**Cell Adhesion Receptor GPR133 Couples to Gs Protein**

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Adhesion G protein-coupled receptors (GPCR), with their very large and complex N termini, are thought to participate in cell-cell and cell-matrix interactions and appear to be highly relevant in several developmental processes. Their intracellular signaling is still poorly understood. Here we demonstrate that GPR133, a member of the adhesion GPCR subfamily, activates the Gs protein/adenyl cyclase pathway. The presence of the N terminus and the cleavage at the GPCR proteolysis site are not required for G protein signaling. Gs protein coupling was verified by Goα knockdown with siRNA, overexpression of Goα, co-expression of the chimeric Goα protein that routes GPR133 activity to the phospholipase C/inositol phosphate pathway, and missense mutation within the transmembrane domain that abolished receptor activity without changing cell surface expression. It is likely that not only GPR133 but also other adhesion GPCR signal via classical receptor/G protein-interaction.

Adhesion receptors comprise the second largest subfamily of putatively G protein-coupled receptors (GPCR) with more than 30 members in vertebrates (1, 2). Adhesion GPCR are characterized by long extracellular N termini, which are composed of multiple functional domains, a seven-transmembrane spanning (7TM) domain, and a cytoplasmic tail. Adhesion GPCR are believed to play a role in immune functions (3, 4), angiogenesis (5), cell polarity (6, 7), and development (8, 9).

Mutations in some members of the protein family were identified as responsible for mutations in congenital and complex diseases (10–12). Usher syndrome (VLGR1) (10) and bilateral frontoparietal polymicrogyria (GPR56) (11). Although there is consensus on the fact that this receptor class mediates essential cell-cell and cell-matrix interactions (1, 12), the molecular mechanism of intracellular signal transduction of adhesion GPCR remains obscure.

There are only a few studies on intracellular signaling mechanisms of adhesion GPCR. Latrophilin 1, the prototype of adhesion GPCR, induces intracellular Ca2+ signaling upon interaction with the exogenous ligand α-latrotoxin (13, 14). GPR56 appears to activate the G12/13 protein/Rho pathway after stimulation with an antibody against the ectodomain (15). BAII recognizes phosphatidylserine and can directly recruit a Rac guanine nucleotide exchange factor (Rac-GEF) complex to mediate the uptake of apoptotic cells (16). The cytoplasmic domain of BAII interacts with GA-binding protein γ, and GA-binding protein-α/γ or GA-binding protein-α/β work as transcriptional repressors of VEGF (17). However, clear evidence of intracellular signaling for most adhesion GPCR via G proteins is still missing (12).

Genetic variations in the GPR133 gene, also a member of the adhesion GPCR family, were associated with adult height (18) and the RR interval duration in electrocardiograms (19). GPR133 is expressed in CNS (20) and other tissues; its endogenous agonists and the signal transduction are unknown. Here we demonstrate that GPR133 is coupled to the Gs protein/adenyl cyclase pathway. This proves that this adhesion receptor is indeed a G protein-coupled receptor.

**EXPERIMENTAL PROCEDURES**

**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).

**Methods**

**Generation of Wild-type GPR133 and Mutants**—Full-length human (NM_198827) and mouse (BC158001) GPR133 sequences were directly cloned from human monocytes and mouse urinary bladder cDNA libraries (primers: human forward, ACTTGGCTCCGAGCCTGAC, and reverse, CAAAGGTGGGCACTTACATT; and mouse forward, AGAAGTTCCCCCTGAAGCTTTGAC, and reverse, TCTGCTCCAGGGACACT) and inserted into the mammalian expression vector pCDs. Human and mouse GPR133 (see Fig. 1A) were N-terminally tagged after the initial signal peptide at amino acid position 31 with a hemagglutinin (HA) epitope followed by a sequence encoding the N-terminal 20 amino acids of bovine rhodopsin N terminus (as described in Ref. 21) and C-terminally with a FLAG epitope by a PCR-based site-directed mutagenesis and fragment replacement strategy.

**Assays to Determine GPR133 Function**—COS-7 cells were grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 7% CO2 in a humidified atmosphere. Cells were split into 12-well plates (105 cells/well, for inositol phosphate (IP) assay) and 48-well plates (3 × 104 cells/well for cAMP assay) and transfected with Lipofectamine™ 2000 (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. To measure IP formation, transfected COS-7 cells were incubated with 2 μCi/ml myo-[3H]inositol
Intracellular IP levels were determined by anion-exchange chromatography as described (22). IP accumulation data were analyzed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). For cAMP measurements, 48 h after transfection, cells were incubated with 3-isobutyl-methyl-xanthine (1 mM)-containing medium for 1 h. Incubation was stopped by washing with ice-cold PBS. Cells were lysed in Ll buffer (PerkinElmer Life Sciences, Monza, Italy) and 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) for 1 h at 37 °C. The protocol was also used for transient transfection. One day prior to transfection, cells were split into 96-well cell culture plates (2.5 × 10^4 cells/well), and 24 h later, cells were co-transfected (75 ng of each) with the GPR133 expression plasmid and the CRE-SEAP reporter plasmid (Clontech, Saint-Germain-en-Laye, France). Lipofectamine™ 2000 (Invitrogen) was used for transient transfection. One day after transfection, serum-free DMEM or serum-free DMEM with 5 mM streptomycin at 37 °C in a humidified 7% CO_2 incubator. Incubation was stopped by washing with ice-cold PBS. Cells were lysed in Ll buffer (PerkinElmer Life Sciences, Monza, Italy) and frozen at −20 °C until measurement.

To estimate cell surface expression of receptors carrying an N-terminal HA tag, we used an indirect cellular enzyme-linked sandwich ELISA was used and performed as described previously (24).

RESULTS AND DISCUSSION

GPR133 Displays Increased Basal Activity in the G Protein/Adenylyl Cyclase Pathway—According to the current model of GPCR function (25, 26), receptor overexpression can result in a constitutive activation of signaling pathways, which are normally activated after agonist stimulation (27–30). Thus, the coupling abilities of several receptors, including “orphan” receptors, have been characterized by overexpression in the absence of an agonist (31–33). For example, the wild-type adenyly cyclase constitutive activator (ACCA), an orphan GPCR, stimulates the Gi protein/adenyl cyclase system to some extent when expressed in COS-7 cells (31). Following this strategy, the human and mouse GPR133 were transiently expressed in HEK293 cells and tested in AP1-, nuclear factor of activated T-cells (NFAT)-, serum responsive element (SRE)-, and CRE-SEAP reporter gene assays. Interestingly, GPR133-transfected cells displayed elevated basal activities when compared with mock-transfected cells in the CRE-SEAP reporter assay (Fig. 1B), indicating that GPR133 activity may increase intracellular cAMP levels. After the initial screen in reporter gene assays, we focused on the signal transduction of the human GPR133 in further functional experiments. First, to verify the basal activation of the Gi pathway, we directly measured cAMP formation in COS-7 cells transfected with the human GPR133. Indeed, cAMP formation was significantly increased in cells transfected with GPR133 (~4-fold) when compared with mock-transfected COS-7 cells (Fig. 1, C and D). Second, because constitutive GPCR activity directly correlates with cell surface expression levels (34), we performed receptor amount titration experiments. As shown in Fig. 1C, increasing amounts of transfected GPR133 plasmid produced increased cell surface expression and cAMP levels, proving that cAMP levels depend on expression of the constitutively active GPR133.

Structural Requirements for G Protein Coupling of GPR133—To control whether the high basal activity of the human GPR133 is specific, several GPR133 mutants were generated and tested in functional assays. Mutations were generated at amino acid positions highly conserved during GPR133 evolution, and we therefore speculated that mutation of these residues may have an effect on receptor function. In contrast to several other mutations that abolished GPR133 basal activity (data not shown), L808T did not significantly affect cell surface expression (Fig. 1, D and E). This indicates that elevated cAMP levels were directly linked to the properly folded structure of the 7TM domain of GPR133. It was shown for many rhodopsin-like GPCR that mutations can induce structural changes in the 7TM domain modulating the constitutive G protein-coupling activity of the receptor (34, 35).

Many adhesion GPCR undergo a self-catalytic cleavage at the GPCR proteolysis site (GPS) to form a heterodimeric complex containing the N terminus and 7TM domain (36–39). To test whether GPS cleavage is required for G protein-signaling of GPR133, we mutated position −2 of the cleavage site (−H↓L↓T−) to an arginine. This change has been shown to abolish autocalytic activity without disrupting protein
expression on the cell surface (40). As expected, GPR133 containing H543R was expressed normally and yet displayed increased basal activity (Fig. 1, D and E). Experiments with a GPR133 where the N terminus is completely exchanged by the N terminus of bovine rhodopsin showed increased basal activity in the CRE-SEAP assay (Fig. 1B). Therefore, the presence of the N-terminal domain and the cleavage at GPS are not required for G protein coupling. These features are very similar to the group of glycoprotein hormone receptors, such as thyrotropin and lutropin receptors. Mutagenesis studies with these glycoprotein hormone receptors have shown that the large ectodomain is not required for ligand- and mutation-induced signaling of the 7TM domain (41, 42).

**G Protein Coupling Specificity of GPR133**—It has been demonstrated that replacement of the four or five C-terminal amino acids of Goq with the corresponding residues of other Go subunits confers the ability to stimulate the phospholipase C-β pathway onto e.g. Gi-coupled receptors using a Goα44 construct (43). We co-expressed GPR133 with a Goα44 construct (kindly provided by Prof. Dr. Evi Kostenis, Institute of Pharmaceutical...
Biologically, University of Bonn, Bonn, Germany) where Gα coupled receptors can be rerouted to the phospholipase C/β/inositol phosphate pathway. As expected, aMSh stimulation (10 μM aMSh) of the Gα coupled melanocortin type 4 receptor (MC4R) co-transfected with Gαq4 resulted in a robust increase in intracellular IP levels (Fig. 2). Note that MC4R-transfected cells alone produce some IP response via Gi coupling (44), whereas control siRNA had no effect (Fig. 2). This experiment clearly indicates that GPR133 must functionally interact with a G protein to mediate intracellular IP formation.

Because increased activity of cAMP-dependent protein kinase is only one of several ways to activate cAMP-response element-binding protein (CREB) (45) and some adenylyl kinase is only one of several ways to activate cAMP-response formation.

The results of these experiments demonstrate that GPR133 couples to the Gs protein, consistent with the known role of cAMP as a regulator of this function. Furthermore, known occurrences of activating Gα mutations in growth hormone-secreting pituitary tumors may provide a rationale for the association between genetic variations of GPR133 and adult height reported previously (18). The high expression of GPR133 in the pituitary gland (20) is consistent with this notion. Recently, an orphan receptor of the adhesion GPCR family, GPR126, has been shown to play an essential role in the myelination of peripheral nerves by neural crest-derived Schwann cells in the zebrafish Danio rerio (49).
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Interestingly, elevation of cAMP by forskolin in GPR126 mutants could restore myelination. It needs therefore to be tested in future experiments whether not only GPR133 but also other adhesion GPCR signal via classical receptor/G protein-interaction.

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