of its role in regulating receptor activities [17, 24, 26, 27]. How the GAIN domain-mediated autoproteolysis may regulate receptor signaling and function will be discussed in the later sections.

1.2 Other Autoproteolytic Cleavages of aGPCRs

Apart from the GPS autoproteolysis, additional autoproteolytic reactions were noted for certain aGPCRs. Abe et al. showed that Ig-Hepta (GPR116/ADGRF5) undergoes two specific proteolytic events in the extracellular region [28]. One of the proteolytic sites identified is at the GPS motif, while the other is at the SEA module located at the N-terminus of the receptor (Fig. 1). Identified first in three different proteins (sea urchin sperm protein, enterokinase, and agrin) [29], the SEA module is a conserved extracellular protein motif of ~80–110 residues usually found in O-glycosylated mucin-like membrane proteins such as MUC1, MUC3, MUC12, MUC13, and MUC17 [30–32]. A highly conserved G↓S[V/I]VV sequence is identified as the SEA domain proteolysis site (SPS) [32].

Interestingly, the SEA module-mediated proteolysis shares many similar characteristics with GPS autoproteolysis. First, although some SEA module-containing molecules are soluble proteins, the SEA module is mostly found in cell-surface proteins and is located at the extracellular region of the molecule, near or close to the TM region [32]. Second, the proteolytic modification takes place within the ER during early protein biosynthesis. Third, proteolysis only proceeds when the P^{+1} cleavage site is a residue containing a terminal hydroxyl group (Ser and Thr) or thiol group (Cys) [33]. Fourth, proteolysis at the SEA domain is an autocatalytic intramolecular reaction likely mediated by a series of nucleophilic attacks and the formation and hydrolysis of an ester intermediate via an N→O acyl shift and H_2O, respectively [33–35]. Fifth, the autoproteolytic reaction is achieved by conformational strain and requires strict and proper protein folding [34, 36, 37]. Finally, the resulting cleaved fragments remain associated non-covalently following proteolysis [33, 35].

Two aGPCRs, GPR110/ADGRF1 and GPR116, are known to contain both the SEA module and the GAIN domain [1]. Indeed, multiple proteolytic modifications of GPR116 have been identified, leading to the formation of a mature receptor with many non-covalently associated fragments [28, 38]. GPR116 has been linked to a

number of physiological and pathological processes such as pulmonary surfactant homeostasis, insulin insensitivity, and breast cancer metastasis [39–44]. However, the role of autoproteolysis in the SEA module and GAIN domain in GPR116 function has yet to be investigated. Little is known regarding the proteolytic modification of GPR110.

1.3 Cleavage of aGPCRs by Other Proteases

With aGPCR research on the rise, more and more homologs are identified as targets of classical proteases such as furin or matrix metalloproteinase (MMP; Fig. 1). These include BAI1/ADGRB1, BAI2/ADGRB2, GPR116, GPR126/ADGRG6, and Latrophilin-1/ADGRL1 [38, 45–48]. Furin, a subtilisin-like proprotein convertase, is a calcium-dependent serine endoprotease enriched in Golgi and is involved predominantly in intracellular protein processing within the secretory pathway [49]. Consistent with previous reports, the proteolytic site of BAI2, GPR116, and GPR126 by furin was identified right after an Arg residue of a consensus furin-cleavage sequence [38, 47, 48]. Interestingly, these furin-cleavage sites are all located at the extracellular region N-terminal to the GPS and SEA domain. One exception is the furin processing of Latrophilin-1, which occurs before an Arg residue located C-terminal of the GPS motif within the CTF [46]. The furin-cleaved aGPCR fragment was shown to either remain associated with the rest of the molecule (GPR116) or released to the extracellular milieu (BAI2, GPR126, Latrophilin-1). The functional significance of the furin-mediated proteolysis of aGPCRs is currently unknown, but additional functions exerted by the shed receptor ectodomain remain a possibility. Modulation of aGPCR activity by furin-mediated shedding is also an alternative.

BAI1, initially identified as a brain-specific p53-regulated gene, is highly expressed in normal but not tumor brain cells [50, 51]. GPS proteolysis of BAI1 released a 120 kDa thrombospondin type-1 repeat (TSR)-containing "vasculostatin" fragment with anti-angiogenic and anti-tumorigenic function [50]. Later studies revealed another extracellular cleavage mediated by MMP-14 at a more N-terminal region, producing a 40 kDa (vasculostatin-40) fragment also with very potent anti-angiogenic activity [45]. In fact, the second cleavage of BAI1 is processed by a two-step protease activation cascade in which the latent MMP-14 is activated by furin [45]. Interestingly, the generation of vasculostatin-120 by GPS autoproteolysis is not a prerequisite for vasculostatin-40 production by MMP-14. Hence, intra- and extracellular proteolytic processing of BAI1 to distinct ectodomain fragments by GPS autoproteolysis and MMP-14, respectively, represents important activation and regulatory mechanisms for the BAI1 receptor function [45].

Another interesting example of aGPCR cleavage involving a sheddase is the dissociation of a CIRL/Latrophilin-1 two-subunit complex at the cell surface that results in the secretion of its ectodomain that contains the intact GAIN domain (Fig. 1). About 5 % of the endogenous brain-expressed CIRL/latrophilin undergoes

this processing. The soluble receptor form is comprised of NTF linked to a small peptide fragment of CTF. This peptide was identified by mass spectrometry indicating the location of the second cleavage site at the border between the GAIN domain and the 7TM core. Similar processing was also shown for CIRL-2/ADGRL2 [46].

2 Biological Effects Controlled Through aGPCR Proteolysis

The consequences and roles of the autoproteolytic processing of aGPCRs have been under intense scrutiny since its discovery. Multiple experimental approaches have been implemented to grasp this biochemical peculiarity of aGPCRs, and several conclusions have been drawn from the results. We will discuss the most popular ones below.

2.1 Trafficking

Several cell physiological consequences have been ascribed to the autoproteolytic processing of aGPCRs at the GPS. Insights into these features derived from studies of aGPCR and polycystin-1 homologs, in which the consensus site was mutated at different positions in order to disable the autocatalytic reaction. The Latrophilin-1 homolog with a GPS disrupting mutation was the first receptor that was scrutinized this way. It was noted that the GPS-deficient Latrophilin-1 variant did not traffic to the cell surface lending support to a model, in which the posttranslational cleavage event may function as a maturation signal during the biosynthesis of the receptor molecule in the ER [15]. Later on, this hypothesis was further explored in several other aGPCRs and polycystins returning mixed results: while impeded surface expression was found for proteolysis-deficient versions of Latrophilin-1 [15] and GPR126 [52], no such effect was noted for polycystin-1 [17], GPR133/ADGRG1 [53], and the nematode latrophilin homolog LAT-1 [54]. Also Latrophilin-1 was reprobed and several GPS cleavage mutations did not affect cell-surface transport of the receptor [19].

Also the GAIN-mediated cleavage of polycystin-1 has drawn interest to its physiological requirement, and its investigation contributed insights into the role of the proteolysis event. An allele of *PKD1*, which encodes for a cleavage-deficient polycystin-1 product, leads to strong hypomorphic phenotypes that manifested through defects in the development of kidney tubules [18] (see below). Follow-up work on this effect suggests that the CTF of polycystin-1 may act as a cofactor that is required for membrane trafficking of the NTF. The NTF subsequently detaches from the CTF, but remains associated to the membrane, probably through other surface receptors [55]. Similar findings were obtained for the NTF of the aGPCR Latrophilin-1, whose CTF may also exist as a separate protomer at the cell surface [56].

Hence, it remains controversial whether GPS autoproteolysis is functioning as a gatekeeping step in the biosynthesis and maturation of aGPCRs. One solution to this puzzle may be offered by the observation that several potentially GPS-disabling mutations rather lead to reduced stability and unfolding of the GAIN domain and consequently do not traffic properly to the cell membrane. Further, GPS cleavage appears to be dependent on cell context and other posttranslational modifiers such as glycosylation [17, 20]. Therefore, recombinant expression of aGPCRs in heterologous cell lines—the classical test system utilized for cleavage assays—may not provide the necessary cofactors or conditions that are required for efficient GAIN proteolysis.

2.2 Terminating Adhesion

An obvious role for the autoproteolytic cleavage of aGPCRs is one that has remained unexplored thus far. Movements during proliferation, migration, polarity establishment, but also postmitotic motion of cells or their context impose considerable forces on cells, which are counteracted by adhesion molecules such as cadherins, laminins, or integrins [57].

In this vein, aGPCRs possess an extensive repertoire of adhesion domains that are located in the ectodomain of most of the receptor homologs (see also [23]). aGPCRs are exposed to and likely engage in binding events with adhesive partner molecules that are affixed either within the extracellular matrix lattice or anchored on opposite cell surfaces [25]. Thus, autoproteolytic cleavage of aGPCRs may determine a threshold for forces transmitted onto the receptor expressing cells, above which the NTF and CTF are separated and relieved of their adhesive interaction. Such a role was suggested for other surface-mounted molecules such as mucins (see above), which line the surface of mucous epithelia. By means of an autocatalytically active SEA domain, potentially damaging shear forces that endanger the epithelial barrier are limited to the energy that is necessary to split the two non-covalently bound cleavage fragments of mucins [35].

2.3 Triggering Metabotropic Signaling

With the advent of molecular models on the activation mechanism of aGPCRs, and their suspected role as mechanoreceptors, receptor autoproteolysis receives increasing attention as a potentially crucial component in these processes.

As discussed in detail in [58, 59], several aGPCRs possess a tethered agonist that is an integral part of the receptor molecule. Structure-function studies of GPR56 implied that the NTF of an aGPCR exerts an inhibitory role on the metabotropic and biological activity of its CTF. This conclusion was based on receptor variants that either contained a shortened or no NTF at all, which displayed increased activation of cellular behaviors [44] and downstream effectors [60], respectively. These observations were explained by two models: either the NTF directly suppresses

metabotropic activity of the CTF consistent with the function of a tethered inverse agonist, or alternatively, the NTF counteracts the activity of a tethered agonist of the CTF [61]. Both models account for the disinhibiting effects of NTF removal. Studies on LAT-1 (see also [62]) provided evidence for the latter model. A panel of LAT-1 receptor variants was scored for their capacity to rescue the penetrant developmental lethality caused through removal of the *lat-1* gene in *C. elegans*. In the course of this study, it was noted that neither a receptor that lacks the 7TM domain nor a full-length chimeric version containing a foreign GPS motif of the GAIN domain was able to remedy the lethal effects of *lat-1* deletion. However, when both receptor variants were co-expressed, they complemented each other intermolecularly to reestablish the full biological functionality of the wild-type receptor. The conclusion drawn from this set of experiments suggested that the GPS motif interacts with the 7TM domain in an agonistic fashion [54].

Further investigations unveiled the molecular underpinnings of this effect and supplied further evidence for the model that aGPCR signaling can be activated through a tethered agonist. The stalk region that links the GPS with the first TM helix, a peptide of approximately 15–25 amino acids in length depending on individual receptor homologs, comprises an agonistic activity that stimulates metabotropic signaling of aGPCRs. When truncated receptor versions that lack the NTF are expressed, the agonist (termed *Stachel*; German: sting, or alternatively *stalk*) is exposed and conceivably interacts with the 7TM continuously leading to high signaling activity as observed before. Receptor layouts that lack the entire ECD (i.e., including the *Stachel*/*stalk*), however, are muted, but can be reactivated by high amounts of soluble *Stachel*/*stalk* peptide indicating that the tethered agonist is necessary and sufficient for receptor activation. This was shown first for GPR126 and GPR133 [52] and subsequently for additional receptors including GPR56 [63], GPR64/ADGRG2 [64], GPR114/ADGRG5 [65], Latrophilin-1, and LAT-1 [66].

Interestingly, the agonistic property of the peptide appears to reside in its N-terminal half [65], which also represents the last beta-sheet of the GAIN domain that is severed through the autocatalytic event from the much larger rest of the fold. In cleavage-competent receptor homologs, the *Stachel*/*stalk* therefore constitutes the very beginning of the CTF, which also mediates the non-covalent lock between NTF and CTF that results in the heterodimeric configuration in which aGPCRs are found on the cell membrane [19].

How is exposure of the *Stachel*/*stalk* enacted under physiological conditions? As the agonist is buried inside the GAIN domain, the simplest mode would see the NTF removed through a combination of firm ligand engagement with the extracellular adhesion domains through which mechanical force is transmitted onto the NTF that pulls it off the CTF. This way, the *Stachel*/*stalk* sequence would become instantly exposed. Corroboration of the interplay between mechanical challenge and transmembrane signal transduction has recently been found in EMR2/ADGRE2: Boyden et al. identified two kindreds that displayed symptoms of severe vibratory urticaria, a condition associated with degranulation of mast cells upon dermal challenge with physical force. In this study, an autosomal-dominant missense mutation in EMR2/ADGRE2 was shown to underlie these effects. In vitro

experiments with mast cells transfected with the mutated receptor variant indicated that removal of the NTF through vibratory shear stress was increased [67]. This is consistent with model in which elevated exposure of the tethered agonist (*Stachel*/stalk) triggers subsequent downstream signaling events and is further discussed in [68]. Also for other protease-activated membrane receptor systems, e.g., the Notch-DSL pathway (see below), similar mechanisms, executed through proteolysis by an exogenous protease, were proposed [69].

In this context, GAIN autoproteolysis would be an essential precondition for the liberation of the tethered agonist upon mechanical stimulus encounter and a satisfactory explanation for its evolutionary conservation. However, also non-cleavable aGPCRs appear to possess agonistic activity in the *Stachel*/*stalk* peptide and are sensitive to mechanical stimulation, at least in vitro, as recently shown for GPR114 [65]. To complicate matters, recent studies indicate that aGPCR engage in *Stachel*-independent metabotropic (CTF-dependent) signaling [70], which is discussed in detail in [59].

Currently, there is no obvious explanation for how the encounter between the agonist and the 7TM may be facilitated assuming that the available GAIN domain structures are representing the physiological conformation of the fold (see also [58, 71]). Alternatively, there exist steric layouts of the GAIN domain that allow access of the *Stachel*/*stalk* to its cognate 7TM interface even if the agonist is an integral part of a contiguous polypeptide chain rather than released through the autoproteolytic cleavage. Such conformations are subject to future investigations and will help answering the question for the role of aGPCR autoproteolysis.

2.4 Liberation of NTF for Cell-Non-autonomous Effects

An interesting addition to the cell-autonomous information fed into the Notch-expressing cell, the Notch-DSL interactions also appear to drive cell-non-autonomous events in the ligand-expressing cells. Also this feature of the Notch pathway may compare to properties of several aGPCR homologs and their capacity to not only act as signal sensors but also senders of information. A well-studied example is the effect of the N-terminal fragment (NTF) of Gpr126/ADGRG6 during mouse and zebra fish heart development [72, 73]. Gpr126 is expressed by endocardial cells but not cardiomyocytes and is essential for cardiac mitochondrial function and trabeculation of the heart. Genetic structure-function studies have indicated that the C-terminal fragment of Gpr126, which contains the metabotropic signaling unit of the aGPCR [52], is dispensable for these effects while they critically depend on the NTF of Gpr126. Interestingly, this requirement is shared by endocardial cells and cardiomyocytes, of which the latter do not express the receptor molecule. Immunolocalization studies further detailed that Gpr126 may work in a paracrine mode to exert its function on cardiomyocytes, possibly by shedding its NTF and thereby governing the development of these cells in a cell-non-autonomous fashion [72].

2.4.1 Split Personality Hypothesis

While the NTF and CTF of most aGPCRs are associated non-covalently, it was found that the two fragments could also be expressed separately on the cell surface as independent entities in some aGPCRs such as Latrophilin-1 and EMR2/ADGRE2 [56, 74, 75]. The so-called split personality hypothesis was coined to reflect the fact that the NTF remains free even though the CTF is pulled down exhaustively by immunoprecipitation [56, 76]. Furthermore, expression of the CTF-truncated recombinant Latrophilin-1 was found to remain tethered on the cell membrane. Most interestingly, the NTF could be efficiently removed from the membrane without solubilizing any CTF when cells were treated with perfluorooctanoic acid, a weak detergent that does not disrupt the lipid bilayer. These results strongly suggest that some NTF is self-anchored on the membrane independently of the CTF.

Indeed, subsequent studies showed that membrane localization of the two fragments does not completely overlap and the fragments could even be internalized independently [56]. Further, it is possible to detect ligand-induced interaction of individual NTF and CTF from the same receptor molecule fused with different tags (so-called homogeneric heterodimers), or even from two distinct aGPCRs (heterogeneric heterodimers; e.g., $NTF^{Latrophilin\text{-}1}$::$CTF^{GPR56}$ or NTF^{EMR4}::CTF^{EMR2}) [56, 74, 75]. For EMR2, it was shown that the NTF and CTF were differentially distributed in lipid raft microdomains and ligation of the NTF by EMR2-specific monoclonal antibodies induced the translocation and interaction of NTF with CTF to the lipid rafts for receptor activation and signaling [74]. Consistent with these findings, GPS proteolysis of aGPCRs could possibly create diverse functional receptor complexes by cross association of independent NTFs and CTFs of different aGPCRs.

Several possibilities exist as to how such molecular cross-chimerization may come about. Receptor fragments may either recombine after GAIN cleavage at the GPS. For this scenario GPS cleavage is absolutely necessary. Alternatively, aGPCRs may form heterodimers at the level of the 7TM domain, ECD, or ICD that may lead to crosswise pulldown results interpreted as heterogeneric heterodimer formation. Only in one study thus far, these possibilities were tested by the use of GPS cleavage-incompetent receptor forms, which still showed co-immunoprecipitation [77]. The authors thus concluded that homo- and heterogeneric cross talk of aGPCRs is likely the result of receptor oligomerization that does not involve NTF-CTF re-pairing at the GPS, but rather the lateral interaction of several aGPCR molecules.

Future investigations will need to further define the properties of GPS proteolysis for separate fates and biological activities of aGPCR fragments.

3 Similarities and Differences to Other Proteolysis-Dependent Signaling Pathways

aGPCRs are by far not the only group of biomolecules whose actions are controlled through proteolytic cleavage (Fig. 3, Table 1). Here, we only concentrate on those that are governed by the proteolytic processing of surface receptors. However, we note that also a wealth of other biological signals depend on the proteolytic activation of precursor states of intracellular or secreted substrates. This includes the shedding of N-terminal signal peptides through signal peptide peptidases or the functionalization of prohormones and proenzymes into active molecule species, such as proinsulin in pancreatic β cells or serine proteases in the gastrointestinal system, respectively.

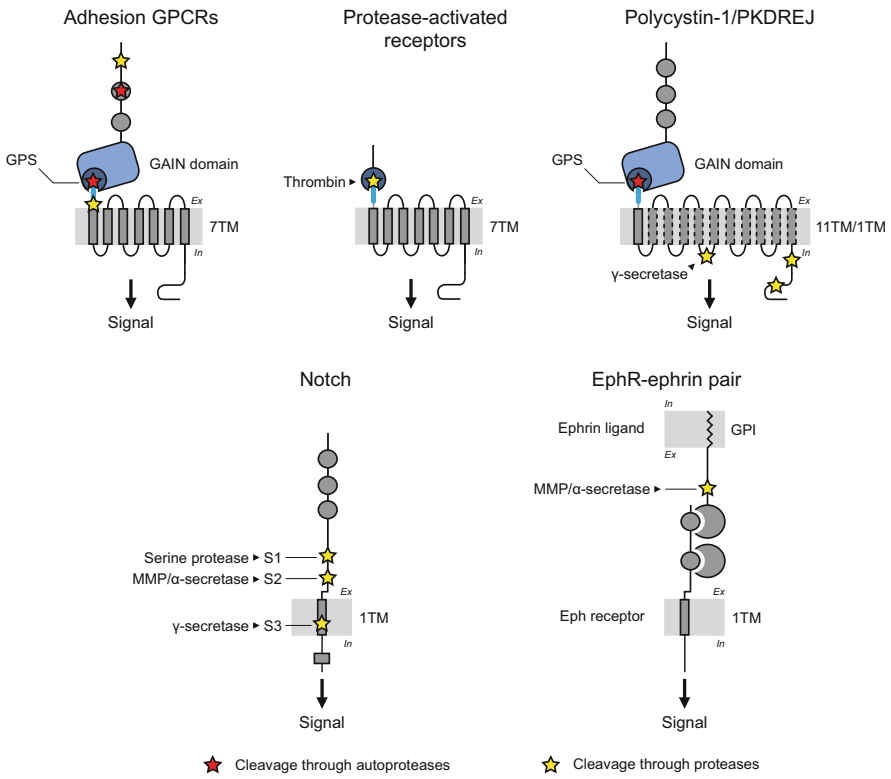

Fig. 3 Molecular pathways controlled though proteolytic processing. aGPCR processing through self-cleavage and cleavage by proteases is implicated in several biological properties of these receptors including critical steps in their signaling cascade. In this respect, aGPCRs may share signaling principles with other receptor systems that rely on proteolysis to trigger and/or transduce extracellular events into intracellular information. These encompass, among others, the protease-activated receptor group of GPCRs, polycystin-1/PKDREJ, Notch, and ephrin receptor families of cell-surface receptors

Table 1 Signaling pathways executed and/or modulated through non-GAIN-domain-mediated proteolysis

Substrate	Serine protease	MMP/ α-secretase	β-secretase	γ-secretase	Selected physiological effects of proteolysis
EGF, TGF-α, neuregulins, etc.		●			Shedding of substrate for EGFR: cell growth, migration, proliferation, differentiation [92, 93]
TNF-α		●			Shedding of substrate for cytokine receptor: inflammation, apoptosis, necrosis [94, 95]
APP		●		●	Physiological tasks: learning, memory, synaptic plasticity (LTP) [96–98]
			●	●	Pathological effects: accumulation of Amyloid-β protein in Alzheimer's Disease [98, 99]
Notch	●	●		●	Binary developmental commands involved in cell fate decisions; NCID: transcriptional regulation [100] of inner ear development [101], pancreatic β cell production [102], specification of goblet cells in the intestine [103], various functions in B and T cell development [104, 105], etc.
N-cadherin		●		●	Cell-cell adhesion [106]; β-catenin transcriptional control: neuronal differentiation, synaptogenesis, axonal remodeling (synaptic plasticity) [107]; cell proliferation and cell survival (cancer) [106]
Ephrin-A2		●			Contact-mediated axon repulsion [108, 109]
Neuroligin-1		●		●	Negative regulation of spine remodeling at excitatory synapses [110]
Neurexin-3β		●		●	Glutamatergic synapse assembly and possibly also function [111, 112]
PAR1	●				Fibrin conversion (platelet aggregation), endothelial cell activation, vasoconstriction/vasodilation, immune regulation [81, 82, 113]
BAI1/ADGRB1		●			Release of vasculostatin-40: inhibition of angiogenesis [45]
BAI2/ADGRB2	●				Unknown [48]
GPR116/ADGRF5	●				α-Fragment: unknown role in cellular signaling [38]
LPHN1/ADGRL1	●				Regulation of surface expression, generation of soluble receptor forms [46, 77]
GPR126/ADGRG6	●				Release of secreted ligand which regulates myocardial energy metabolism, contractility, and trabeculation [47, 72]

3.1 Protease-Activated Receptors (PARs)

Apart from aGPCRs, there are also other members of the GPCR superfamily that require cleavage for their biological activity, e.g., for the initiation of their signaling cascade. Thus far, four protease-activated receptors have been identified, PAR1–4 [78], each following a canonical activation principle. PAR1 is considered the prototype receptor of the family. It is activated by the serine protease thrombin, a key regulator of platelet aggregation, endothelial cell activation, and further vascular effects [79–81].

For activation of human PAR1, thrombin cleaves the ectodomain of the receptor at a specific recognition site (LDPR↓S) that is located at position 41 of the receptor molecule [82]. The resulting new N-terminus contains a tethered agonist that becomes unmasked upon the proteolytic event (Fig. 3, Table 1). The agonist physically interacts with the 7TM domain of PAR1 and activates its signaling cascade. Furthermore, synthetic peptides comprising the first six residues of the unmasked tethered agonist are capable of activating PAR1, even without prior receptor cleavage [82]. Additionally, genetic exchange of the cleavage site, e.g., to a trypsin cleavage site, resulted in receptor activation through trypsin under heterologous expression conditions [83, 84], confirming the hypothesis that the role of thrombin comprises the exposure of the receptor's tethered agonist [85, 86].

An activation mechanism which shares similarities with the proteolytic activation of PAR1 has recently been unraveled for GPR126, GPR133 [52], and further aGPCRs [63–65] (see above). However, several differences to PAR activation have to be considered: while PAR1 processing through the exogenous thrombin protease directly leads to the exposure of its tethered ligand, GAIN domain-mediated GPS cleavage of aGPCRs alone may not be sufficient to unmask the *Stachel*/*stalk* agonist of selected aGPCRs as the cleavage fragments remain attached to each other. Further structural changes in their extracellular domain, e.g., through ligand binding to the adhesion domains within the receptor ectodomain similar to the situation of the Notch receptor, and/or mechanical removal of the NTF may be required for *Stachel*/*stalk* exposure and aGPCR activation [87–89].

However, studying properties of PAR receptors may reveal additional parallels to the signaling paradigm(s) utilized by aGPCRs. For example, rapid phosphorylation-dependent internalization of activated PAR molecules and subsequent lysosomal degradation terminate PAR1 signaling [90, 91]. At least one aGPCR study suggests that ligand contact and mechanical challenge of CD97 trigger removal and degradation of the receptor's CTF, thereby providing means to quench signaling through aGPCRs [87].

3.2 Notch

Developmental signals governed through the activation of the Notch receptor are arguably the best researched and understood functions that result from proteolytic processing of a receptor molecule. The Notch receptor consists of the single-pass

transmembrane protein and contains a species-specific array of up to 36 epidermal growth factor-like (EGF) repeats strung along the length of its extensive ectodomain. Through the ectodomain, the receptor interacts with DSL ligands (Delta, Serrate/Jagged, LAG-2), which themselves are large type I transmembrane molecules mounted on neighboring cell surfaces to the Notch-bearing cell [114, 115], thereby resembling the interaction scenario of several aGPCRs and their cellular ligands, e.g., CD97 with CD55 [116] or Latrophilins with FLRTs, teneurins, and neurexins [117–119].

An important consequence imposed by the positional Notch receptor-ligand configuration is the restriction of signaling events to cellular neighbors [120], which may also figure in the physiological roles of aGPCRs. This restriction is critical for the developmental switches governed by the Notch pathway, as it regulates binary cell fate decisions during embryogenesis, organogenesis, and cell differentiation, and many examples across the tree of metazoan life bear witness to the generality of this concept [104, 121]. In the classical paradigm of the Notch receptor-DSL ligand interplay, two daughter cells deriving from a precursor blastomere inherit equal amounts of both Notch and DSL. Engagement of Notch and ligand at the cell contact faces initiates an iteratively looping feedback cycle, which culminates in downregulation of the receptor in only one of the two cells. In the 'winner cell', the intracellular actions of Notch repress a proneural gene battery and drive it into the epidermal cell lineage. Conversely, the cell that has lost Notch on its surface becomes a neuronal precursor cell [122].

Intriguingly, the activation of the Notch receptor molecule is the consequence of a cascade of at least four cleavage events that sequentially process the receptor molecule along its N→C axis (Fig. 3, Table 1). First, after biosynthesis and en route to the cell surface, the receptor is cleaved by a furin-like convertase at the S1 cleavage site severing most of the receptor's ectodomain including the ligand-binding EGF repeats from a fragment holding the juxtamembrane, transmembrane, and intracellular receptor portion. S1 cleavage, however, does not result in physical separation of the cleavage fragments as they form a heterodimer held together through non-covalent interactions [123, 124], resembling the situation of aGPCRs that have undergone GAIN autoproteolysis but appear as heterodimers at the plasma membrane [4, 125].

The second proteolytic step occurs at the S2 site, which is positioned C-terminal to the S1 site just above the transmembrane helix. Before activation, the S2 site is protected by an arrangement of three LIN-12/Notch (LNR) repeats that are grouped around the cleavage site blocking access for the cognate S2 metalloproteases Kuzbanian and TACE/ADAM10 [126, 127]. When Notch engages with its DSL ligand, DSL endocytosis is thought to generate mechanical forces pulling at the receptor molecule, which eventually leads to conformational unwinding of the LNR repeats and exposure of the S2 site and its cleavage [128, 129]. While S1 cleavage is dispensable for Notch function [130], S2 cleavage appears as the gatekeeping step in Notch activation rendering the pathway a developmental command control system that may be triggered by mechanical input and may thus share similarities with the role of aGPCRs in development.

After S2 proteolysis, the remaining transmembrane-intracellular fragment of the Notch receptor [Notch extracellular truncated (NEXT)] undergoes further regulated intramembrane cleavage (RIP) catalyzed by the γ-secretase complex. This large multi-protein enzyme cleaves the NEXT intermediate at two further sites inside the membrane (S3 and S4 sites) [126, 127, 131, 132]. Ultimately, S3/S4 proteolysis results in the release of the Notch intracellular domain (NICD), which heteromerizes with DNA-binding and transcriptional activation partners and travels into the nucleus, where the complex controls the expression of target genes [133–136].

3.3 Ephrins

Apart from Notch, proteolysis through ADAM10 assumes a central position in the processing of a number of other neuronal proteins like APP, N-cadherin, neuroligins, or ephrins [96, 106, 108, 110]. Among those, the proteolytic activation and physiological relevance of the signaling mode of ephrin-A2 are exemplary (Fig. 3, Table 1).

Ephrin-A2 is a GPI-anchored molecule that is cleaved by ADAM10 upon binding its endogenous receptor EphA3. The binding and cleavage event consequently disrupts the cell-cell contact mediated through the Eph/ephrin interaction [108]. Upon the formation of the ligand-receptor complex, the molecular recognition motif in ephrin-A2 is rendered accessible for ADAM10, which then associates with this complex and cleaves ephrin-A2 in a *trans* mode, as protease and substrate are expressed in different cells [137, 138]. It appears that this mechanism ensures the exclusive cleavage of receptor-bound ligands [137]. Following the proteolytic rupture of the intercellular connection, the Eph/ephrin complex is rapidly internalized into the receptor expressing cell [138], which has been shown for ephrin-A5, a related member of the ephrin family [139]. Blocking the Eph/ephrin complex binding site of ADAM10 using specific monoclonal antibodies resulted in impaired internalization and EphA3-mediated cell function, suggesting a physiological role for the cleavage [140].

Ephrins and their Eph receptors are generally involved in the guidance of cell migration and neural development, tissue separation, and synaptic plasticity [139, 141], but also in extraneuronal processes including vascular development, epithelial cell response, and inflammation [142–144]. One particular physiological function of ephrin-A2 is the control of axon guidance [108] and involves proteolysis through ADAM10. Migrating EphA3-presenting axons come in contact with cells expressing ephrin-A2. Upon this encounter, the EphA3-positive neurites are actively repelled by the proteolytic disruption of the Eph/ephrin connection and thereby lead to axon withdrawal and precise spatio-mechanical control of neurogenesis [108, 145]. Inside the cell, regulation of this signal is mainly communicated through the intrinsic tyrosine kinase activity of ephrin receptors. Phosphorylation-dependent activation of EphA3 triggers a conformational change shifting the kinase domain away from the plasma membrane into its active form

[146, 147], where it no longer obstructs the alignment with ADAM10 and therefore allows ephrin shedding [146, 148]. Accordingly, EphA3 mutants carrying a constitutively released kinase domain showed increased ephrin cleavage by ADAM10, even when kinase function was disabled [146]. Thus, tyrosine kinase activity of ephrin receptors is an intracellularly regulated means to switch between cell-cell repulsion (high activity) and cell-cell adhesion (low activity) [146, 148, 149].

This binary signaling of Eph receptors may bear functional and cell biological similarities to aGPCRs. Their variety of extracellular adhesion motifs are predestined for intercellular cell-cell interactions like those observed for Eph/ephrin, although the majority of aGPCRs are still orphaned without known ligands or intracellular interactors [1]. aGPCR-ligand complexes could conceivably be shed involving an exogenous protease (see discussion about furin-mediated cleavages of individual aGPCR homologs above). Equally possible, mechanical force may solely govern receptor fragment (NTF-CTF) separation at the breakpoint originating from receptor autoproteolysis at the GPS. This way, aGPCR-expressing cells may be able to switch from an adhesion to signaling state.

3.4 Polycystins

PKD1 and *PKD2* are genes encoding polycystins, which are multitransmembrane proteins with a large amino-terminal extracellular domain [150]. *PKD* mutations have been demonstrated to cause one of the most common genetic diseases worldwide, the autosomal-dominant polycystic kidney disease (ADPKD) [151], which is characterized by the formation of multiple fluid-filled cysts that lead to renal failure in patients [152]. Loss-of-function mutations in polycystin-1 (*PKD1*) are responsible for a vast majority of ADPKD cases with physiological functions of *PKD1* found in cell adhesion and cell junction formation [153, 154]. Consistent with these findings, polycystin-1 appears involved in mechanical coupling between cells and in the regulation of tubular lumen diameter along the nephron [155].

Interestingly, polycystin-1 shares several structural features with aGPCRs (Fig. 3). They possess a multi-pass transmembrane domain (with 11 instead of 7 helices), an extended ectodomain with arrays of PKD motifs, and most notably a GAIN domain [19]. Similar to aGPCRs, polycystin-1 undergoes autoproteolysis resulting in the generation of an NTF and CTF [17], which remain non-covalently attached after cleavage. The physiological role of the polycystin-1 GPS cleavage is unknown. However, cleavage-deficient mutants exhibit impaired function in vitro [17] and in vivo [18]. A *PKD1* GPS proteolysis-deficient mouse mutant shows abnormal renal development after the first days of postnatal life apparent in reduced size and weight, as well as grossly enlarged cystic kidneys. Furthermore, the mutation is lethal within 6 weeks after birth presumably due to renal insufficiency [18]. This is only partly compatible with defects displayed by $PKD1^{-/-}$ mice, which show severe embryonic phenotypes and die already a few hours after birth [156]. Therefore, it was concluded that GPS cleavage of polycystin-1 is required for postnatal renal maturation, while it is not essential for

embryonic nephrogenesis [18]. This is supported by the fact that cleaved and uncleaved polycystin-1 can coexist under physiological conditions [155]. Interestingly, polycystin-1 is also substrate to proteolytic events in addition to GAIN domain autoproteolysis, as it, too, is cleaved by the γ-secretase complex, resembling S3 and S4 proteolyses of the Notch receptor [157, 158].

4 Conclusions

aGPCRs are by far not the only group of biomolecules whose actions are controlled through proteolytic cleavage. Here, we only concentrated on those that are governed by the classical proteases and autoproteolytic events. As shown here, autoproteolysis and proteolytic cleavage seem neither mutually exclusive nor are their functional implications in aGPCRs sufficiently understood. Considering the tremendous number of surface receptors controlled through proteolysis, and the requirements for their function, there is no doubt that elucidation of the physiology of aGPCRs requires further investigation of their proteolytic properties. This should include a better understanding how GAIN domain-mediated cleavage is involved in receptor signaling and resolve the question if and how it may be modulated, e.g., through allosteric mechanisms. Further interest should be directed toward the study of other aGPCR domains that entertain non-GAIN domain autoproteolytic steps and the role of other proteases in the processing of the receptor molecule and pin down their physiological roles in receptor trafficking, cell adhesion, metabotropic, and non-cell-autonomous signaling. The structural and physiological properties of other surface molecule systems including the PAR, Notch, ephrin, or polycystin pathways should be considered as examples of how proteolytic processing can shape the function of receptor modules. The extensive body of work accumulated on their biological significance can instruct new experimental avenues and working models that should be explored in the quest to elucidate the interplay between the proteolytic processing of aGPCRs and their diverse signaling profiles.

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