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RESEARCH PAPER

The GPCR-associated sorting protein 1 regulates ligand-induced down-regulation of GPR55

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BACKGROUND AND PURPOSE
Many GPCRs, including the CB_1 cannabinoid receptor, are down-regulated following prolonged agonist exposure by interacting with the GPCR-associated sorting protein-1 (GASP-1). The CB_1 receptor antagonist rimonabant has also recently been described to be an agonist at GPR55, a cannabinoid-related receptor. Here we investigated the post-endocytic properties of GPR55 after agonist exposure and tested whether GASP-1 is involved in this process.

EXPERIMENTAL APPROACH
We evaluated the direct protein-protein interaction of GPR55 with GASP-1 using (i) GST-binding assays and (ii) co-immunoprecipitation assays in GPR55-HEK293 cells with endogenous GASP-1 expression. We further tested the internalization, recycling and degradation of GPR55 using confocal fluorescence microscopy and biotinylation assays in the presence and absence of GASP-1 (lentiviral small hairpin RNA knockdown of GASP-1) under prolonged agonist [rimonabant (RIM), lysophosphatidylinositol (LPI)] stimulation.

KEY RESULTS
We showed that the prolonged activation of GPR55 with rimonabant or LPI down-regulates GPR55 via GASP-1. GASP-1 binds to GPR55 *in vitro*, and this interaction was required for targeting GPR55 for degradation. Disrupting the GPR55-GASP-1 interaction prevented post-endocytic receptor degradation, and thereby allowed receptor recycling.

CONCLUSION AND IMPLICATIONS
These data implicate GASP-1 as an important regulator of ligand-mediated down-regulation of GPR55. By identifying GASP-1 as a key regulator of the trafficking and, by extension, functional expression of GPR55, we may be one step closer to gaining a better understanding of this receptor in response to cannabinoid drugs.

LINKED ARTICLES
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Abbreviations
7TM/GPCR, 7 transmembrane spanning/GPCR; CB_1, cannabinoid 1 receptor; CB_2, cannabinoid 2 receptor; D_2, dopamine 2 receptor; DMSO, dimethylsulphoxide; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GASP-1, GPCR-associated sorting protein 1; GPR55, GPCR 55; GPR55-HEK, stable HEK293 cells expressing FLAG-GPR55; HA, haemaglutinin; HRP, horseradish peroxidase; LAMP1/2, lysosomal-associated membrane protein 1/2; LPI, L-α-lysophosphatidylinositol; MCF-7, human breast cancer cell line; PNGase, N-glycosidase; shRNA, small hairpin RNA;

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SR141716A, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; TBS, Tris-buffered saline; U2OS, human osteosarcoma cell line; WIN55-212-2 (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

Introduction

The endocannabinoid system consists of endogenous cannabinoids, two well-defined seven transmembrane spanning/GPCRs (7TM/GPCRs), the cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptors (Pertwee, 1997), as well as enzymes synthesizing and degrading endocannabinoids. The recently de-orphanized GPCR 55 (GPR55) has been implicated as a novel cannabinoid receptor, since besides being activated by the endogenous lipid ligand L-α-lysophosphatidylinositol (LPI), several synthetic CB_1 inverse agonists/antagonists, such as AM251, AM281 and rimonabant (SR141716A, RIM), have been shown to activate this receptor (Oka *et al*., 2007; Ryberg *et al*., 2007; Henstridge *et al*., 2009; 2010; Kapur *et al*., 2009; Yin *et al*., 2009; Brown *et al*., 2011). Rimonabant (Acomplia ®, Sanofi-Aventis) has further attracted attention since it was marketed to induce weight loss and reduce smoking. However, due to severe side effects, such as the development of anxiety and depression, rimonabant was withdrawn from the market (Christensen *et al*., 2007).

Depending on the cellular background, activated GPR55 has been shown to couple to a variety of G-proteins (Ryberg *et al*., 2007; Lauckner *et al*., 2008; Schroder *et al*., 2010; Sharir & Abood, 2010) and to activate signal cascades involving small GTPases, oscillatory Ca^{2+}-release, MAP kinases and a variety of transcription factors (Henstridge *et al*., 2009; 2010; Balenga *et al*., 2011a). However, the pharmacology and signalling properties of this receptor remain controversial (Pertwee *et al*., 2010). Accumulating evidence suggests that GPR55 plays a role in diverse physiological systems (Balenga *et al*., 2011b), including cancer development (Ford *et al*., 2010; Andradas *et al*., 2011; Pineiro *et al*., 2011), bone formation (Whyte *et al*., 2009), pain regulation and inflammation (Staton *et al*., 2008; Pietr *et al*., 2009; Balenga *et al*., 2011a). For instance, it has been reported that GPR55 is highly expressed in malignant human tumours (Andradas *et al*., 2011) and cancer cell lines (Ford *et al*., 2010; Pineiro *et al*., 2011), and its expression level is directly correlated to the aggressiveness of the tumours (Andradas *et al*., 2011). Likewise, GPR55 was shown to be expressed in human osteoclasts, and its activation to stimulate osteoclast polarization and resorption *in vitro* (Whyte *et al*., 2009). In addition, $GPR55^{-/-}$mice have been shown to be resistant to inflammatory and neuropathic pain (Staton *et al*., 2008), and GPR55 may play a role in inflammatory response of microglial cells (Pietr *et al*., 2009).

GPCR-mediated signalling is extensively regulated to guarantee an appropriate cell surface receptor density in a given physiological setting. One of these regulatory mechanisms is that of receptor endocytosis. Many GPCRs are endocytosed by a mechanism involving receptor phosphorylation, interaction with β-arrestins and concentration in clathrin-coated pits (Ferguson *et al*., 1998). However, the functional consequences of GPCR endocytosis through this conserved cellular mechanism are diverse. Trafficking of internalized GPCRs by a rapid recycling pathway restores the complement of functional receptors in the plasma membrane and promotes resensitization of receptor-mediated signal transduction. In contrast, the sorting of internalized GPCRs to lysosomes promotes proteolytic down-regulation of receptors, leading to a prolonged attenuation of cellular signal transduction (Tsao *et al*., 2001). Several proteins have recently been identified that specifically target GPCRs – typically by interaction with their C-terminal domains – to either recycling or degradative pathways (Hanyaloglu and von Zastrow, 2008). One such protein is the GPCR-associated sorting protein 1 (GASP-1) (Whistler *et al*., 2002).

GASP-1 was originally found to target δ-opioid receptors (DOP) to lysosomes and hence a degradative pathway (Whistler *et al*., 2002). GASP-1 has since been shown to interact with the carboxyl-termini of many GPCRs (Heydorn *et al*., 2004) and to regulate the trafficking properties of many of these, both *in vitro* and *in vivo* (bu-Helo and Simonin, 2010; Moser *et al*., 2010). For instance, experiments examining the function of the dopamine D_2 receptor both *in vitro* and *in vivo* revealed that GASP-1 plays a crucial role in regulating agonist responses via this receptor (Bartlett *et al*., 2005; Boeuf *et al*., 2009; Thompson *et al*., 2010). When slices from the rat ventral tegmental area (VTA) were pretreated with the D_2 receptor agonist quinpirole, the receptors failed to recover from desensitization, which was consistent with the ability of the D_2 receptor to degrade after endocytosis. However, disrupting the D_2 receptor /GASP-1 interaction with an inhibitory antibody allowed recovery of functional D_2 receptor responses (Bartlett *et al*., 2005). Along these lines, repeated treatment of wild-type – but not GASP-1 knockout – mice with cocaine lead to a down-regulation of D_2 receptors in mouse striatum (Thompson *et al*., 2010). In addition, GASP-1 knockout mice showed reduced locomotor sensitization to cocaine (Thompson *et al*., 2010) and reduced acquisition of cocaine self-administration (Boeuf *et al*., 2009).

In addition, we have previously reported that the CB_1 receptor is targeted to lysosomes and the degradative pathway via GASP-1 after prolonged stimulation with the agonist WIN55,212–2 *in vitro* (Martini *et al*., 2007). This CB_1/GASP-1 interaction has important physiological consequences; i.e. the down-regulation of CB_1 receptors via GASP-1 is a key determinant in the development of analgesic tolerance to cannabinoid drugs *in vivo* (Tappe-Theodor *et al*., 2007; Martini *et al*., 2010). Importantly, GASP-1 was recently found to be a crucial determent in directly regulating not only the trafficking but also the signalling capacity of a GPCR (e.g. the viral receptor US28) (Tschische *et al*., 2010).

Little is known regarding the internalization and post-endocytic trafficking properties of GPR55. Without ligand stimulation, GPR55 is predominantly located on the cell surface and internalizes following agonist stimulation in different cell models, such as HEK293, U2OS and MCF-7 cells (Henstridge *et al*., 2009; 2010; Kapur *et al*., 2009; Ford *et al*., 2010). Here we characterize the internalization and post-endocytic sorting properties of GPR55 after stimulation with the cannabinoid drug rimonabant as well as the endogenous ligand LPI and demonstrate that GASP-1 is crucially involved in regulating the trafficking of GPR55.

Methods

DNA constructs

The human GPR55 (nomenclature follows Alexander *et al.*, 2011) was tagged on the N-terminus with the FLAG epitope (DYKDDDDA) and inserted into a pcDNA3.1(+)vector. The cloning of the FLAG-δ-opioid and FLAG-μ-opioid receptors was previously described in Whistler *et al.* (2002). The GPR55-GST-fusion protein was constructed by subcloning the last 34 carboxyl-terminal amino acids of the human GPR55 into the pGEX-4T-1 plasmid. The generation of the GST-fusion protein constructs of the delta-opioid-receptor (DOP), the μ-opioid receptor (MOP) and the HA-tagged GASP-1 construct (HA-GASP-1) were as previously described (Whistler *et al.*, 2002). The generation of the lentivirus constructs encoding small hairpin RNA (shRNA) against GASP-1 (shGASP-1) or scrambled shRNA (shScr) are described in Tschische *et al.* (2010). All DNA constructs were verified by sequencing.

Cell culture and stable cell lines

HEK293 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2, humidified atmosphere. HEK293 cells stably expressing the human FLAG-GPR55 (GPR55-HEK) were generated by selection with zeocin containing medium. GPR55-HEK cells were deprived of serum overnight in Opti-MEM before all experiments.

Co-immunoprecipitation

FLAG-GPR55 was co-immunoprecipitated with endogenous GASP-1 as previously described (Whistler *et al.*, 2002). Briefly, HEK293 cells were transiently transfected with FLAG-GPR55 using Lipofectamine 2000, and 48 h post-transfection experiments were performed. Cells were washed twice with ice-cold PBS and lysed in IPB buffer (0.3% Triton X-100, 150 mM NaCl, 25 mM KCl, 1 mM $CaCl_2$, 10 mM Tris–HCl, pH 7.4, supplemented with complete protease inhibitors). Lysates were centrifuged, and supernatant was incubated with 20 μL of anti-FLAG M2 monoclonal antibody affinity matrix over night at 4°C. 30 μL of lysates were kept for FLAG, GASP and β-actin control blots. Precipitates were washed, deglycosylated with PNGase, resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked, probed with either anti-FLAG M2 antibody (1:500), anti-GASP antibody (1:1000) or β-actin monoclonal antibody (1:1000) and immunoblotted with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody. Blots were visualized with ECL Western Blotting Substrate.

GST-fusion protein-binding assay

GST-fusion protein-binding assays were carried out as described in Tschische *et al.* (2010). GST-fusion proteins were expressed in *Escherichia coli* and bound to glutathione–agarose. Fusion proteins on beads were incubated in blocking buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 5% BSA), while ^{35}S-methionine-labelled HA-GASP-1 was synthesized using a TNT T7 Coupled Reticulocyte Lysate System and subsequently incubated with the fusion proteins in wash buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100) for 1 h at 4°C. Probes were washed and resolved on a SDS/PAGE. Gels were stained with PAGEblue, dried and exposed to X-ray films.

Lentivirus production and shRNA knock-down of GASP-1

GASP-1 knockdown experiments were performed as previously described (Tschische *et al.*, 2010; Thompson and Whistler, 2011). Virus was produced in HEK293T cells and harvested 48 h post-transfection. Knockdown of GASP-1 levels in GPR55-HEK cells was induced by infection with lenti-shGASP-1 (shGASP-1) or lenti-shScrambled (shScr) virus for 48 h. Enhanced green fluorescent protein (EGFP) was used to determine successful lentivirus infection.

Biotin internalization and protection/degradation assays

GPR55-HEK cells were infected with either shScr or shGASP-1 virus, and experiments were conducted as previously described (Whistler *et al.*, 2002; Tschische *et al.*, 2010). In brief, cells were incubated with disulphide cleavable biotin for 10 min at 4°C and washed with TBS (25 mM Tris base, 135 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, pH 7.4). For internalization assays, cells were placed in warm medium for 0–45 min in the presence of ligands (RIM or LPI) /vehicle (DMSO or H_2O) to allow receptor endocytosis. Remaining cell surface biotin was stripped (50 mM glutathione, 75 mM NaCl) (except 100% plates) and quenched (50 mM iodoacetamide, 1% BSA in PBS), followed by cell lysis (0.3% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, 1 mM $CaCl_2$, 5 mM iodoacetamide, pH 7.4, with complete protease inhibitors). Protection/degradation assays were performed as described above. After quench, cells were placed in warm Opti-MEM for the indicated time points to allow receptor degradation (except 100% and Strip) before cell lysates were prepared. Samples of cleared lysates were taken for control blots (anti-GASP, anti β-actin), and the remainder were immunoprecipitated with anti-FLAG M2 affinity matrix. Precipitates were washed, deglycosylated, resolved by SDS-PAGE and visualized with Vectastain ABC. Blots were quantified using IMAGEJ Software.

Recycling experiments

ShScr or shGASP-1 virus-infected GPR55-HEK cells were grown on poly-D-lysine-coated coverslips. Living cells were incubated with the Ca^{2+}-sensitive anti-FLAG M1 antibody and stimulated with agonist (RIM or LPI) /vehicle (DMSO or H_2O) for 45 min. Remaining surface receptors – except those at 0 min and 45 min time points – were stripped (0.04% PBS–EDTA), and warm Opti-MEM was added for up to 90 min. Cells were fixed in 3.7% formaldehyde, blocked (50 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$ and 3% milk) and stained with Alexa647-conjugated IgG2b under non-permeabilizing conditions. Receptors were visualized using a laser-scanning confocal imaging system (Zeiss LSM510).

Statistical analysis

Statistical analysis was performed using ANOVA for comparisons between multiple groups followed by a Bonferroni's *post hoc* test using GraphPad Prism and Mirocal Origin software. A *P*-value of <0.05 was considered significant.

Materials

Mouse M1 and M2 monoclonal antibodies, β-actin antibody, anti-FLAG M2 affinity matrix, BSA, L-glutathione, iodoacetamide, Triton X-100, poly-D-Lysine, Kodak BioMax light films, pysophosphatidylinositol (LPI) and Tween 20 were purchased from Sigma Aldrich (Vienna, Austria); 4–20% Tris–glycine gels, cell culture reagents, Lipofectamine 2000, Alexa Fluor647nm-conjugated IgG2b were from Invitrogen (Lofer, Austria). HRP-conjugated antibodies were obtained from Jackson Immuno Research (Dianova, Germany). Generation of anti-GASP-1 antibodies has been previously described (Whistler *et al.*, 2002). Complete Protease inhibitor cocktail tablets were from Roche (Vienna, Austria) and PNGaseF from New England Biolabs (Frankfurt, Germany). Immobilon-P Transfer Membrane was purchased from Millipore (Vienna, Austria). ECL Western Blotting Substrate was from Pierce (THP, Austria). Vectashield mounting medium and Vectastain ABC Kit were purchased from Vector Laboratories (Szabo-Scandic, Austria). EZ-Link Sulpho-NHS-SS-Biotin was from Thermo Scientific (Histocom, Austria). NaOH, KCl and $CaCl_2*2H_2O$ were obtained from Merck (Vienna, Austria), NaCl, Tris and formaldehyde were from Roth (Lactan, Austria). TNT T7 Coupled Reticulocyte Lysate System was purchased from Promega (Mannheim, Germany). EasyTag [^{35}S]-methionine was obtained from PerkinElmer (Vienna, Austria) and PAGEBlue from Fermentas (St. Leon-Rot, Germany). SR141716A (rimonabant, RIM) was from Sanofi-Synthélabo Recherche (Montpellier, France). LPI was dissolved in H_2O and SR141716A in DMSO.

Results

GPR55 interacts with GASP-1

In HEK293 cells expressing GPR55, the receptor co-immunoprecipitated with endogenous GASP-1 (Figure 1A; upper panel, GPR55). Many GPCRs interact with GASP-1 through their cytoplasmic tails (Heydorn *et al.*, 2004). This was also the case for GPR55. Specifically, a purified recombinant glutathione *S*-transferase (GST)-fusion protein containing the last 34 carboxyl-terminal amino acids of GPR55 bound GASP-1 *in vitro*, similar to the C-terminus of the δ-opioid receptor (Figure 1B; upper panel, GPR55 and DOP) (Whistler *et al.*, 2002), which served as positive control. Neither the control GST protein nor the carboxyl-terminus of the μ-opioid receptor (Whistler *et al.*, 2002) significantly bound GASP-1 (Figure 1B; upper panel, MOP and GST).

The internalization of GPR55 is not regulated by GASP-1

In various recombinant cell lines – including HEK293, U2OS and MCF-7 cells – GPR55 is predominantly located on the cell surface but internalizes following agonist stimulation (Henstridge *et al.*, 2009; 2010; Kapur *et al.*, 2009; Ford *et al.*, 2010). To examine whether GASP-1 affects the internalization of GPR55, we quantified the rate of internalization using a biotinylation protection assay in HEK293 cells where GASP-1 levels were knocked down with a shRNA lentivirus (Tschische *et al.*, 2010). This assay allows the selective monitoring of the endocytic fate of a pool of receptors that is expressed on the cell surface and is subsequently stimulated with agonists. In brief, receptors that reached the cell surface of intact cells were labelled with thio-cleavable disulphide-linked biotin (Figure 2A and B; upper panel, 100%). Cells were then incubated with agonists in warm media to allow biotinylated receptors to internalize for up to 45 min. After this incubation step, cell surface remaining biotin was cleaved with a membrane impermeable reducing agent, and the 'protected' internalized biotinylated receptor pool was immunoprecipitated and detected by streptavidin overlay. The shRNA caused efficient knockdown of GASP-1 protein expression 48 h after infection (Figure 2B; GASP-1), while scrambled shRNA virus (shScr) had no effect on GASP-1 levels (Figure 2A; GASP-1). In both, shScr (Figure 2A) and shGASP-1 (Figure 2B) infected cells, biotinylated GPR55 internalized following treatment with either 2.5 μM rimonabant (Figure 2A and B; left panels, RIM and 2C) or 2.5 μM LPI (Figure 2A and B; right panels, LPI and 2C); 45 min of vehicle (DMSO, final concentration 0.025%) treatment did not lead to significant receptor internalization in the presence and absence of GASP-1, respectively (Figure S2). Lysates were immunoblotted for GASP-1 and β-actin (Figure 2A and B; lower panels). GASP-1 did not appear to affect internalization of GPR55.

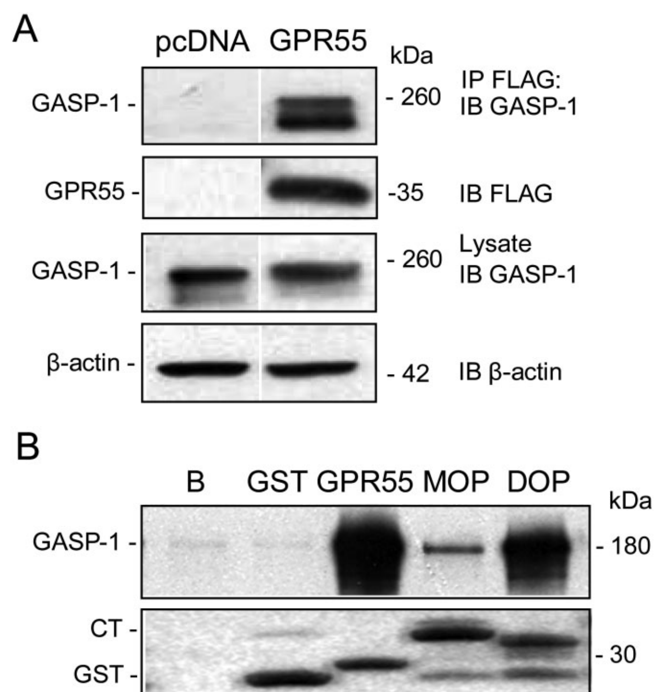

Figure 1

GPR55 interacts with GASP-1 *in vitro*. (A) pcDNA3.1 (pcDNA) or FLAG-GPR55 (GPR55) were immunoprecipitated with anti-FLAG affinity matrix (IP) and immunoblotted (IB) for GASP-1 (first panel) and receptor (second panel). Lysates were probed for GASP-1 (third panel) and β-actin (fourth panel). (B) GST-fusion proteins containing the c-tail (CT) of GPR55 and the δ-opioid receptor (DOP) bind [^{35}S]-methionine-labelled recombinant GASP-1. In contrast, GASP-1 did not bind to empty beads (B), the GST-protein (GST) alone or GST-μ-opioid receptor (MOP). Total protein levels are shown below the autoradiograph. Blots are representative of four independent experiments.

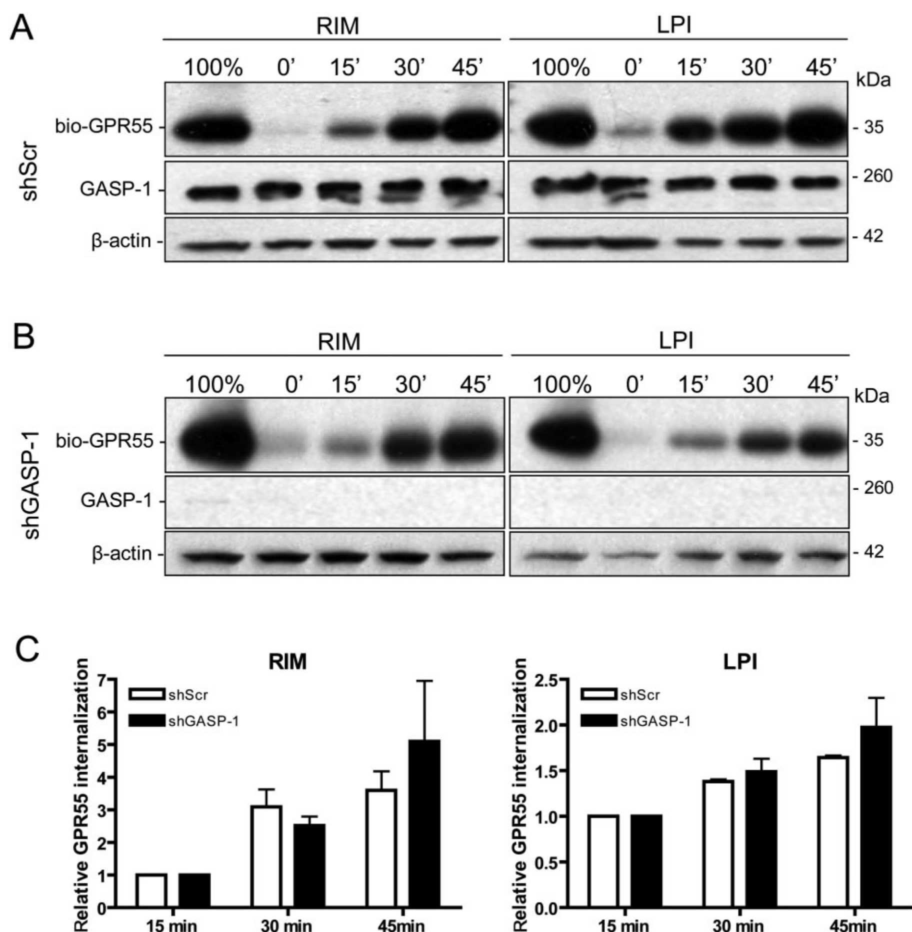

Figure 2

Knockdown of GASP-1 does not impair GPR55 internalization. GPR55-HEK cells were infected with either (A) shScr or (B) shGASP-1 lentivirus. Internalization of biotinylated FLAG-GPR55 (bio-GPR55) was monitored for the indicated time points (0′, 15′, 30′ and 45′). GPR55 internalized after stimulation with 2.5 μM RIM (left panels) or 2.5 μM LPI (right panels) in both, shScr- and shGASP-1-infected cells. The lower panels show the corresponding lysate samples immunoblotted for GASP-1, indicating successful GASP-1 knockdown, and β-actin. (C) Bio-GPR55 bands were normalized to β-actin, and 15 min values were set at 1. Data are means of three independent experiments ± SEM.

GASP-1 promotes the degradation of GPR55 in response to LPI and rimonabant

Since GASP-1 has been reported to specifically target GPCRs for degradation in lysosomes after their internalization (Moser *et al.*, 2010), we next assessed whether GASP-1 alters the post-endocytic targeting of GPR55. Consistent with its ability to bind GASP-1, GPR55 was targeted to lysosomes after internalization (Figure S1). To quantify the post-endocytic fate of GPR55, we next monitored the stability of a pool of endocytosed GPR55 in the presence or absence of GASP-1 using shRNA knockdown of GASP-1 and a biotin protection/degradation assay. Biotinylated GPR55 receptors were stimulated with either 2.5 μM rimonabant (Figure 3A and C; RIM) or 2.5 μM LPI (Figure 3B and C; LPI) for 45 min to allow receptor internalization (45′). Remaining biotin on the cell surface was stripped, and the internalized 'protected' pool of receptors was monitored for up to 180 min (Figure 3A, B and C; +30′, +90′ and +180′). Lysate samples immunoblotted for β-actin (Figure 3A and B; lower panels) served as controls for protein level.

In GPR55-HEK cells infected with shGASP-1 lentivirus (Figure 3A and B right panels, and C), biotinylated, internalized GPR55 was significantly more stable than in cells infected with the scrambled shScr lentivirus (Figure 3A and B left panels, and C). Taken together, these results indicate that GASP-1 plays a crucial role for the sorting of GPR55 to the lysosomes for degradation after endocytosis.

The recycling of GPR55 is promoted in the absence of GASP-1

In cells devoid of GASP-1, the degradation of some GPCRs is disrupted. In some cases, receptors are recycled back to the cell surface (Whistler *et al.*, 2002; Enquist *et al.*, 2007; Martini *et al.*, 2007; Tschische *et al.*, 2010), while in others the receptors are neither degraded nor recycled (Thompson *et al.*, 2007; 2010) but retained in intracellular compartments. Hence, we next tested whether disrupting the GPR55/GASP-1 interaction facilitated recycling of GPR55.

To assess receptor recycling, GPR55-HEK cells were infected with shScr (Figure 4A and B; upper panels) or

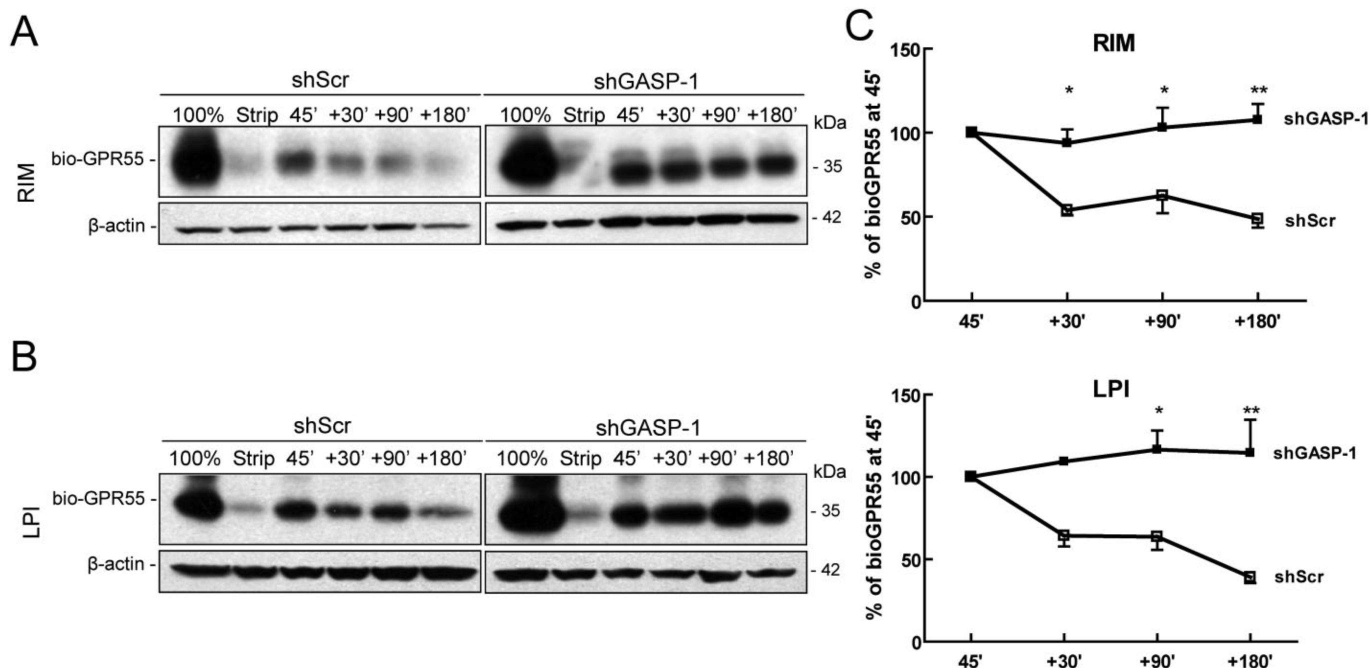

Figure 3

Disruption of the GASP-1/GPR55 interaction inhibits the degradation of GPR55. GPR55-HEK cells were infected with shScr virus (left panels) or shGASP-1 virus (right panels). Biotinylated GPR55 (bio-GPR55, 100%) was allowed to internalize in the presence of (A) 2.5 μM RIM or (B) 2.5 μM LPI for 45 min (45′) before cells were stripped of surface-biotin (Strip). The degradation of GPR55 was monitored for an additional 180 min (+30, +90 and +180). GPR55 degradation was observed in shScr (A and B; left panel, C), but not in shGASP-1 (A and B; right panel; C) infected cells. (C) Quantification of biotinylation assays in (A) and (B). Biotinylated GPR55 bands from shScr or shGASP-1 infected cells were normalized to β-actin and 45 min values were set at 100%. Data are means of three independent experiments ± SEM, **P* < 0.05, ***P* < 0.01.

shGASP-1 (Figure 4A and B; lower panels) lentivirus. Surface receptors were labelled with anti-FLAG antibody (0 min), and cells were then stimulated with 2.5 μM rimonabant (Figure 4A) or 2.5 μM LPI (Figure 4B) for 45 min to allow receptor internalization (45 min). Since cells stimulated with vehicle (DMSO or H_2O, final concentration 0.025%) did not internalize (see Figure S2), recycling could not be monitored. Cells were stripped of the FLAG-antibody (Strip), and receptor trafficking was monitored for up to 90 min (+30 min, +60 min and +90 min). Cells were fixed, and any recycled receptor was detected by a fluorescent antibody. Before agonist stimulation, GPR55 was detected primarily on the cell surface (Figure 4A and B, 0 min) but internalized rapidly following treatment with 2.5 μM rimonabant (Figure 4A, 45 min) or 2.5 μM LPI (Figure 4B, 45 min). Following the antibody strip (Strip), no recycled receptor was detected in shScr-infected cells treated with either rimonabant (Figure 4A; upper panel) or LPI (Figure 4B; upper panel) even after 90 min, consistent with the targeting of GPR55 to the lysosome for degradation (Figure 3 and S1). In contrast, knockdown of GASP-1 with shGASP-1 facilitated recycling of GPR55 back to the cell surface as early as 30 min following the strip (Figure 4A and B, lower panel, +30, +60, +90 min).

Discussion

The sorting of GPCRs after receptor activation is a highly regulated process and includes receptor degradation or recycling back to the cell surface. One regulatory mechanism to guarantee appropriate receptor expression levels in physiological conditions is that of down-regulating GPCRs via sorting proteins, for example GASP-1, thus leading to an attenuation of cellular signalling events (Moser *et al*., 2010).

This study demonstrates the importance of GASP-1 as a cellular regulator for the post-endocytic sorting of GPR55 to both the exogenous agonist drug rimonabant and the endogenous ligand LPI. GPR55 binds directly to GASP-1 (Figure 1A and B), and this interaction is essential for targeting the receptor to the degradative pathway (Figure 3A, B and C; shScr). Upon stimulation with the GPR55 agonists rimonabant and LPI, GPR55 co-localizes with the lysosomal markers LAMP1/2 in the presence of GASP-1 (Figure S1; shScr). In the absence of GASP-1, rapid recycling of GPR55 can be observed (Figure 4A and B; shGASP-1); however, the agonist-induced internalization of GPR55 is not altered in the absence of GASP-1 (Figure 2B and C). Taken together, these results implicate GASP-1 as a key player to target GRP55 to the lysosomes/degradative pathway upon agonist exposure.

The endogenous ligand LPI is up to a 100-fold more potent than rimonabant in activating intracellular signalling cascades via GPR55 (Henstridge *et al*., 2010). Interestingly, we could not observe any differences regarding the internalization and post-endocytic sorting properties of GPR55 when the cells were stimulated with equimolar doses of the endogenous ligand LPI or the diarylpyrazole rimonabant. However, these two ligands are similarly potent (i.e. only a threefold

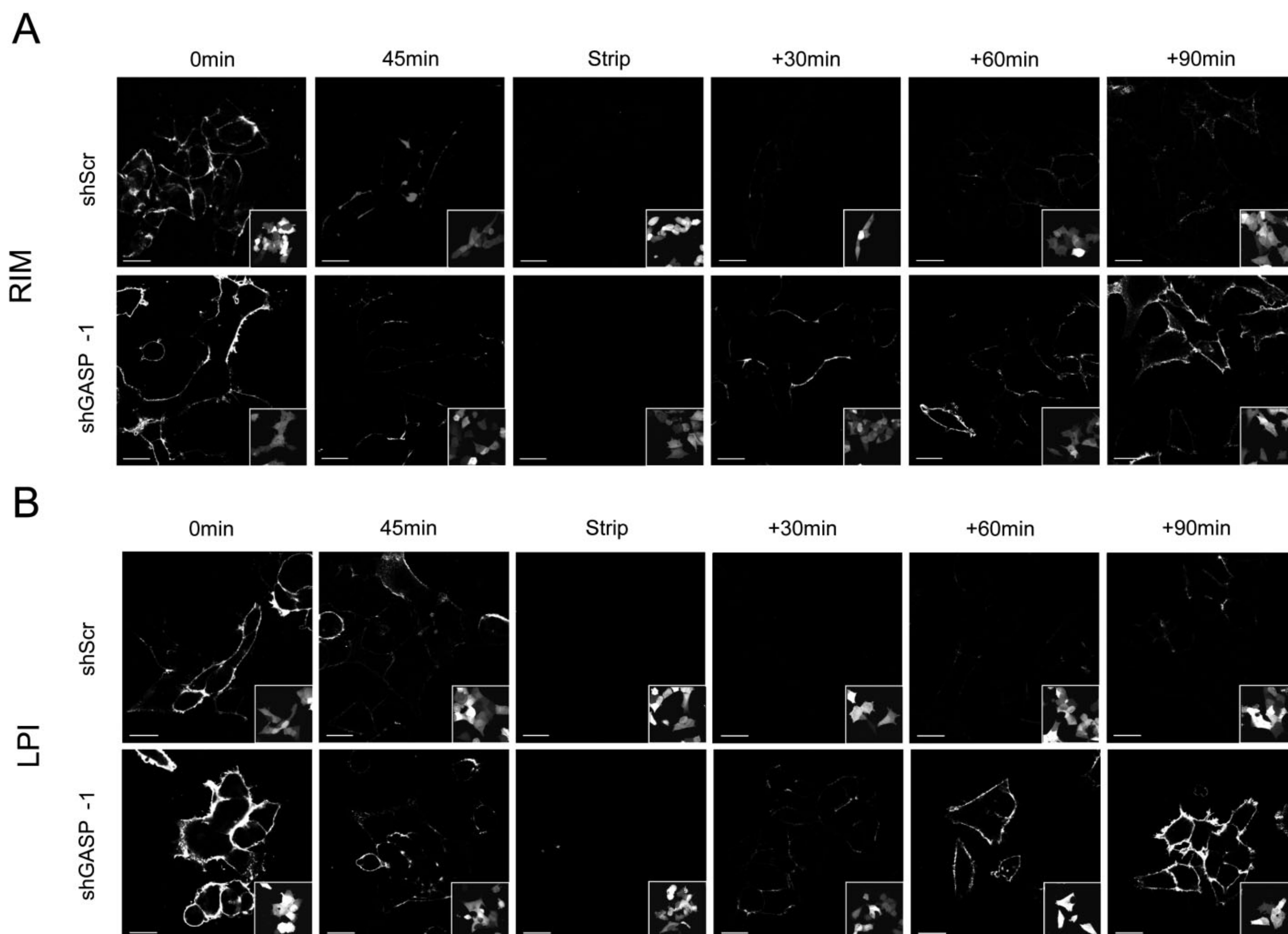

Figure 4

Recycling of GPR55 is enhanced in the absence of GASP-1. shScr (upper panels) or shGASP-1 (lower panels) infected GPR55-HEK cells were incubated with anti-FLAG antibody for 45 min (0 min) to label surface receptors. Cells were incubated with (A) 2.5 μM RIM or (B) 2.5 μM LPI for 45 min (45 min) to allow internalization and then stripped of surface antibody (Strip). Cells were incubated in warm medium for up to 90 min (+30 min, +60 min, +90 min) to allow degradation. In contrast to shScr-infected GPR55-HEK cells (A and B; upper panels), GPR55 recycled in shGASP-1-infected cells (A and B; lower panels). Insets indicate EGFP-shRNA-virus expression. Scale bar = 20 μm.

difference between LPI and rimonabant) in recruiting β-arrestin to GPR55 (Kapur *et al.*, 2009).

For centuries, cannabinoids have played an important role in medicine due to their psychoactive, analgesic and anti-inflammatory properties. Since then, many natural and synthetic compounds, which have the ability to bind CB_1 and CB_2 receptors, have been discovered. Recently, the GPCR GPR55 was 'de-orphanized' and characterized as a novel, non-CB_1/CB_2 cannabinoid receptor. Rimonabant – developed as a CB_1 receptor inverse agonist/antagonist – has recently attracted significant attention, since it was marketed for weight loss and smoking cessation. Due to adverse side effects, including the development of anxiety and depression in patients (Christensen *et al.*, 2007), rimonabant was withdrawn from the market. Recently, rimonabant has been shown to be not only an antagonist/inverse agonist at the CB_1 receptor but also an agonist at GPR55 (Ryberg *et al.*, 2007; Kapur *et al.*, 2009; Henstridge *et al.*, 2010), a potential off target effect that could be contributing to the adverse events. Although much higher concentrations of rimonabant than typically used for CB_1 receptor antagonism (5–15 nM) (see Pertwee *et al.*, 2010) are required to elicit an effect on GPR55 (Ryberg *et al.*, 2007; Henstridge *et al.*, 2010), a role for GPR55 activation needs to be considered where micromolar concentrations of rimonabant are present (i.e. during long-term treatment and/or accumulation in fatty tissues) such as the brain.

Intriguingly, the side effects of rimonabant were pervasive only after prolonged drug use, suggesting that they were not a consequence of acute activation/inhibition of GPR55/CB_1 receptors, but could be a consequence of prolonged agonist exposure. For instance, a prominent example of how prolonged agonist stimulation of a GPCR results in the development of adverse effects both *in vitro* (Finn and Whistler, 2001) and *in vivo* (Kim *et al.*, 2008) is that of the μ-opioid receptor in response to chronic morphine. As outlined in the introduction, prolonged exposure to agonists also results in the GASP-1-mediated down-regulation of D_2 (Bartlett *et al.*, 2005; Boeuf

et al., 2009; Thompson *et al.*, 2010) and CB_1 receptors (Martini *et al.*, 2007; 2010; Tappe-Theodor *et al.*, 2007) *in vitro* and *in vivo* and dramatically changes the behavioural responses to these drugs.

Here we used a model HEK293 cell culture system exogenously expressing FLAG-tagged GPR55 and showed that, likewise, GPR55 is down-regulated after exposure to rimonabant within 3 h, and that this process is dependent on GASP-1 (see Figure 3A). It is tempting to speculate that prolonged exposure to rimonabant would cause a down-regulation of GPR55 *in vivo* and thereby may contribute to the adverse side effects of this drug. However, in light of the ambidextrous role of rimonabant – i.e. being an antagonist of CB_1 and an agonist of GPR55 receptors – the relative effect of rimonabant on GPR55 and CB_1 receptors after prolonged use, and ultimately the adverse effects of this drug, have yet to be verified *in vivo*.

Recently, GPR55 has been shown to be highly expressed in malignant human tumours (Andradas *et al.*, 2011) and cancer cell lines (Ford *et al.*, 2010; Pineiro *et al.*, 2011), and its expression is correlated to tumour aggressiveness (Andradas *et al.*, 2011). Hence, by identifying GASP-1 as a key regulator of the trafficking and, by extension, functional expression of GPR55, we may be one step closer to gaining a better understanding of this receptor in response to cannabinoid drugs and its significance in pathogenesis.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 GASP-1 promotes the sorting of GPR55 to lysosomes. (A) GPR55-HEK cells infected with shScr lentivirus were fed anti-FLAG antibody and were either left untreated (0 min) or treated with 2.5 µM of RIM or LPI for 30 or 90 min. Receptors (green) were analysed for co-localization with the lysosomal markers LAMP1/2 (red) (B) GPR55-HEK cells were infected with shGASP-1 and treated as in panel A. No co-localization was observed for GPR55 (green) and LAMP1/2 (red) in shGASP-1 cells, but receptors were predominantly found on – or in vesicles close to – the cell surface. Inserts in FLAG-GPR55 panels indicate EGFP-shRNA-virus expression. Scale bars = 10 µM.
Figure S1 GPR55 does not internalize in response to vehicle. GPR55-HEK cells were infected with shScr (left panel) or shGASP-1 (right panel) lentivirus. In the presence and absence of GASP-1 biotinylated GPR55 (bio-GPR55, 100%) internalized in response to 45 min of RIM, but not vehicle (DMSO), stimulation.
Table S1 Endogenous GPR55 and GASP-1 expression in different cell lines. Undifferentiated (uHL60), differentiated (dHL60) human promyelocytic leukemia cells (HL60) and several colon cancer cell lines (HT-29, CaCo-2, DLD-1, SW480, SW620) were screened for endogenous GPR55 and GASP-1 expression by using either (i) real-time PCR or (ii) Western Blot techniques. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was obtained with High Capacity cDNA Transcriptase Kit (Applied Biosystems, Vienna, Austria). Real-time PCR was performed by using Fast SYBR Green PCR Master Mix (Applied Biosystems). GPR55 protein expression was determined by Western Blot analysis using a specific GPR55 antibody (Cayman, VWR Scientific, Vienna, Austria)

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