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Identification of the tethered peptide agonist of the adhesion G protein-coupled receptor GPR64/ADGRG2

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ABSTRACT

The epididymis-specific adhesion G protein-coupled receptor (aGPCR) GPR64/ADGRG2 has been shown to be a key-player in the male reproductive system. As its disruption leads to infertility, GPR64 has drawn attention as potential target for male fertility control or improvement. Like the majority of aGPCRs GPR64 is an orphan receptor regarding its endogenous agonist and signal transduction. In this study we examined the G protein-coupling abilities of GPR64 and showed that it is activated through a tethered agonist sequence, which we have previously identified as the *Stachel* sequence. Synthetic peptides derived from the *Stachel* region can activate the receptor, opening for the first time the possibility to externally manipulate the receptor activity.

1. Introduction

Among the superfamily of G protein-coupled receptors (GPCRs) the class of adhesion GPCRs (aGPCRs) is the second largest [1,2], yet the most neglected one. Although increasing information about their relevance is available from gene-deficient animals [3–5], human diseases [6,7] and variant-associated phenotypes [8–10] surprisingly little is known about the molecular function of aGPCRs. With up to 6300 amino acids aGPCRs are among the largest proteins in nature composed of a long extracellular domain (ECD), a seven-transmembrane domain (7TM) and an intracellular C-terminal tail (ICD) [11,12]. A hallmark of this receptor group is the highly conserved GPCR autoproteolysis inducing (GAIN) domain which contains the GPCR proteolysis site (GPS) where the receptor is processed into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (Fig. 1A).

The mode of signal transduction is an essential piece in understanding the receptor function but this is still unsolved for most aGPCRs. Until recently it was uncertain whether aGPCRs couple to G proteins at all. Recently, more direct evidence for G_s-protein coupling was provided measuring intracellular cAMP levels induced by basal activity of the aGPCRs GPR133 and GPR126 [13–15]. Increased receptor activity was described after autoproteolytic cleavage at the GPS and removal of the resulting NTF [16–18] leading to the assumption that the NTF contains an inverse agonist. Using these active CTF mutants fused with the N terminus of the human $P2Y_{12}$ receptor to ensure membrane expression (Fig. 1A) we have shown that GPR126 and GPR133 contain a tethered peptide agonist in the very N terminus of the CTF [19]. Peptides derived from this region, called *Stachel* sequence, were able to activate G protein-mediated signal transduction *in vitro* and *in vivo*. Recently, the concept of a tethered peptide agonist was independently confirmed on the aGPCR GPR56 and GPR110 [20].

Expression of the orphan GPR64/ADGRG2 is normally restricted to the epididymis [21] where it is essential for maintaining male fertility [22,23]. This observation has sparked marked interest in this receptor as a potential target for male contraception [24,25] or fertility improvement. GPR64 was found to be over-expressed in human cancer as in Ewing's sarcoma, where it increases malignancy of the tumor [26,27]. Therefore, identifying the mode of signaling and modulators of GPR64 activity is of high interest. Here, we characterize the G protein-mediated signal transduction of GPR64 and describe a *Stachel* sequence-derived agonistic peptide.

Abbreviation: 7TM, seven-transmembrane spanning domain; CTF, C-terminal fragment; ECD, extracellular domain; eV, empty vector; DMEM, dulbecco minimum essential medium; ELISA, enzyme-linked immunosorbent assay; GAIN, GPCR autoproteolysis inducing; GPCR, G protein-coupled receptors; GPS, GPCR proteolysis site; HA, hemagglutinin epitope; ICD, intracellular domain; NTF, N-terminal fragment; SP, signal peptide; TM, transmembrane helix; wt, wild type.

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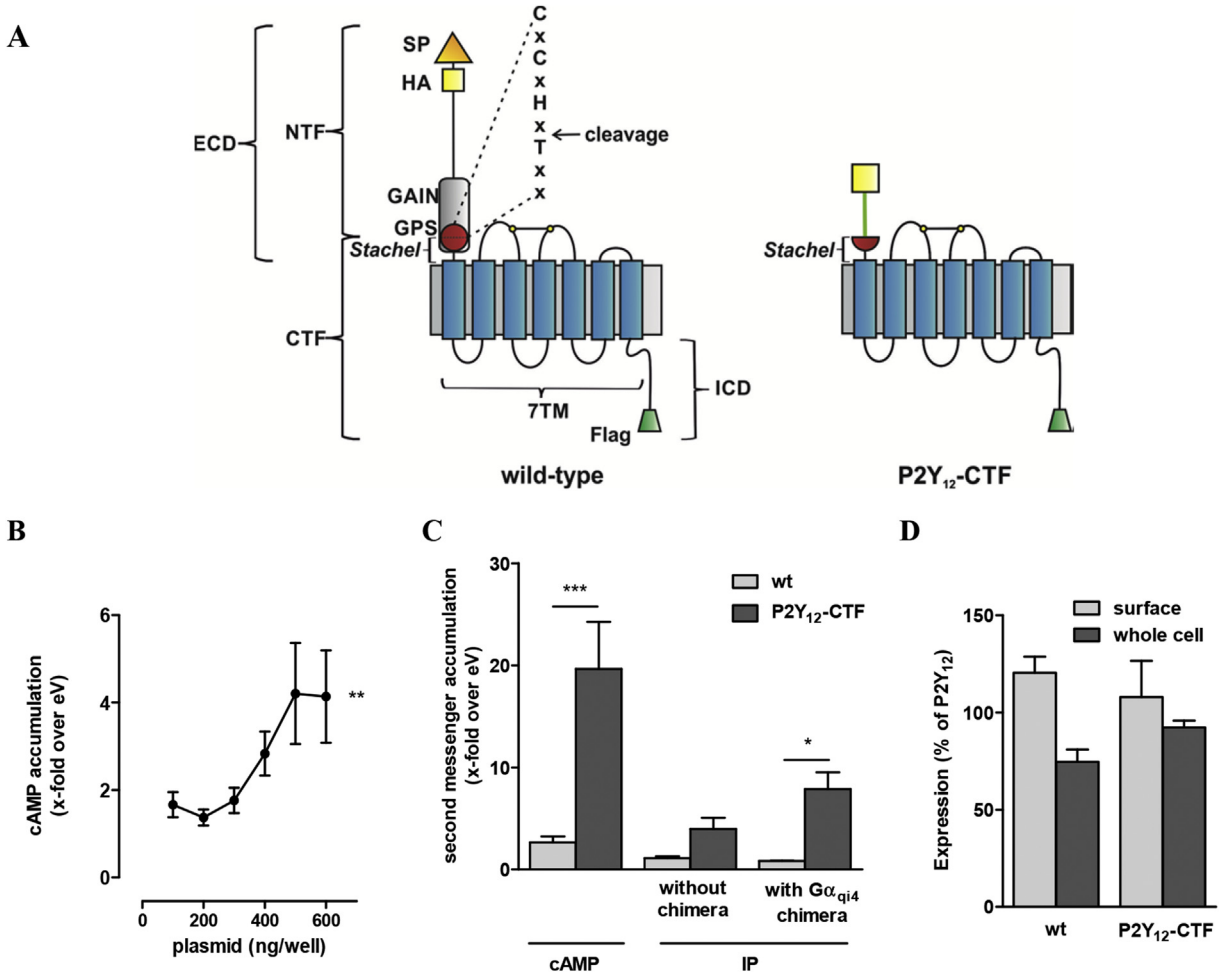

Fig. 1. Schematic structure of wt and chimeric aGPCR structure and their basal signal transduction. (A) The structural architecture of a prototypic aGPCR is shown. In general, one can distinguish an extracellular domain (ECD) with a signal peptide (SP, yellow triangle) and the GAIN (gray rectangle)/GPS (red circle) domain, the 7TM domain and the intracellular domain (ICD). The previously reported *Stachel* sequence is located between the cleavage site and TM1. Autocatalytic-cleavage at the GPS results in an N-terminal fragment (NTF) and a C-terminal fragment (CTF). For immunological detection all constructs were epitope-tagged with an N-terminal HA epitope (yellow square) and a C-terminal Flag epitope (green trapezoid). Further, the global structure of the chimeric $P2Y_{12}$-CTF constructs is depicted. HA- and Flag epitope are marked as yellow square and green trapezoid, respectively. The half red circle symbolizes the GPS at its cleavage site. A green line represents the N terminus of the human $P2Y_{12}$ receptor fused onto the given aGPCR mutant. (B) COS-7 cells were transfected with increasing amounts of wt GPR64 plasmid. Basal cAMP levels were determined as described in *Methods*. Data are given as x-fold over empty vector (eV), which served as negative control (pcDps; cAMP level: 3.68 ± 2.54 nM/well). Statistics were performed analyzing linear regression for each curve: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (C) COS-7 cells were transfected with 500 ng for cAMP assay or 1500 ng for IP assay of the given wt and CTF constructs and 100 ng of the chimeric $G\alpha_{qi4}$. Basal cAMP and IP levels were determined as described in *Methods*. Empty vector served as negative control (for IP: pcDps level: 429 ± 182 cpm/well, pcDps + $G\alpha_{qi4}$: 357 ± 150 cpm/well; for cAMP: pcDps level: 11.37 ± 2.67 nM/well). Data are given as x-fold over eV. (D) For expression studies, ELISAs were used to measure cell surface and total cellular expression levels of wt and CTF mutants. COS-7 cells were transfected with 500 ng of CTF constructs. Specific optical density (OD) readings (OD value of double HA/Flag-tagged aGPCR constructs minus OD value of mock-transfected cells) are given as percentage of the human $P2Y_{12}$ receptor, which served as positive control. For the cell surface ELISA, the non-specific OD value (pcDps = empty vector, eV) was 0.03 ± 0.03 (set 0%) and the OD value of $P2Y_{12}$ was 1.30 ± 0.24 (set 100%). OD readings of 0.08 ± 0.04 (set 0%) and 2.22 ± 0.73 (set 100%) were found in sandwich ELISA (total expression) for the negative control (eV) and positive control ($P2Y_{12}$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Materials

If not stated otherwise, all standard substances were purchased from Sigma–Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), and C. Roth GmbH (Karlsruhe, Germany). Cell culture material and primers were obtained from Invitrogen (Darmstadt, Germany).

2.2. Methods

Generation of wild type and mutant GPR64 constructs - Full-length mouse GPR64 (isoform 4: NM_001079848.1) sequence was amplified from mouse testis cDNA library (primer: forward 5′cacacggagtttcctcccta-′/reverse 5′-tcctttcgaggttgctgaat-3′), and directly cloned into the mammalian expression vector pcDps [28]. For detection purposes a hemagglutinin (HA) epitope was inserted directly downstream the predicted signal peptide (SignalP 4.1 server; http://www.cbs.dtu.dk/services/SignalP) of GPR64 by a PCR-based site-directed mutagenesis and fragment replacement strategy. Further, a Flag epitope was introduced at the very C terminus.

Receptor chimeras and changes in receptor architecture were generated by PCR and homologous recombination in *E. coli* from Invitrogen (Darmstadt, Germany). The correctness of the sequences of wild type (wt) and derived constructs were verified by sequencing.

Functional assays – GPR64 constructs were heterologously expressed in COS-7 cells grown in Dulbecco's minimum essential

medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. For assays cells were split into 12-well plates (1×10^5 cells/well, for inositol phosphate (IP) assay) and 48-well plates (3×10^4 cells/well for cAMP assay) and transfected with Lipofectamine™ 2000 (Invitrogen, Paisley, UK) according to the manufacturer's protocol. For 48-well and 12-well plates 600 ng and 1500 ng, respectively, of plasmid DNA/well were used. To measure IP formation, transfected COS-7 cells were incubated with 2 μCi/ml myo-[^{3}H]inositol (18.6 Ci/mmol, PerkinElmer Life Sciences) for 16 h. Thereafter, cells were washed once with serum-free DMEM containing 10 mM LiCl followed by incubation with serum-free DMEM containing 10 mM LiCl. Empty vector (eV) transfected cells stimulated with 10 μM of forskolin and the M3 muscarinic acetylcholine receptor (M3R) [29] transfected cells stimulated with 10 μM of carbachol, for 1 h at 37 °C served as positive controls for the cAMP and IP assays, respectively. Intracellular IP levels were determined by anion-exchange chromatography as previously described [30]. For cAMP measurements, 48 h after transfection, cells were incubated with 3-isobutyl-methyl-xanthine (1 mM)-containing medium. Cells were lysed in LI buffer (PerkinElmer Life Sciences, Monza, Italy) and kept frozen at −20 °C until measurement. To measure cAMP concentration, the Alpha Screen cAMP assay kit (PerkinElmer Life Sciences) was used according to the manufacturer's protocol. The accumulated cAMP was measured in 384-well white OptiPlate microplates (PerkinElmer Life Sciences) with the Fusion AlphaScreen multilabel reader (PerkinElmer Life Sciences). Second messenger assay data was analyzed using GraphPad Prism version 6.0 for Windows (GraphPad Software). To estimate cell surface expression of receptors carrying an N-terminal HA tag, an indirect cellular enzyme-linked immunosorbent assay (ELISA) was used [31]. To assess the amounts of full-length HA/Flag double-tagged constructs in the cell a sandwich ELISA was performed as previously described [32].

Peptide synthesis – Solid phase peptide synthesis of the peptides was performed on an automated peptide synthesizer MultiPep from Intavis AG (Köln, Germany) using standard Fmoc-chemistry. The final side chain deprotection and cleavage from the solid support employed a mixture of TFA, water and thioanisole (95:2.5:2.5, Vol %) for the peptides. The peptides were purified to >95% purity using preparative RP-HPLC (Shimadzu LC-8, Duisburg, Germany) equipped with a PLRP-S column (300 × 25 mm, Agilent, Waldbronn, Germany). For both analytical and preparative use, the mobile phases were water (A) and acetonitrile (B), respectively, each containing 0.1% TFA. Samples were eluted with a linear gradient from 5% B to 90% B in 30 min for analytical runs and in 90 min for preparative runs. Finally, all peptides were characterized by analytical HPLC (Agilent 1100) and MALDI-MS (Bruker Microflex LT, Bremen, Germany), which gave the expected $[M+H]^+$ mass peaks.

Statistics and data presentation – If not indicated otherwise, all data are given as means ± SEM of three independent experiments each performed in triplicates. Statistics were performed by applying a one-way ANOVA in combination with Bonferroni as post-hoc test: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

Wildtype and chimeric GPR64 show increased basal activity in G protein-dependent pathways – Clarifying the signal transduction of an unknown GPCR is a central issue in its deorphanization. Despite many efforts over the past years the signaling mode of many aGPCRs including GPR64 remained enigmatic. The enormous size of aGPCRs and the absence of agonists have long hampered efforts to characterize the signaling modes of this receptor class. Based on the commonly accepted concept in which GPCRs exist in an equilibrium between active and inactive conformations [33] it is possible to detect intracellular signaling of GPCRs even in the absence of an agonist by overexpressing them [34,35]. Additionally, the observation that CTF-mutants of aGPCRs display increased receptor activity can be used to analyze the signal transduction pathways. Therefore, we used overexpression approaches to identify basal signal transduction of wt and mutant GPR64 constructs (Fig. 1A) by expressing them in COS-7 cells. Taken that constitutive GPCR activity should directly correlate with cell expression levels we applied receptor amount titration experiments. Increasing amounts of transfected wt GPR64 led to a significant increase in cAMP levels (Fig. 1B). However, no significance was achieved in inositol phosphate (IP) accumulation assay with or without co-transfection of a chimeric $G\alpha_{qi4}$ protein, which can redirect Gi-coupled receptors to the PLC-β/inositol phosphate pathway [36] (data not shown). When comparing wt to the chimeric $P2Y_{12}$-CTF GPR64 mutant in the same experimental setup, the mutant showed significant increase in cAMP levels and IP accumulation upon co-transfection with the $G\alpha_{qi4}$ chimera indicative of a promiscuous coupling to Gs and Gi (Fig. 1C). This increase in activity cannot be explained by a similar increase in cell membrane expression (Fig. 1D). Assuming that the CTF mutant of GPR64 also represents the activated form of the receptor the following two conclusions can be drawn. First, GPR64 can signal through several second messenger pathways most likely downstream of Gs and Gi and second, the constitutive activity of the CTF mutant is likely mediated through a tethered peptide agonist, called the *Stachel* sequence, as has been shown for other aGPCRs [19,20].

The aGPCR GPR64 is activated through a tethered peptide agonist – A peptide library derived from the homologous *Stachel* sequence, varying in length from 11 to 20 amino acids was tested in cAMP assays. All peptides started with the +1 position after the natural cleavage site and were then sequentially C-terminally truncated (Fig. 2). A peptide with the length of 15 amino acids (p15) induced highest cAMP accumulation (Fig. 2A) and was therefore chosen for further studies. This observation corresponds to a high conservation of the GPR64 *Stachel* sequence among mammalian orthologs (Fig. 2B), which will likely allow for cross-species activation of this peptide agonist, as it has been shown for GPR126 between human and zebrafish orthologs [19]. Peptides of a length between 13 and 17 amino acids exhibited similar activation potential as p15 indicating that the activating portion of p15 actually lies within the first 13 amino acids. Concentration-response curves with p15 showed an EC_{50} value of 77.2 μM (Fig. 2C) which is considerably lower than EC_{50} values found for GPR126 and GPR133 (>400 μM) in the same second messenger assay [19]. Since GPR64 has been reported to couple to Gq proteins [22] we tested p15-induced activation in IP accumulation assay although we found no basal activity in this pathway (Fig. 1C). A relatively low amount of p15, 500 μM, was sufficient to induce significant IP accumulation in GPR64-transfected cells (Fig. 2D). This indicates that synthetic peptides can induce activation more efficiently than deletion of the NTF or overexpression of the wt. Therefore, the complete repertoire of signaling abilities may become only visible following proper agonist stimulation. Activation through p15 is specific since no significant cAMP increases were observed exposing GPR133- or GPR110-transfected cells and empty vector-transfected cells to p15 (Fig. 2E).

4. Discussion

GPR64 is a receptor with strong clinical implications as it is tightly linked to male fertility and cancer progression. Considering that GPCRs are in general ideal targets for pharmaceutical

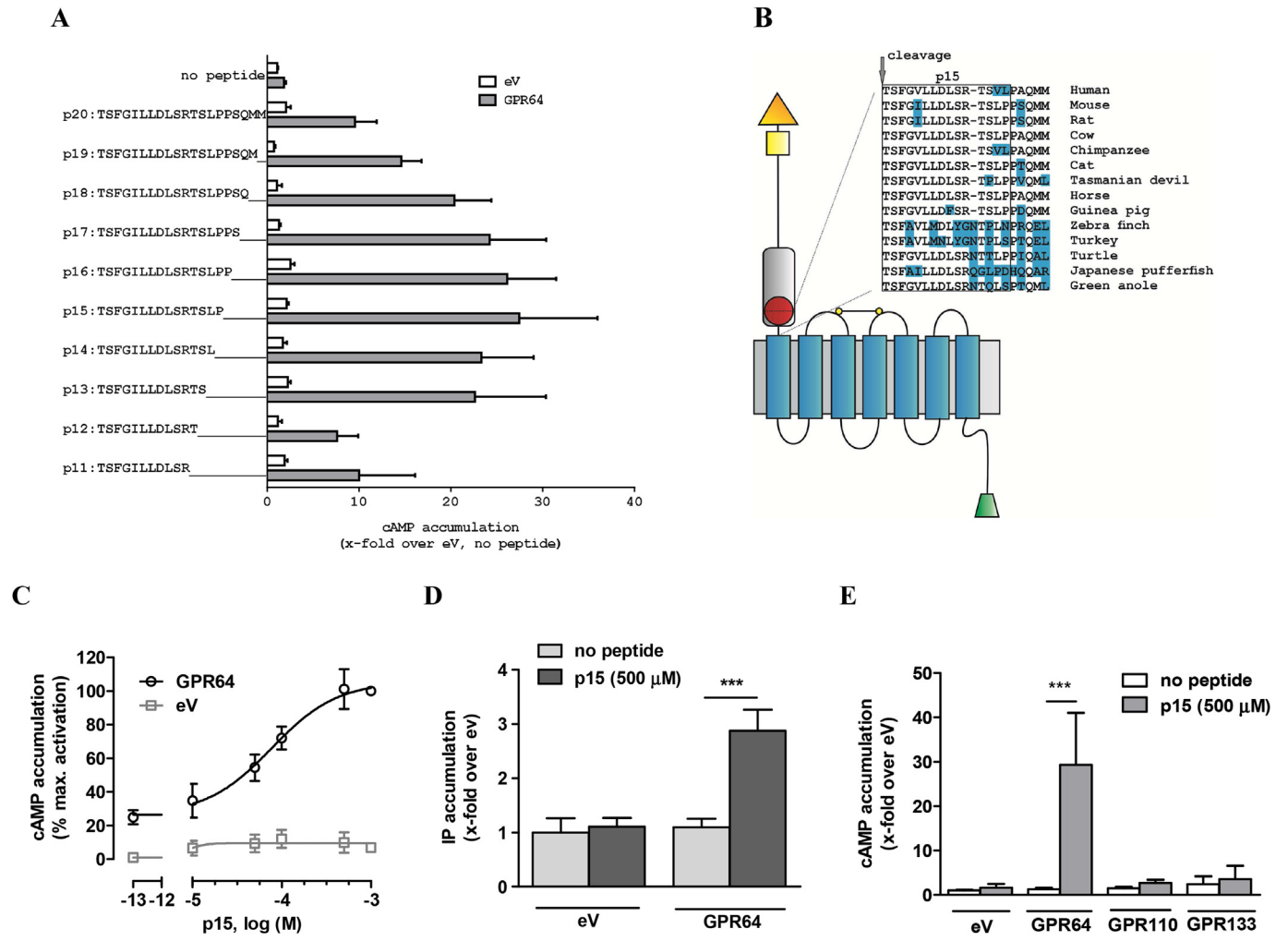

Fig. 2. GPR64 is activated by peptides derived from a tethered agonist sequence (*Stachel*). (A) Application of peptides (1 mM) of different lengths derived from the highly conserved C-terminal part of the GPS beginning at the cleavage site of GPR64 revealed agonistic properties as measured by cAMP accumulation. The highest agonistic efficacy was detected for a peptide containing 15 amino acids (p15). Empty vector (eV) served as negative control and data is shown as x-fold over eV. Basal cAMP levels were 50.3 ± 13.9 nM/well. (B) Alignment of representative ortholog sequences shows high conservation of the region p15 is derived from. The sequences were taken from UniProt (www.uniprot.org): human (*Homo sapiens*) Q8IZP9, mouse (*Mus musculus*) Q8CJ12, rat (*Rattus norvegicus*) Q8CJ11, cow (*Bos taurus*) F1MRZ3, chimpanzee (*Pan troglodytes*) H2R2N5, cat (*Felis catus*) M3WP79, Tasmanian devil (*Sarcophilus harrisii*) G3VGM9, horse (*Equus caballus*) F6YN77, Guinea pig (*Cavia porcellus*) H0VID5, Zebra finch (*Taeniopygia guttata*) H0ZBF4, turkey (*Meleagris gallopavo*) G1NP98, turtle (*Pelodiscus sinensis*) K7FFN2, Japanese pufferfish (*Takifugu rubripes*) H2RXI7, Green anole (*Anolis carolinensis*) G1KLX3. (C) Different p15 concentrations were tested on eV and wt GPR64 transfected cells. Concentration-response curve of p15 at wt GPR64 revealed an EC50 value of 77.2 µM. Basal eV levels were 6.0 ± 0.6 nM/well (D) COS-7 cells were transfected with 500 ng of eV and of wt GPR64 constructs. Basal IP levels were determined as described in *Methods*. Addition of 500 µM of p15 led to a 2.6-fold increase in GPR64-mediated IP levels. Empty vector served as negative control (pcDps: level: 766.3 ± 203.0 cpm/well). Data are given as x-fold over eV as means ± SEM of three independent experiments performed in duplicates. (E) COS-7 cells were transfected with pcDps (eV), GPR64, GPR110 and GPR133, respectively, and cAMP accumulation was determined as stimulated response in x-fold over eV with 500 µM of GPR64-specific p15. Empty vector served as negative control (eV: 34.0 ± 3.7 nM/well). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment, deciphering the signal transduction and activation mode of GPR64 is a decisive step towards future therapeutic applications. We show that GPR64 signals via downstream effectors of Gs and Gi in addition to its already identified Gq-protein coupling [22]. However, in our experimental setup the full length receptor shows only elevated activity in cAMP production, while IP formation depends on overexpression of the CTF mutant. Multiple coupling abilities are common among GPCRs [37] and dissecting the relevance of activating the individual pathways is an ongoing task in GPCR research [38].

Activation of GPR64 can be also achieved through peptides that are derived from the encrypted tethered agonist sequence of the *Stachel* region. The most efficient peptide, p15, displays a relatively low EC_{50} value when compared to GPR126-and GPR133-derived peptides in the same assay. p15 does not activate control aGPCRs (GPR110, GPR133); thereby displaying apparent specificity for the receptor it originated from. In contrast to other peptidergic GPCRs, *Stachel* sequence-derived peptides have an apparently low affinity to the 7TM. One can assume that, because of the covalent integration of the *Stachel* sequence, the 1:1 stoichiometry between the agonist and the 7TM compensates for low affinity. Currently, two models are discussed how the *Stachel* sequence triggers 7TM activation: 1. Structural changes in the ectodomain upon ligand binding expose the *Stachel* sequence to the 7TM and 2. the *Stachel* sequence preoccupies the 7TM binding pocket and isomerizes into an activating structure upon ligand binding to the ectodomain. Indeed, activating ligands, e.g. collagen and laminin, have been identified for other aGPCRs [39,40]. The identification of an extracellular ligand for GPR64 and, therefore, the trigger exposing or isomerizing the GPR64 *Stachel* sequence awaits discovery.

In sum, GPR64 is yet another aGPCR that is activated through *Stachel* sequence-derived peptides and signals via multiple G protein-signaling cascades. It is very likely that this is a general activation mechanism for the whole receptor class.

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