FEBS Letters 586 (2012) 1214–1219

journal homepage: www.FEBSLetters.org

Signaling property study of adhesion G-protein-coupled receptors

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ARTICLE INFO

Article history:
Received 9 January 2012
Revised 9 March 2012
Accepted 9 March 2012
Available online 21 March 2012

Edited by Lukas Huber

Keywords:
Adhesion
G-protein-coupled receptor
GPR97

ABSTRACT

Adhesion G-protein-coupled receptors (GPCR) are special members of GPCRs with long N-termini containing multiple domains. We overexpressed our collection of receptors together with G-proteins in mammalian cell lines and measured the concentrations of intracellular signaling molecules, such as inositol phosphate and cAMP. Our results show that a subset of tested adhesion GPCRs has constitutive activities and is capable of coupling to a variety of G-proteins. In addition, we have identified a small molecule compound that specifically activates one of the subfamily members, GPR97, and the activation was confirmed by an independent GTPγS assay. These findings suggest classical GPCR screening assays could be applied to de-orphanize these receptors, and provide pharmacological tools to improve understanding of the physiological functions of these receptors.

1. Introduction

The superfamily of G-protein-coupled receptors (GPCRs) is one of the largest and most studied families of proteins in the mammalian genome. The ligands for this superfamily are extremely diverse. GPCRs have been very successful pharmaceutical targets, with around 50% of all newly introduced drugs targeted against this superfamily [1]. There are over 800 GPCRs in the human genome, and they form five major subfamilies [1]. One rather new and peculiar subfamily of GPCRs is adhesion GPCRs. The name "adhesion" relates to the N-termini, which contain adhesion-like motifs such as EGF-like repeats, cadherin domains, or leucine-rich repeats that are likely to participate in cell–cell adhesion [1–4]. Although adhesion GPCRs share the same seven-transmembrane topology with other GPCRs [1–4], there are large variations among their long extracellular N-termini. In humans, adhesion GPCRs are expressed in a variety of tissues including the immune system, central nervous system, and endocrine organs, suggesting that they can take part in a variety of physiological functions. However, the large size and the genomic complexity of these proteins have made them difficult to study.

Signaling associated with most GPCRs has been well studied and usually involves coupling to heterotrimeric G-proteins in the cell. The activated GTP-bound α-subunit dissociates from the receptor and activates specific effectors such as adenylate cyclase and phospholipases. A majority of GPCRs couple to three types of G_{α}-proteins: $G_{\alpha s}$, $G_{\alpha i/\alpha o}$ and $G_{\alpha q}$. $G_{\alpha s}$ and $G_{\alpha i/\alpha o}$ stimulate or inhibit adenylate cyclase activity, respectively, whereas $G_{\alpha q}$ activates phospholipase C (PLC) [5]. However, the majority of adhesion GPCRs are orphans, and their coupling mechanisms are unknown. Although, CD97, one of the most studied receptors in this family, has been shown to interact with CD55 [6,7], and a glycosaminoglycan (chondroitin sulfate) has been shown recently to be a ligand for EMR2 [8], no G-protein-mediated signaling events (such as calcium mobilization or inositol phosphate production) have been reported for those two receptors. Latrophilin was able to induce intracellular calcium signaling upon binding to α-latrotoxin, but very few reports have shown any G-protein coupled signaling for any other receptors in this subfamily [9]. Adhesion GPCRs belong to a relatively new GPCR subfamily about which there is limited functional information. Identifying their signaling pathways and ligands will provide a path toward understanding their physiological functions.

In this study, we measured the constitutive activities of our in-house collection of adhesion GPCRs and performed ligand hunting for one of the receptors as a proof-of-concept study. Several receptors displayed constitutive activity in our in vitro assays. Our results provide compelling evidence that this subfamily of GPCRs can also signal through classical GPCR signaling pathways.

2. Experimental procedures

2.1. Materials

[^{35}S] guanosine 5′-[γ-thio]triphosphate (GTPγS; 1250 Ci/mmol) was purchased from Perkin–Elmer Life and Analytical Sciences (Waltham, MA). Unlabeled GTPγS was obtained from Roche Molecular Biochemicals (Indianapolis, IN). GDP was purchased from Sigma (St. Louis, MO). PTX was obtained from Calbiochem. Beclomethasone dipropionate was obtained from Prestwick

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.03.014

Chemical (France). Beclomethasone dipropionate was completely soluble in dimethyl sulfoxide (DMSO) and was stable as a 10 mM stock. The 10 mM stock solution was then subsequently diluted into media or buffer to final concentrations as indicated in the figures. The final DMSO concentration in the media of all cell-based assays was 0.1% (v/v) and in GTPγS binding assay was 1% (v/v). The same final DMSO concentrations were also maintained in no compound control cells or reactions that were set up to eliminate any potential solvent effects. No precipitation of compounds were observed either in media or in reaction buffers under the conditions described here.

2.2. Methods

2.2.1. Cloning and cell culture

Full length adhesion GPCRs were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Cellgro) containing 10% fetal bovine serum and antibiotics. HEK293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. All cell lines were cultured at 37 °C with 5% CO_2. Cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.2.2. Inositol phosphate accumulation assay

HEK293 cells seeded in 96-well plates were transfected with adhesion GPCRs (100 ng/well) and small chimeric G-proteins (G_{qi5}, G_{qi9}, G_{qo3}, G_{qo5}, $G\alpha_{15}$, $G\alpha_{16}$ or G_{myri5} 20 ng/well). After labeling with [^{3}H]myoinositol (Amersham Biosciences) for 16 h, cells were treated with Hanks' buffered salt solution, 25 mM Hepes (pH 7.4), 10 mM LiCl, 0.01% bovine serum albumin at 37 °C for 2 h. Cells were lysed using 20 mM formic acid at 4 °C for 4 h. Ysi-SPA beads (Amersham Biosciences) were added to the cell lysates and incubated overnight in the dark. Radioactivity was measured on a Topcount 96/384 scintillation counter (Packard).

2.2.3. cAMP assay

Control HEK 293 cells and HEK 293 cells transiently transfected with 100 ng/well adhesion GPCRs were transferred to 96-well plates (approx 1.0–1.5 × 10^4 cells per well). Cells were then incubated with 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. Reactions were terminated, and stimulation of adenylyl cyclase was measured using the Tropix cAMP Screen system (Applied Biosystems). For siRNA experiments, cells were co-transfected with 100 ng/well GPCR construct and 1 pmol/well $G\alpha_s$ siRNA (sc-29328, Santa Cruz) or control siRNA (sc-37007, Santa Cruz).

2.2.4. Aequorin assay

For each 10-cm CHO cells dish, 5 μg of GPR97, 5 μg of aequorin reporter plasmids and 2 μg of G-proteins were used using for transient transfection. Twenty-four hours after transfection, cells were harvested and resuspended in Hanks' buffered salt solution containing 0.01% bovine serum albumin and 20 mM HEPES (Cellgro), loaded with 1 μg/ml coelenterazine (P. J. K. Industrievertetungen, Handel, Germany) at room temperature for 1 h, and stimulated with various compounds. Ligand-induced calcium mobilization, as indicated by an increase in aequorin luminescence, was recorded over a period of 20 s with a Microlumat luminometer (Berthold).

2.2.5. Flow cytometry analysis

Transiently transfected CHO cells were stained with anti-FLAG M2 monoclonal antibody in staining buffer (FBS) (BD Pharmigen) for 1 h at 4 °C. After extensive washing, cells were incubated with goat F(ab')2 anti-mouse IgG-APC secondary antibody (Caltag) for 30 min. Flow cytometry analysis was carried out using FACSCalibur (BD Biosciences).

2.2.6. Transient transfection and membrane preparation

For each 15-cm cells dish, 10 μg of $G_{o\alpha}$ and 10 μg of $G_{o\beta}$ expression constructs were used for transfection into HEK293-GPR97 stable cells or parental HEK293 cells. Membranes were harvested 48 h after transfection. Pertussis toxin (100 ng/ml) was added 16 h before harvesting. All the membrane preparation steps were performed at 4 °C. In brief, cells were harvested by centrifugation (10 min at 10,000×g), washed once with phosphate-buffered saline, resuspended in lysis buffer (10 mM Tris–HCl, pH 7.4, with 1 mM EDTA and protease inhibitors) and lysed using 25 strokes of a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation (5 min at 500×g). The supernatant was centrifuged again (30 min at 40,000×g) and the resulting pellet was washed once with 20 ml of lysis buffer (10 mM Tris–HCl, pH 7.4, alone). Membranes were resuspended at 1.5–2.0 mg/ml protein in binding buffer (20 mM HEPES pH 7.5, 5 mM $MgCl_2$) and stored at −80 °C until use.

2.2.7. [^{35}S]GTPγS binding

The optimal experimental conditions for the concentrations of GDP, $MgCl_2$, and NaCl in the assay buffer were initially determined. The assay was performed in assay buffer [20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 0.1% (w/v) BSA] with 100 mM NaCl, 1 μM GDP, and 20 μg of membranes/well. No NaCl was included for determining constitutive activity of the receptor. The reaction was initiated by addition of 0.4 nM [^{35}S]GTPγS in the absence or presence of beclomethasone dipropionate and incubated at room temperature for 90 min. Non-specific binding was determined in the presence of 100 μM GTPγS and was always less than 0.2% of total binding. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by filtration through GF/B filters followed by five washes with 200 μl of ice-cold assay buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

3. Results

Knowledge of downstream signaling can be gained by evaluating whether an orphan receptor is constitutively active. $G_{\alpha s}$- and $G_{\alpha q}$-coupled receptors can be evaluated for constitutive activity via the measurement of cAMP or inositol phosphate accumulation in cells transfected with high levels of receptor expression vectors. For $G_{\alpha i}$- or $G_{\alpha o}$-coupled receptors, chimeric G-proteins can be used to convert signals to $G_{\alpha q}$ – mediated signaling pathways. Chimeric G-proteins are created by replacing the C-terminal amino acids of $G_{\alpha q}$ with the corresponding amino acids from $G_{\alpha i}$- or $G_{\alpha o}$-proteins (Fig. 1A). This allows measurement of receptor activity using an inositol phosphate accumulation assay, which is generally more robust than traditional adenylate cyclase assays [10].

We transfected our collections of adhesion GPCRs (listed in Table 1) together with each individual chimeric G-proteins into HEK293 cells and performed inositiol phosphate accumulation assays. $G_{\alpha 15}$ and $G_{\alpha 16}$ were also included since they have been shown to couple to a variety of $G_{\alpha s}$, $G_{\alpha i/\alpha o}$ and $G_{\alpha q}$ dependent receptors. Four receptors showed constitutive activities (Fig. 1B). Among them, GPR115 and EMR2 are active when cotransfected with $G_{\alpha 15}$. GPR110 is constitutively active even in the absence of any G-proteins, suggesting that it couples to endogenous $G_{\alpha q}$-protein. GPR97 requires chimeric protein G_{qo3} to induce inositol phosphate accumulation, indicating it primarily couples to $G_{\alpha o}$-protein. We also transfected adhesion GPCRs into HEK293 cells and measured the cAMP accumulation after 24 h. Among them, GPR114 and GPR133-transfected cells showed high levels of cAMP compared with the transfection control and other receptors (Fig. 2A), suggest-

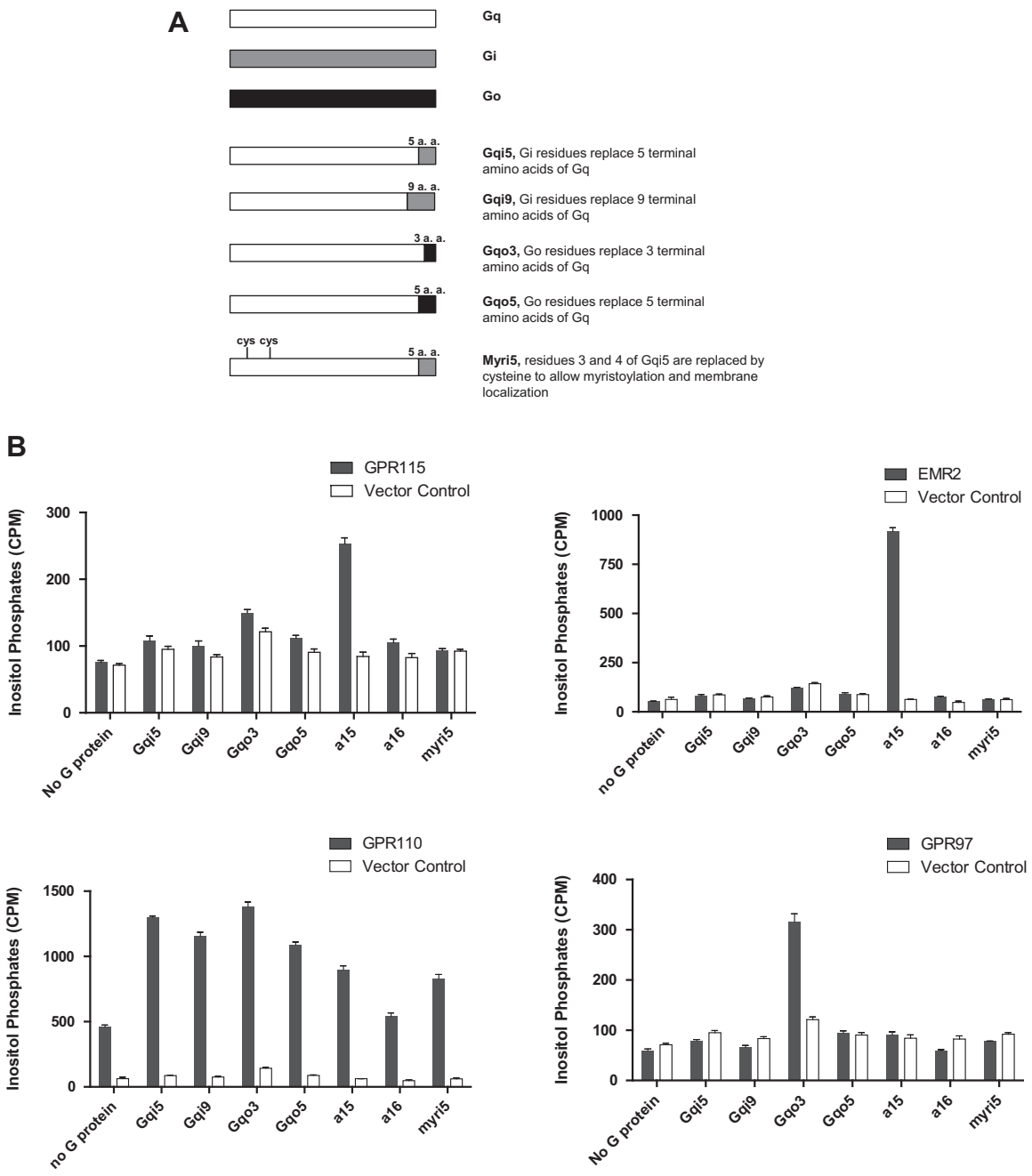

Fig. 1. Constitutive activity in the inositol phosphate accumulation assay. (A) Schematic depiction of the chimeric G-proteins used in this study. (B) HEK293 cells were transiently transfected with GPCR plasmid alone or co-transfected with various chimeric G-proteins. Control vector containing aequorin cDNA served as a negative control. Twenty-four hours after transfection, the cells were incubated with HBSS containing 10 mM LiCl for 1 h. Total inositol phosphates were determined and results presented as the mean of quadruplicate determinations. The results have also been confirmed by multiple independent experiments.

ing that they can couple to endogenous $G_{\alpha s}$-proteins. To confirm $G_{\alpha s}$-proteins are directly involved in GPR114 and GPR133 signaling, we used siRNA to specifically knock down the endogenous $G_{\alpha s}$-proteins. Co-transfection of siRNA and GPR114 or GPR133 abolished the increases in cAMP levels, while control siRNA has no effect (Fig. 2B), clearly demonstrating GPR114 and GPR133 can signal through $G_{\alpha s}$ coupling. GPR133 coupling to $G_{\alpha s}$-proteins has also been confirmed recently in an independent study [11].

GPR97 belongs to an interesting cluster of adhesion GPCRs enriched in immune cells [12]. To test whether adhesion GPCRs can also activate intracellular signaling upon small molecule ligand binding, we started a proof-of-concept study using GPR97 and screened the Prestwick Chemical Library (Prestwick Chemical, France) for activators of Ca^{2+} mobilization using an aequorin assay. CHO cells were transiently transfected with GPR97, aequorin reporter, and G_{qo3}, which will convert the $G_{\alpha o}$-coupled signaling to Ca^{2+} signaling. Beclomethasone dipropionate evoked a specific rise in $[Ca^{2+}]_i$ in cells expressing human GPR97 and G_{qo3}, with a median effective concentration (EC_{50}) of 46 nM (Fig. 3). No specific response was observed in control cells not co-transfected with either GPR97 or G_{qo3} (Fig. 3). Beclomethasone dipropionate (structure shown in Fig. 3) is a potent glucocorticoid steroid from Prestwick Chemical Library. But the fact that beclomethasone dipropionate only activates cells when both GPR97 and G_{qo3} were expressed and the acuteness of the response time suggest the signals from the aequorin assay are unlikely due to the activation of the

Table 1
List of adhesion GPCRs tested in this study.

GPCR	Species
CD97	Human
EMR2	Human
ETL1	Human
BAI1	Human
BAI2	Mouse
BAI3	Human
CELSR3	Human
GPR97	Human
GPR110	Mouse
GPR114	Human
GPR115	Mouse
GPR116	Human
GPR123	Mouse
GPR124	Human
GPR125	Human
GPR126	Human
GPR133	Human
GPR56	Human

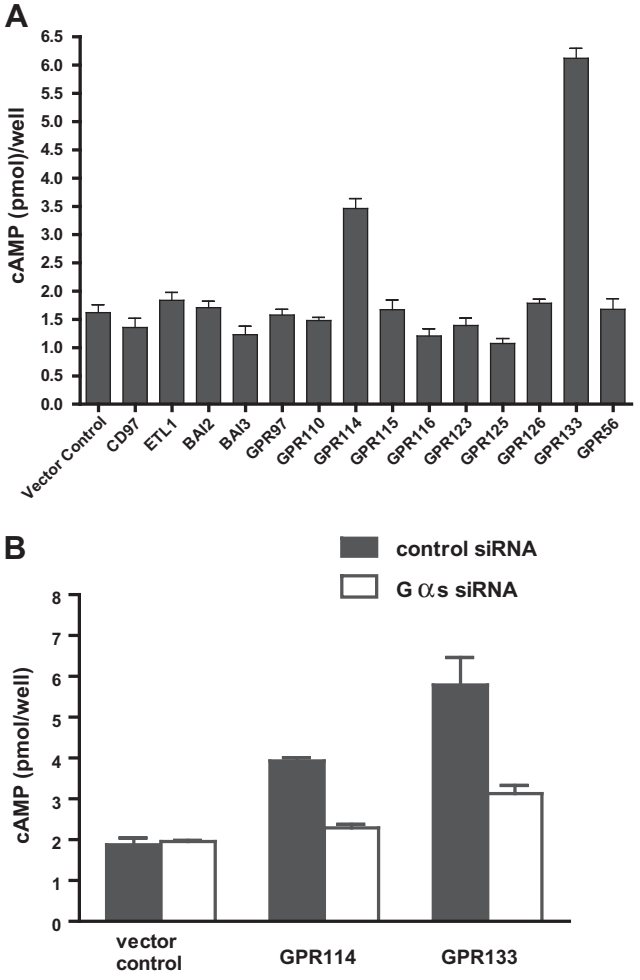

Fig. 2. Constitutive activity in cAMP accumulation assay. (A) HEK293 cells were transiently transfected with adhesion GPCR plasmid, and aequorin cDNA was used as vector control. After 24 h, cells were incubated with 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. Reactions were terminated and concentration of adenylyl cyclase was measured. Results are presented as the mean of quadruplicate determinations. The results have also been confirmed by multiple independent experiments. (B) HEK293 cells were co-transfected with GPR114, GPR133 or vector control together with $G_{\alpha s}$ siRNA or control siRNA. After 48 h, cells were incubated with 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. Reactions were terminated and concentration of adenylyl cyclase was measured.

glucocorticoid receptor. A more extensive screening from a bigger library in the future should let us identify more specific compounds for GPR97 without other cellular targets.

To ensure that the observed activity was mediated by cell surface expressed GPR97, N-terminal FLAG-tagged GPR97 was generated to study the cellular localization of GPR97. FLAG-GPR97 behaved similarly to wild-type GPR97 in both the inositiol phosphate accumulation assay and the aequorin assay (data not shown). Flow cytometry analysis confirmed cell surface expression of GPR97 on CHO cells transfected with FLAG tagged GPR97, and co-transfection of chimeric G-protein G_{qo3} had no effect on its surface expression (Fig. 4).

To further confirm that GPR97 indeed couples through G_o-proteins upon activation, we performed an [^{35}S]GTPγS incorporation assay using membrane preparations from HEK293–GPR97 stable cells transiently transfected with $G_{o\alpha}$ and $G_{o\beta}$ G-proteins, and parental HEK293 cells transfected with $G_{o\alpha}$ and $G_{o\beta}$ were used as a negative control. Membranes from HEK293–GPR97 stable cells showed constitutive activity of about 10% over control cells ($P < 0.0128$, unpaired T-test, two tailed) (Fig. 5A). Similar to the results from the aequorin assay, we also found that beclomethasone dipropionate stimulated G-protein coupling to the GPR97 receptor in a concentration-dependent manner (Fig. 5B). The EC_{50} for beclomethasone dipropionate-induced activation of human GPR97 in [^{35}S]GTPγS binding assay was 3.1 nM. The discrepancy of the EC_{50} numbers between the aequorin assay and the [^{35}S]GTPγS binding assay could be due to the different receptor coupling efficiency between chimeric G-protein G_{qo3} and $G_{o\alpha\beta}$ G-proteins. This agonist-induced effect was abolished upon treatment with pertussis toxin (PTX), indicating that GPR97 couples to PTX-sensitive G_i/G_o G-proteins (data not shown). No significant response was obtained using membranes from parental HEK293 cells transfected with $G_{o\alpha}$ and $G_{o\beta}$ G-proteins (Fig. 5B). Our results provided strong evidence that GPR97 can be activated by a small molecule compound, beclomethasone dipropionate, through coupling to G_o protein.

4. Discussion

G-protein-coupled receptors (GPCRs) share a common molecular architecture comprising a seven-transmembrane domain. They also share a common signaling mechanism of interacting with G-proteins (heterotrimeric GTPases) in regulating intracellular secondary messengers such as cyclic AMP, inositol phosphates, and calcium ions [5]. Adhesion GPCRs belong to a large subfamily of GPCRs whose members are potentially involved in a variety of physiological activities [1]. However, functional data for the majority of adhesion GPCRs are very limited due to the lack of proper tool compounds to probe their functions. We tested a large number of our in-house collection of adhesion GPCRs to understand their signaling mechanisms and found several to be constitutively active and capable of coupling to different G-proteins. Such information could shed light on the possible physiological roles played by those receptors. For example, the fact that GPR133 is enriched on beta cell surface with constitutive $G_{\alpha s}$-protein coupling activity to activate cAMP production raises the interesting possibility for its involvement in insulin secretion. EMR2, GPR114 and GPR97 are all expressed in the immune cells, so it is reasonable to speculate that their signaling activities might contribute to immune responses such as cytokine release and cell migration, as demonstrated by many canonical GPCRs enriched in the immune system. Other separate studies from recent literature also support our finding that adhesion GPCRs can signal through classical G-protein-coupled pathway [11,13]. The actual number of the receptors having constitutive activities might be even higher since we

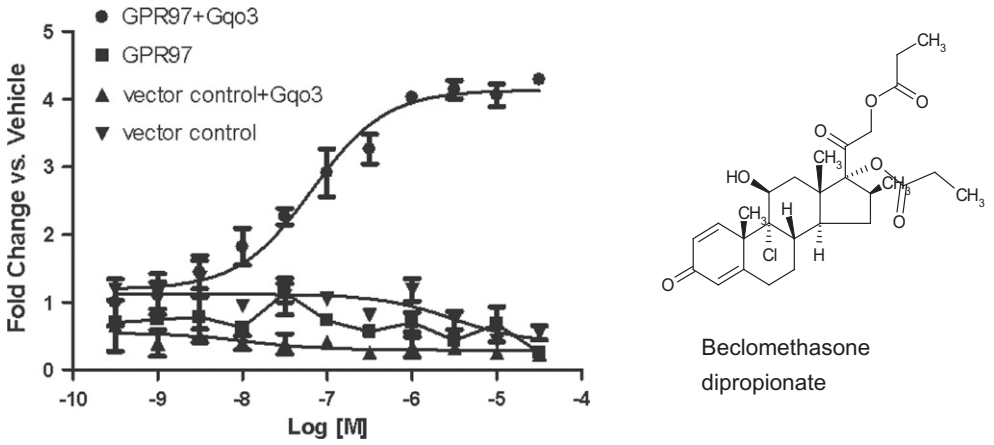

Fig. 3. Identification of beclomethasone dipropionate as a ligand for GPR97. Dose-dependent activation of human GPR97 by beclomethasone dipropionate. CHO cells were transfected with an aequorin expression vector and human GPR97 or a vector control in the presence or absence of plasmids expressing the chimeric G-protein, G_{qo3}. Ligand-induced [Ca^{2+}] increase was recorded as the aequorin luminescence signal for 20 s after the compounds were added and data were plotted as fold change over vehicle treated cells. The results presented as the mean of duplicated determinations and have been confirmed by multiple independent experiments. The structure of beclomethasone dipropionate is shown on the right.

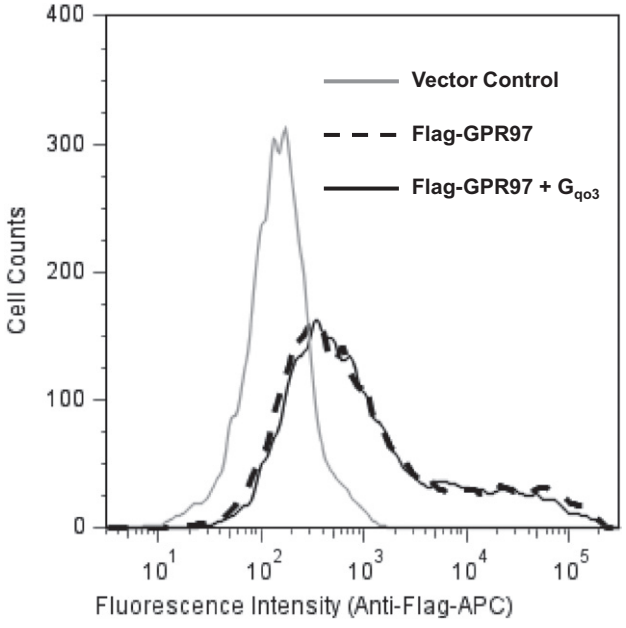

Fig. 4. Cell surface localization of GPR97. CHO cells were transiently transfected with vector control, flag-GPR97, or flag-GPR97 + G_{qo3}. After 48 hours, cells were harvested and incubated with anti-FLAG antibody and goat F(ab')2 anti-mouse IgG-allophycocyanin (APC) secondary antibody before flow cytometry analysis.

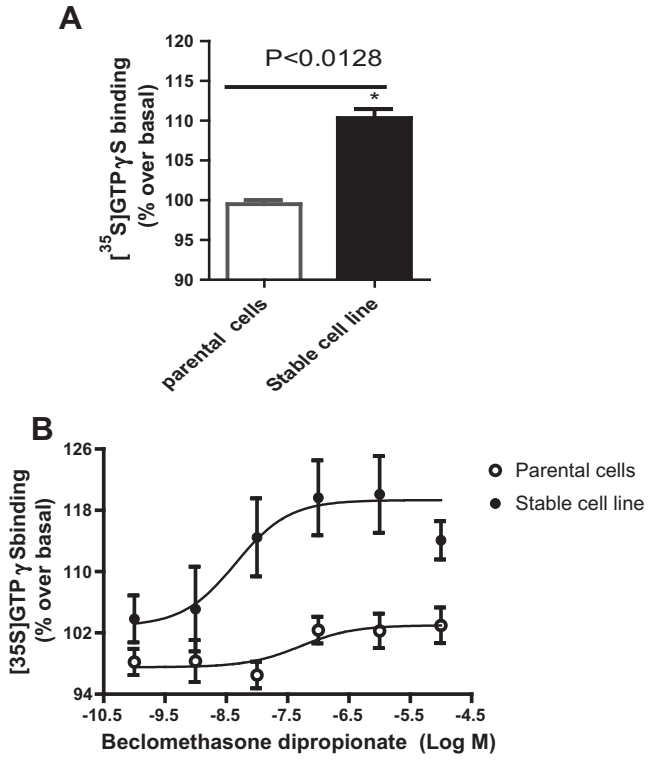

Fig. 5. [^{35}S]GTPγS incorporation assay. (A) Constitutive activity of GPR97 in GTPγS binding assay (black bar) and control (white bar). Data are the mean of two independent experiments. Control activity (considered 100%) is from parental HEK293 cells co-transfected with $G_{o\alpha}$ and $G_{o\beta}$-proteins. (B) Concentration-dependent increase of GTPγS binding by beclomethasone dipropionate from HEK293–GPR97 stable cell membranes. GTPγS binding assay was carried out as described in the Section 2. Basal (100%) represents response in the absence of any ligand. Data shown are the mean ± S.E.M of three to four independent experiments performed in duplicates or triplicates. Data were analyzed by non-linear regression and were best fitted to agonist dose–response curves.

could have missed the receptors that have poor expression levels under our assay conditions.

Furthermore, through screening a small-molecule library, we identified a compound that will activate GPR97 in the presence of the corresponding G-protein. This is the first time that a small chemical compound has been shown to activate a member of the adhesion GPCR subfamily. Our study generated convincing evidence that adhesion GPCRs can signal through G-proteins and activate intracellular secondary messenger production similar to other well-studied classic GPCRs. Using a conventional screening platform, we could potentially identify their agonists and use them as tools for probing the physiological functions of these receptors. For example, GPR97 has been implicated in being involved in early lymphoid development since it is specifically expressed in pre-B cells and thymocytes but not in mature B and T cells. However,

GPR97-deficient mice did not display any significant quantitative or qualitative defects in B- and T-cell development [14]. But that does not exclude a role for GPR97 in B- and T- cell development, as there might be proteins which are functionally redundant carrying out its function, suggesting that loss of function approaches such as gene knockout alone may not be sufficient to elucidate the function of a gene. In this case, having a specific agonist for GPR97 that stimulates the signaling activity can be very useful for the functional study.

The unique property of the adhesion family GPCRs is their long N-termini, which contain multiple domains that may be involved in cell–cell adhesion [1]. A great deal of research has focused on their interactions with other cell surface molecules. For example, CD97 has been shown to interact with the cell surface protein CD55 since CD97-expressing cells are able to adhere to CD55 positive erythrocytes [6,7]. It is not clear whether the adhesion function of this class of GPCRs is independent or related to the intracellular signaling activities. A recent study showed purified cadherin repeats of Celsr2 or Celsr3 can increase the intracellular calcium level in cells expressing those two receptors [15]. Whether this is receptor specific or common among the subfamily requires further investigation.

In summary, we utilized an over-expression system and candidate approach, which have been successfully applied to deorphanize and characterize many canonical GPCRs, to generate convincing evidence for the signaling activities from this subfamily of GPCRs. We believe this strategy is the best option at the current stage to guide the efforts for tool compound generation. Once proper ligands are identified, studies can be carried out under more physiologically relevant settings to validate the signaling results and other molecular biology tools such as siRNA can be used in loss of function studies for further understandings of the biological functions of these receptors.

Acknowledgements

We thank Dr. Jeff Reagan and Angela Fu for support and discussion and Scott Silbiger for editing the manuscript.

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