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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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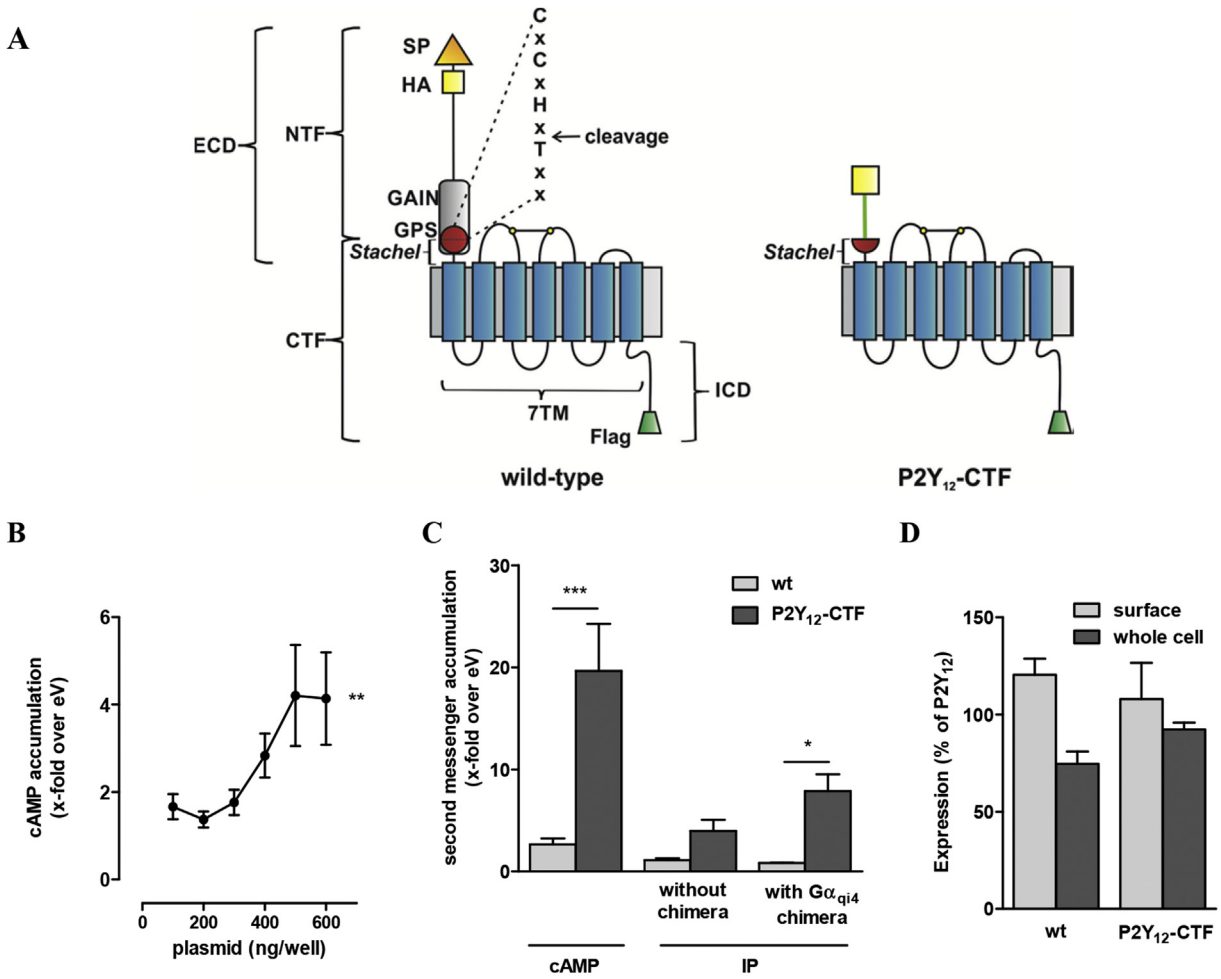

Fig. 1. Schematic structure of wt and chimeric aGPCR structure and their basal signal transduction. (A) The structural architecture of a prototypic aGPCR is shown. In general, one can distinguish an extracellular domain (ECD) with a signal peptide (SP, yellow triangle) and the GAIN (gray rectangle)/GPS (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

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