A tethered agonist within the ectodomain activates the adhesion G protein-coupled receptors GPR126 and GPR133

Ines Liebscher  
*University of Leipzig*

Julia Schon  
*University of Leipzig*

Sarah C. Petersen  
*Washington University School of Medicine in St. Louis*

Liane Fischer  
*University of Leipzig*

Nina Auerbach  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Please let us know how this document benefits you.

**Recommended Citation**

Liebscher, Ines; Schon, Julia; Petersen, Sarah C.; Fischer, Liane; Auerbach, Nina; Demberg, Lilian Marie; Mogha, Amit; Coster, Maxi; Simon, Kay-Uwe; Rothemund, Sven; Monk, Kelly R.; and Schoneberg, Torsten, "A tethered agonist within the ectodomain activates the adhesion G protein-coupled receptors GPR126 and GPR133." Cell Reports. 9, 6. 2018-2026. (2014).  
https://digitalcommons.wustl.edu/open_access_pubs/3632

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Authors
Ines Liebscher, Julia Schon, Sarah C. Petersen, Liane Fischer, Nina Auerbach, Lilian Marie Demberg, Amit Mogha, Maxi Coster, Kay-Uwe Simon, Sven Rothemund, Kelly R. Monk, and Torsten Schoneberg
A Tethered Agonist within the Ectodomain Activates the Adhesion G Protein-Coupled Receptors GPR126 and GPR133

Graphical Abstract

Highlights

- A tethered peptide agonist (termed the Stachel sequence) activates adhesion GPCRs

- The Stachel sequence is highly specific for the given adhesion GPCR

- Structural changes within the ectodomain induce active peptide conformation

- The Stachel sequence is essential for receptor activation in vivo

Authors

Ines Liebscher, Julia Schön, ..., Kelly R. Monk, Torsten Schöneberg

Correspondence

liebscher@medizin.uni-leipzig.de (I.L.), schoberg@medizin.uni-leipzig.de (T.S.)

In Brief

In this article, Liebscher et al. provide in vitro and in vivo proof that members of the adhesion GPCR class are activated through a tethered peptide agonist, which they term the Stachel sequence. This raises the possibility of actively inducing adhesion GPCR function.
A Tethered Agonist within the Ectodomain Activates the Adhesion G Protein-Coupled Receptors GPR126 and GPR133

Ines Liebscher,1,4,5 Julia Schön,1,4 Sarah C. Petersen,2 Liane Fischer,1 Nina Auerbach,1,2 Lilian Marie Demberg,1 Amit Mogha,2 Maxi Cöster,1 Kay-Uwe Simon,1 Sven Rothemund,3 Kelly R. Monk,2 and Torsten Schöneberg1,*

1Institute of Biochemistry, Medical Faculty, University of Leipzig, 04103 Leipzig, Germany
2Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
3Core Unit Peptide Technologies, Medical Faculty, University of Leipzig, 04103 Leipzig, Germany
4Novo Nordisk Center for Basic Metabolic Research, Department of Biomedical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark
5Co-first author
*Correspondence: liebscher@medizin.uni-leipzig.de (I.L.), schoberg@medizin.uni-leipzig.de (T.S.)

http://dx.doi.org/10.1016/j.celrep.2014.11.036

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

Adhesion G protein-coupled receptors (aGPCRs) comprise the second largest yet least studied class of the GPCR superfamily. aGPCRs are involved in many developmental processes and immune and synaptic functions, but the mode of their signal transduction is unclear. Here, we show that a short peptide sequence (termed the Stachel sequence) within the ectodomain of two aGPCRs (GPR126 and GPR133) functions as a tethered agonist. Upon structural changes within the receptor ectodomain, this intramolecular agonist is exposed to the seven-transmembrane helix domain, which triggers G protein activation. Our studies show high specificity of the given Stachel sequence for its receptor. Finally, the function of Gpr126 is abrogated in zebrafish with a mutated Stachel sequence, and signaling is restored in hypomorphic gpr126 zebrafish mutants upon exogenous Stachel peptide application. These findings illuminate a mode of aGPCR activation and may prompt the development of specific ligands for this currently untargeted GPCR family.

INTRODUCTION

Adhesion G protein-coupled receptors (aGPCRs) are among the largest proteins in nature and consist of a long extracellular domain (ECD), a seven-transmembrane domain (7TM), and an intracellular C-terminal tail (ICD) (Figure 1A; Bjarnadottir et al., 2004; McMillan et al., 2002). Another feature of this class is an autoproteolytic cleavage event that occurs at the GPCR proteolytic site (GPS), located within the GPCR autoproteolysis-inducing (GAIN) domain, which cleaves aGPCRs into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (Araç et al., 2012; Figure 1A). aGPCRs play essential roles in controlling cell and tissue polarity (Lawrence et al., 2007) and can modulate synaptic functions (O’Sullivan et al., 2012; Südhof, 2001). Although increasing information about aGPCR relevance is being obtained from mutant animal models, human diseases, and variant-associated phenotypes, little is known about the molecular function, activation, and signal transduction of this receptor class (Langenhan et al., 2013; Liebscher et al., 2013).

The first indirect functional data for G protein coupling by aGPCRs were obtained in studies of GPR56 by knockdown experiments involving G12/13/p115 RhoGEF pathway components (Iguchi et al., 2008). An intriguing observation was reported in a study of gpr126 mutant zebrafish (zebrafish), which exhibit defects in peripheral myelination (Monk et al., 2009). This phenotype was reversible through forskolin-induced cyclic AMP (cAMP) elevation, suggesting Gα-protein coupling. Other studies provided direct evidence of Gα-protein coupling by measuring intracellular cAMP levels induced by basal activity of the aGPCRs GPR133 (Bohnekamp and Schöneberg, 2011) and GPR126 (Mogha et al., 2013). Furthermore, experiments with chimeric G proteins, stoichiometric titrations of the Gαs subunit and receptor, and Gαs subunit knockdown experiments (Bohnekamp and Schöneberg, 2011) strongly support G protein coupling for GPR133.

Although it is now clear that aGPCRs couple to G proteins, it remains unclear whether endogenous binding partners can induce activation of aGPCRs. Interestingly, several studies have described increased receptor activity when an N-terminal deletion mutant was expressed (Okajima et al., 2010; Paavola et al., 2011, 2014; Yang et al., 2011; see Figure 1A). These observations led to the assumption that the ectodomain functions as an inverse agonist, although at least two scenarios for aGPCR activation have been proposed (Liebscher et al., 2013): (1) the ectodomain contains an inverse agonist that inhibits 7TM signaling; and (2) ligand binding at the ECD or NTF removal changes the conformation of an aGPCR and exposes a tethered agonist (Figures S1A and S1B).

To test these two models, we used the human (h) GPR126 and GPR133 to analyze the contribution of the ECD to receptor basal activity, because Gα-protein coupling has been experimentally suggested for these aGPCRs (Bohnekamp and Schöneberg, 2011; Gup te et al., 2012; Mogha et al., 2013). Systematic
mutagenesis studies revealed tethered peptide sequences within the most C-terminal part of the ECD, which specifically activates G protein signaling via 7TM interactions in vitro. Finally, we performed loss-of-function and rescue experiments in zf gpr126 mutants to confirm the in vivo and evolutionarily conserved significance of the tethered agonist. Together, our results define a mechanism of aGPCR activation.

RESULTS

ECD Deletion Activates GPR126 and GPR133
First, we deleted the ECDs of hGPR126 and hGPR133 at their natural GPS cleavage sites and tested the mutants in cAMP assays. In these constructs, termed CTF(GPR126) and CTF(GPR133), the NTF between the signal peptide and the GPS cleavage site was removed, but the ECD part located C-terminally to the GPS cleavage site remained attached to the 7TM. All mutants lacking the ECD displayed significantly increased basal activities in cAMP assays (Figure 1C), consistent with results from other NTF-deficient aGPCRs. Both mutants were poorly detected at the cell surface via hemagglutinin (HA)-tag staining (Figure S1C). Accordingly, total ELISA and confocal imaging revealed an absence of the HA tag in CTF(GPR126) constructs. However, confocal imaging of the C-terminal FLAG tag showed specific membrane fluorescence (Figure S1D). We therefore speculate that the HA tags in the CTF mutant constructs are processed during intracellular protein maturation, thereby precluding detection. Because the N termini of rhodopsin-like receptors can improve cell-surface expression and detection of other GPCRs (Bohnekamp and Schöneberg, 2011; Stäubert et al., 2010), we added an HA-tagged P2Y12 N terminus to the residual ECD of the CTF mutants. This generated
chimeric P2Y12-CTF(GPR126) and P2Y12-CTF(GPR133) receptors (Figure 1B), which enabled proper plasma membrane detection via HA-tag visualization (Figure S1C). As observed for the CTF constructs, P2Y12-CTF(GPR126) and P2Y12-CTF(GPR133) displayed high constitutive activity (Figure 1C). These results demonstrate that deletion of the NTF activates hGPR126 and hGPR133.

The ECDs of GPR126 and GPR133 Contain Agonistic Domains

We generated GPR126 and GPR133 mutants in which the entire ECD, including the entire GPS motif, was deleted or replaced by the N terminus of P2Y12 (ΔGPS-CTF; P2Y12-ΔGPS-CTF; Figure 1B). None of the constructs displayed constitutive activity (Figure 1C), although these chimeras were expressed at the cell surface (Figure S1C). These results argue against the inverse agonist model of aGPCR activation because constitutive activity caused by the release of an inverse agonist would not depend on the presence of the residual GPS motif. These results point toward an activation model that requires the residual GPS motif, and we hypothesized that the GPS sequence downstream of the cleavage site contains determinants required for receptor activation.

To identify this potential tethered agonist, we sequentially deleted amino acids (aa) C-terminal to the GPS cleavage site in GPR126. Functional analysis showed that while the most N-terminal aa (Thr813; Figure 1B) was not essential for receptor activation (Figure 1D), deletion of the first two, as well as larger deletions that removed aa following Thr813, abolished basal receptor activity. This abolishment was not due to expression changes, since total and cell-surface expression levels were not significantly different between the constructs (Figure S1E).

To maintain correct aa length C terminal to the cleavage site, we exchanged several positions with alanine. Again, mutants with an exchange of position 813 retained constitutive activity, whereas the exchange of positions 815, 818, and 819 abolished activity in P2Y12-CTF(GPR126) (Figure 1E), but expression levels were not affected (Figure S1F). Mutagenesis studies at corresponding positions in P2Y12-CTF(GPR133) revealed almost identical results (Figures 1E and S1F). These experiments support the existence of a defined agonistic region C-terminal to the GPS.

A Tethered Peptide Activates GPR126

To demonstrate that the aa sequence C-terminal to the GPS cleavage site has agonistic properties, we tested peptides derived from this domain on P2Y12-ΔGPS-CTF(GPR126). Interestingly, systematic truncation of the peptide’s C terminus revealed several agonistic peptides (Figure 2A). The most efficient peptide, p16 (16 aa long), was used for further structure-function studies. N-terminal deletion of the first two aa abolished the agonistic abilities of p16 (p16-1 and p16-2; Figure 2A). This does not contradict the results of Figures 1D and 1E, because in the original CTF mutants, the first aa were replaced by the P2Y12 N terminus or by alanine. Thus, these changes are tolerated, whereas the deletions in p16 are not. N-terminal extension beyond the cleavage site by one (p16+1) or two to four (p16+2 to p16+4) aa showed reduced or no agonistic activity of p16, respectively (Figure 2A). This indicates that noncleaved aa upstream of Thr813 are not part of the agonistic structure. In concentration-response curves, p16 displayed low potency (EC50 > 400 μM) on both P2Y12-ΔGPS-CTF(GPR126) and wild-type (WT) GPR126 (Figure 2B), which can be explained by the natural 1:1 stoichiometry of the covalently bound agonist in its natural conformation. The higher cell-surface expression of WT GPR126 compared with P2Y12-ΔGPS-CTF(GPR126; Figure S1C) explains the increased efficacy of p16 on WT GPR126 activation. Time-course analyses of cAMP accumulation (Figure S2A) and GTPγS binding assays (Figure S2B) in response to p16 supported p16-induced G protein coupling in GPR126-transfected cells. Note that eV-transfected cells showed residual cAMP accumulation (Figure 2C) and GTPγS binding, indicating endogenous expression of GPR126 in COS-7 cells. This was confirmed by RT-PCR (Figure S2C), cAMP assays (Figure 2C), and kinetic dynamic mass redistribution (DMR) measurements (Epic; Corning Life Sciences) with small interfering RNA (siRNA)-mediated knockdown of the endogenous GPR126 (Figures S2D and S2E).

The endogenous expression of GPR126 and the high sensitivity of the DMR technology enabled us to test p16 apart from heterologous overexpression systems. As shown in Figure 2D, p16 induced concentration-dependent cellular responses very similar to those found with isoprenaline and β-adrenergic receptor endogenously expressed in COS-7 cells. Mutation of position 6 (Leu6Ala) in p16 abolished the response (Figure 2D), confirming specificity. To identify functionally relevant positions in the peptide, we performed a systematic alanine scan (Figure 2E). As expected from our receptor mutagenesis data (Figures 1D and 1E), the more N-terminal aa (positions +2 to +7) were required for agonistic activity, whereas positions +8, +10, +12, and +14 to +16 could be replaced with Ala and still show agonistic properties. These data are in line with a high evolutionary conservation of the N-terminal portion of this peptide sequence (Figure S2F).

Interestingly, the peptide p16 Gly7Ala blocked p16-induced GPR126 activation at double concentration (Figure 2F), indicating that p16 Gly7Ala can compete with the p16 binding site. Together, these data support the notion that the tethered peptide p16 activates GPR126.

A Tethered Peptide Activates GPR133

To determine whether activation by a tethered peptide is common for aGPCRs, we performed similar studies with GPR133. The P2Y12-ΔGPS-CTF(GPR133) could be activated by a peptide derived from the 13 aa (p13) downstream of the putative cleavage site (Figure 3A). Concentration-response measurements of p13 revealed specific activity on P2Y12-ΔGPS-CTF(GPR133) and WT receptor (EC50 > 400 μM; Figure 3B). The derived peptides were highly specific for the aGPCR from which they originated: GPR133 p13 did not activate GPR126, and GPR126 p16 did not activate GPR133 (Figure 3C). Because the importance of GPS cleavage for aGPCR expression and activity has been the subject of controversy (Liebscher et al., 2013), we tested two cleavage-deficient mutants: GPR126T841A (Moriguchi et al., 2004) and GPR133H540R (Bohnkamp and Schöneberg, 2011). Both mutants were expressed and activated by their respective peptides (Figures S2G–S2I), indicating that cleavage...
at the GPS is not required for aGPCR activation by the tethered agonistic peptides. These data demonstrate that the tethered peptide p13 activates GPR133. Together with our analysis of GPR126, these studies suggest that tethered peptide activation is a common signaling modality for the aGPCR class.

**Tethered Peptide Activation of Gpr126 In Vivo**

We next sought to test the in vivo relevance of aGPCR tethered peptide activation. For these studies, we used zf because previous mutant analyses demonstrated that Gpr126 is essential for Schwann cell myelination and ear development and that these physiological functions require cAMP elevation (Geng et al., 2013; Monk et al., 2009). Although several zf gpr126 mutant alleles have been recovered in genetic screens (Pogoda et al., 2006), none specifically affect the tethered agonist sequence. Therefore, we utilized transcription-activator-like effector nucleases (TALENs) to target this region (Figures S3A and S3B). We isolated a mutant, gpr126stl215, which lacks only two codons (Gly831-Ile832) within the tethered agonist sequence (Figures 4A, 4B, and S3C). The gpr126stl215 mutants were grossly normal compared with WT animals (Figure S3D), but developed swollen ears (Figure 4C), failed to express myelin basic protein (mbp, a
marker of mature Schwann cells) along the posterior lateral line nerve (PLLn) (Figures 4D and 4E), and did not myelinate peripheral axons (Figures S3E–S3H). These defects completely phenocopy the previously published **gpr126st49** mutant, which has an early stop codon in the GAIN domain upstream of the GPS motif (Figure 4B; Monk et al., 2009). Importantly, the Gly831-Ile832 deletion introduced by the **gpr126stl215** mutation did not alter the cell-surface expression of the receptor (Figures S4A and S4B). Therefore, we conclude that the phenotypes observed in **gpr126stl215** mutants are caused by loss of a functional tethered agonist.

Finally, we tested whether p16 serves as an agonist for endogenous Gpr126 in vivo, using zf PLLn **mbp** expression as an assay. The **gpr126st63** allele contains a point mutation in the first extracellular loop of the 7TM that converts a conserved cysteine residue to tyrosine (C917Y; Figure 4B; Monk et al., 2009). This mutant receptor shows reduced cell-surface expression compared with the WT (~60% of WT levels; Figure S4A) and a concomitant reduction in basal activity (Figure S4B). In vivo, **mbp** expressed is reduced, but not absent, along the PLLn (Pogoda et al., 2006). In contrast, **mbp** expression is completely absent along the PLLn of the strong loss-of-function **gpr126st49** and **gpr126stl215** mutants (Figures 4D and 4E). Therefore, we predicted that the **gpr126st63** allele produces a hypomorphic Gpr126 protein with reduced signaling capability. Accordingly, our ultrastructural analysis revealed that **gpr126st63** mutants can myelinate axons in the PLLn, although fewer axons are myelinated than in the WT (Figures S4C and S4D) (S.C.P. and K.R.M., unpublished data).

Because we can infer that **gpr126st63** mutants possess a partially functional 7TM, we hypothesized that exogenous addition of p16 could increase the signaling of endogenous hypomorphic Gpr126. This assay is feasible given that small molecules, including peptides, can freely diffuse into the developing larva in the presence of a carrier (Morash et al., 2011), and because the functionally important positions in p16 are almost 100% identical between *Danio rerio* and *Homo sapiens* (Figure S2F). Indeed, p16 was able to activate WT zf Gpr126 in vitro cAMP assays (Figure S4B). Therefore, we treated **gpr126st63** mutants with 100 μM p16 in DMSO from 50–55 hr postfertilization, which constitutes a temporal window in which cAMP elevation by forskolin administration can rescue myelination in **gpr126st49** mutants (Glenn and Talbot, 2013; Monk et al., 2009). We then qualitatively scored **mbp** expression in the PLLn (Figure 4A). As a negative control, we treated siblings with DMSO and observed normal PLLn **mbp** expression in the WT (Figures S4C and S4D) (S.C.P. and K.R.M., unpublished data).

**Figure 3. Tethered Agonistic Peptides Are Receptor Specific**

(A) Application of 1 mM peptides of different lengths derived from the C-terminal part of the GPS, beginning at the cleavage site of GPR133, revealed agonistic properties as measured by cAMP accumulation. The highest agonistic efficacy was detected for a peptide containing 13 aa (p13). Negative controls: eV and GPR126-P2Y12-ΔGPS-CTF mutant. Basal cAMP levels were 5.2 ± 2.0 nM.

(B) The concentration-response curve of the p13 peptide revealed an EC50 > 400 μM. Basal eV levels were 2.9 ± 0.2 nM.

(C) Specificity of the p16 (GPR126) and the p13 (GPR133) peptides were verified using WT P2Y12, WT GPR126, and WT GPR133 as controls. p16 peptide activated WT GPR126 and P2Y12-ΔGPS-CTF(GPR126), whereas it exhibited unspecific activity in control receptors due to endogenous expression of GPR126 in COS-7 cells (Figure S2C). The p13 peptide specifically activated WT GPR133 and P2Y12-ΔGPS-CTF(GPR133). Basal cAMP levels were 3.0 ± 0.8 nM. Data are shown as means ± SEM of three independent experiments, each performed in triplicate. Statistics were obtained by two-way ANOVA and Bonferroni post hoc test: *p < 0.05; **p < 0.01; ***p < 0.001. See also Table S1.
100 μM p16 caused no significant change in WT larvae, but significantly rescued mbp expression in gpr126st49/st63 hypomorphic (0% “strong” or “some” in gpr126st63/st63 + DMSO versus 44% “strong” or “some” in gpr126st63/st63 + p16; Figures 4H, 4J, and 4K). To test whether this effect is specific to Gpr126 signaling, we also assayed strong loss-of-function gpr126st49 mutants, which presumably do not express a 7TM (Patra et al., 2013). Exogenous treatment of gpr126st49 mutants with 100 μM p16 did not rescue mbp expression in the PLLn (Figure 4K), indicating that p16 signals through the 7TM. Together, these loss- and gain-of-function experiments in zf demonstrate the in vivo relevance of tethered peptide activation of aGPCRs.

**DISCUSSION**

We define a common intramolecular agonistic domain for the aGPCRs GPR126 and GPR133 that comprises a sequence between the GPC cleavage site and TM1. Because of its activating nature and its position at the very C terminus of the ECD, we refer to this agonistic sequence as the “Stachel sequence” (Stachel is the German word for stinger). Our analysis of gpr126st63 suggests that Stachel-mediated activation of Gpr126 is essential for Schwann cell myelination in zf (Figures 4C–4E and S3E–S3G); however, the in vivo mechanisms that unmask this tethered agonistic domain are unknown. GAIN domain crystal structures revealed that the Stachel sequence lies buried between two β sheets (Araç et al., 2012). We and others have shown that CTF-only truncation mutant aGPCRs possess increased basal activity (Figures 1C–1E; Okajima et al., 2010; Paavola et al., 2011, 2014; Yang et al., 2011). In all of these studies, the critical GAIN domain β sheets were deleted along with the rest of the NTF, presumably exposing the Stachel sequence. Therefore, structural changes in vivo due to extracellular molecules interacting with the ECD (Langenhan et al., 2013; Liebscher et al., 2013) or even mechanical removal of the NTF may expose the Stachel sequence to activate the 7TM. The low affinity of the Stachel sequence to the 7TM suggests a fast on/off ligand-receptor interaction and supports activation by mechanical signals (Karpus et al., 2013).

Peptide agonists usually bind to their cognate receptor in a sequential two-step mechanism (Monteclaro and Charo, 1996). The first step requires high-affinity interactions with extracellular loop regions, whereas the second step is mediated by low-affinity interactions with the helix bundle, promoting receptor activation. Based on our findings, the first step is not required for aGPCRs, because the activating peptide is part of the receptor’s own ECD and therefore covalently bound to the 7TM. In the second step of our model of aGPCR activation, the Stachel sequence is predicted to interact with extracellular loops and upper helix bundles as in other peptide/peptide-GPCR pairs (Thompson et al., 2012), which requires a low affinity. This model is also consistent with protease-activated receptors in which thrombin cleaves the receptor’s N terminus and exposes an activating tethered agonist (Vu et al., 1991).

Large ECDs are not unique to the aGPCR family. The ectodomains of glycoprotein hormone receptors (the rhodopsin-like GPCR class) are also composed of several hundred aa forming leucine-rich repeat domains. In glycoprotein hormone receptors, a conserved module termed the hinge region (Sangkuhl et al., 2002) connects the ECD to the 7TM in a manner similar to that observed for the GPS domain in aGPCRs. Although the interspaced hinge region does not share the predicted 3D structural identity with the GPS motif, both the hinge region and the GPS motif possess multiple disulfide bonds that form at least two loops of the polypeptide chain (Arac¸ et al., 2012). Interestingly, hinge-region mutations of glycoprotein hormone receptors can activate these rhodopsin-like GPCRs, suggesting an “intramolecular agonistic unit” (Krause et al., 2012). Similarly, mutations in Cys775, Cys794, Cys807, and Cys809 of GPR126, which normally form disulfide bridges in the GAIN domain, displayed constitutive activity in cAMP assays (Figures S4E and S4F). These data provide further evidence that structural changes in the GPS region promote activation via the Stachel sequence.

Our results are compatible with an activation scenario of aGPCRs in which an intramolecular agonistic domain (the Stachel sequence) is unmasked upon structural changes of the ECD, which subsequently triggers 7TM-mediated activation of G protein-signaling cascades (Figures S1B [cis signaling] and S4G). Recent evidence indicates that the ECD of GPR126 and other aGPCRs can mediate biological functions independently of the 7TM (trans signaling) (Patra et al., 2013; Prömél et al., 2012). Our discovery may facilitate attempts to distinguish between trans- and cis-dependent functions; for example, pheno- typic perturbations in model organisms through peptide agonists could be attributed to cis signaling of the receptor (e.g., Figures 4F–4K). Our study defines a signaling modality for aGPCRs and lays the foundation for rational ligand design to promote a deeper understanding of the physiology and therapeutic usefulness of this emerging class of GPCRs.

**EXPERIMENTAL PROCEDURES**

aGPCR Constructs and Functional Assays

Epitope-tagged, full-length human aGPCR sequences were inserted into pcDps, and mutant aGPCRs were generated by PCR (Table S1). For functional assays, transfected COS-7 cells were split into 48-well plates and cAMP concentrations were determined with the Alpha Screen cAMP assay kit (PerkinElmer Life Sciences) according to the manufacturer’s protocol. To measure label-free receptor activation, a DMR assay (Epic biosensor kit (PerkinElmer Life Sciences) with COS-7 cells endogenously expressing GPR126 was performed as previously described (Schröder et al., 2010). To estimate cell-surface and total expression of receptors carrying N-terminal HA and C-terminal FLAG tags, ELISA was used (Schöneberg et al., 1998). Assay data were analyzed with GraphPad Prism version 6.0 for Windows (GraphPad Software) and statistical details are given in each figure legend.

Peptide Synthesis

Solid-phase peptide synthesis of the peptides was performed on an automated peptide synthesizer (MultiPep; Intavis AG) using standard Fmoc chemistry.

Zebrafish Studies

Adult zebrafish were maintained in the Washington University Zebrafish Consortium facility in accordance with institutional animal protocols (http://zebrafish.wustl.edu/husbandry.htm). Embryos were collected from heterozygous gpr126 mutant adults, and mutant larvae were compared with WT siblings for all assays. See the Supplemental Experimental Procedures for details on TALEN mutagenesis, in situ hybridization, transmission electron microscopy, and peptide treatment.
Figure 4. Tethered Agonistic Peptides Function In Vivo

(A) Sequences of WT and stl215 alleles. stl215 is characterized by a 6 bp in-frame deletion, which results in the removal of aa Gly831 and Ile832. The BtsCl restriction enzyme site targeted by the TALEN is underlined.

(B) Schematic representation of Gpr126 showing the stl215 allele compared with st49 and st63 alleles.

(C) Dorsal view of 4 days postfertilization (dpf) larvae. Arrowheads indicate normal ear morphology in the gpr126+/+ larva (WT) and swollen ears in the gpr126stl215/stl215 larva (stl215).

(D) Lateral view of whole-mount in situ hybridization (WISH) of zf larvae at 4 dpf. The posterior lateral line nerve (PLLn) is marked with an arrow; mbp expression in the CNS is indicated with an arrowhead. mbp expression can be observed in the CNS, but not in the PLLn of gpr126sf215/stl215 mutant larvae (st215).

(E) Quantification of the swollen ear phenotype and PLLn mbp expression out of the total number of larvae scored per genotype (WT = gpr126+/+ and gpr126stl215/+).

(legend continued on next page)
Additional details regarding the methods used in this work are available in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.036.

AUTHOR CONTRIBUTIONS

I.L., J.S., L.F., K.-U.S., L.M.D., and T.S. performed the in vitro experiments. S.C.P., N.A., A.M., and K.R.M. performed the zebrafish experiments and S.C.P. and N.A. analyzed the results. I.L., J.S., L.F., and T.S. analyzed the data. S.R. synthesized the peptides. I.L. and T.S. designed the study and wrote the paper with contributions from all of the authors.

ACKNOWLEDGMENTS

We thank Xianhua Piao and Thue W. Schwartz for their very helpful comments and discussion of the paper. We thank Marilyn Levy and Robyn Roth for assistance with transmission electron microscopy. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 610 to T.S. and FOR 2149 to I.L. and T.S.), the BMBF (IFB Adiposity Diseases Leipzig to J.S. and I.L.), the NIH/NINDS (F32NS087786 to S.C.P. and R01NS079445 to K.R.M.), the Boehringer Ingelheim Fonds (MD stipend to N.A.), and the University of Leipzig (Formel 1 startup grant to I.L.).

Received: June 30, 2014
Revised: October 10, 2014
Accepted: November 22, 2014
Published: December 18, 2014

REFERENCES


#J Wish of 5 dpf larvae showing mbp expression in the CNS (arrowhead) and PLLn (arrow). (F) Scoring rubric for PLLn mbp expression; enlarged panels show the PLLn-only key. “Strong” = strong and consistent mbp expression, “some” = weak but consistent mbp expression, “weak” = weak and patchy mbp expression, “none” = no mbp expression. (G–J) The WT larvae treated with DMSO (G) or 100 μM p16 (H) have strong PLLn mbp expression. DMSO-treated gpr126emb153/emb153 mutants have reduced PLLn mbp expression (I), which is significantly rescued with peptide treatment (J).

(K) Quantification of WISH experiments. Bars indicate the proportion of larvae with each PLLn expression (I), which is significantly rescued with peptide treatment (J). **p < 0.0001, combined gpr126emb153/emb153 mutants with “some” and “strong” versus combined gpr126emb153/emb153 mutants with “weak” and “none”; Fisher’s exact test. WT = gpr126emb153/emb153 and gpr126emb153/emb153 siblings of gpr126emb153/emb153 mutants. n = 3 technical replicates, n = 105 WT (51 DMSO-treated, 54 peptide-treated), n = 53 gpr126emb153/emb153 (21 DMSO-treated, 32 peptide-treated), and n = 8 gpr126emb153/emb153 (4 DMSO-treated, 4 peptide-treated).

See also Figures S3 and S4.


