Mapping Pathways Downstream of Sphingosine 1-Phosphate Subtype 1 by Differential Chemical Perturbation and Proteomics\

Pedro J. Gonzalez-Cabrera, Timothy Hla, and Hugh Rosen

From the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037 and the Center for Vascular Biology, University of Connecticut Health Center, Farmington, Connecticut 06030-3501

Sphingosine 1-phosphate subtype 1 (S1P1) receptor agonists alter lymphocyte trafficking and endothelial barrier integrity in vivo. Among these is the potent, non-selective agonist, FTY720-P, whose mechanism of action has been suggested to correlate with S1P1 down-regulation. Discovery of the in vivo active S1P1-selective agonist, SEW2871, has broadened our understanding of minimal requirements for S1P1 function while highlighting differences regarding agonist effect on S1P1 fate, because SEW2871 does not degrade S1P1. To further understand the mechanism of agonist-induced S1P1 down-regulation, we compared signaling and fate of human S1P1-green fluorescent protein (GFP) in stable 293 cells, using AFD-R, a chiral analog of FTY720-P, SEW2871, and S1P. Although all agonists acutely internalized S1P1 to late endosomal vesicles and activated GTPγS binding and pERK to similar maxima, only AFD-R led to significant S1P1 down-regulation, as shown by GFP immunoprecipitation studies. Down-regulation was time- and concentration-dependent, was partially blocked by proteasomal inhibition and reversed by chloroquine and an antagonist to S1P1. All agonists induced a receptor-associated increase in ubiquitination, with AFD-R inducing 3-fold more accumulation than S1P and being 3–4 logs more potent than SEW2871. The formation of AFD-R-receptor ubiquitin complex was inhibited by antagonist and chloroquine and was enhanced by proteasomal inhibition. Identification of proteins by PAGE liquid chromatography-tandem mass spectrometry in cells treated with AFD-R confirmed the co-migration of ubiquitin peptides with those of S1P1 and GFP, relative to vehicle alone. These data suggest that the hierarchy of ubiquitin recruitment to S1P1 (AFD-R > S1P > SEW2871) correlates with the efficiency of lysosomal receptor degradation and reflects intrinsic differences between agonists.

Trafficking of agonist-stimulated G protein-coupled receptors (GPCRs) classically proceeds through time-dependent steps starting by acute (within minutes) internalization of receptors from plasma membrane into cytoplasmic vesicular compartments and followed by receptor sorting into either recycling or degradative pathways, which usually take place within hours of agonist treatment. The long term effects of agonists on GPCR fate are dependent on both the nature of the agonist as well as the cellular environment and are believed to be important determinants of agonist effectiveness. Several mechanisms implicated in early GPCR trafficking have been described and involve phosphorylation of agonist-stimulated receptor by GPCR kinases and subsequent binding of arrestin proteins to phosphorylated receptor sites and to adapter proteins such as clathrin and AP-2, which form coated-pit receptor complexes (reviewed in Refs. 1 and 2). Based on the stability of the arrestin-receptor complexes, GPCRs have been separated into class A receptors, which favor the recycling pathway, and class B receptors, which recycle slowly and are instead destined for degradation (3).

The ubiquitin-proteasome pathway has been shown to be involved in the trafficking and degradation of some GPCRs (reviewed in Refs. 4 and 5). Ubiquitin, a 76-amino acid protein, is known to conjugate via a conserved three-step enzymatic reaction to lysine residues of proteins that are destined for proteasomal degradation (6). As such, ligand-dependent GPCR ubiquitination has been shown to impact the down-regulation of CXCR4, protease-activated receptor-2, V1a vaspressin receptor, and β2-adrenergic receptors, in some cases, by functioning as a sorting signal for lysosomal receptor targeting (7–10).

Sphingosine 1-phosphate (S1P) is a secreted lipid that binds with nanomolar affinity to a family of five GPCRs, referred to as S1P1–5. Receptor coupling for S1P subtypes includes inhibition of adenyl cyclase, activation of the small G proteins Rac and Rho, and activation p42/p44 mitogen-activated protein and AKT kinases and calcium release (reviewed in Ref. 11). The physiological functions of S1P1 extend to multiple systems, including cardiovascular, lymphoid, and auditory, and are being unveiled through both genetic and pharmacological
approaches (11, 12). A well known outcome of S1P1 receptor agonist administration in vivo is the inhibition of lymphocyte egress from lymph node and thymus. Originally described for FTY720 (13), this pro-drug, once converted into its active phosphate-ester form (FTY720-P) by cellular sphingosine kinase 2, acts as a potent and non-selective agonist (activates S1P receptors 1, 3, 4, and 5), which induces and maintains blockade of lymphocyte egress. Studies in the S1P1, genetic knock-out mouse (14) and the discovery of S1P1-selective agonists (15, 16), later singled out the S1P1 receptor as the primary mediator of S1P signals that alter lymphocyte recirculation. In fact, we have provided evidence that administration of the selective S1P1 agonist, SEW2871, discovered from high throughput screening, induces dose-dependent and reversible lymphopenia in mice, with onset kinetics and magnitude similar to the FTY720-P chiral analog, AFD-R (15). Additional studies, intended to compare S1P1 agonist signaling in cell lines stably expressing S1P1, determined that SEW2871 recapitulates S1P effectors signaling and overlaps with S1P for key S1P1 pocket interactions, although at lower potency (17). Interestingly, fate of the receptor was significantly different with different agonists, and while stimulation with the physiological ligand S1P or SEW2871 supported S1P1-GFP recycling, FTY-720-P-treated cells did not lead to recycling, suggesting the existence of ligand-dependent differences in receptor fate within the same cellular environment. In the present study, we have investigated whether differences in agonist-induced receptor ubiquitination could account for this discrepancy in fate.

Here, we provide biochemical and proteomic evidence that S1P1 agonists induce recruitment of ubiquitin to S1P1, although by different magnitudes depending on the choice of agonist and despite similar ligands’ efficacy at acute receptor activation. We found that there is enhanced efficacy of receptor ubiquitination by AFD-R relative to SEW2871 or S1P, and this is strongly associated with receptor sorting to lysosomes and receptor down-regulation. Our model proposes that the extent of ubiquitin recruitment by distinct ligands, and thus receptor degradation, represents a ligand-regulated step in determining the fate of S1P1.

EXPERIMENTAL PROCEDURES

Materials—GTPγS35 was obtained from PerkinElmer Life Sciences. S1P was obtained from Biomol. The selective S1P1 agonist, SEW2871, was from Maybridge. The S1P receptor agonist, AFD-R (the phosphate-ester of the prodrug amino alcohol AAL(R)) and AAL(S) were gifts from Dr. Volker Brinkmann (Novartis Pharma). Anti-GFP antibodies and the mannose 6-phosphate (M6P) receptor antibody were from Abcam, anti-ubiquitin P4D1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and ERK antibodies from Cell Signaling. The proteasomal inhibitors (MG132 and lactacystin) were obtained from Calbiochem. Chloroquine and cycloheximide (CHX) were from Sigma-Aldrich.

Cell Culture—HEK293 cells stably expressing the GFP-tagged human S1P1 receptor (293-S1P1-GFP) and 293-vector-GFP cells (18) were maintained in regular growth medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution and selected with 500 µg/ml G418 (Invitrogen). Prior to every experiment, the growth medium was replaced with 0.2% charcoal-stripped fetal bovine serum (cs-FBS, HyClone) medium, and the cells were incubated overnight.

Detection of S1P1-GFP and Evaluation of Ligand-dependent Down-regulation—Confluent cells expressing S1P1-GFP or vector-GFP control grown in 6-well plates were washed twice in ice-cold PBS, and lysates were obtained by incubation in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton x-100) containing 1 mM NaVO4, 1 mM NaF, 0.5 mM β-glycerol phosphate and protease inhibitor mixture (Roche Applied Science). Cellular lysates were cleared by centrifugation (16,500 × g, 15 min), and the protein concentration of lysates supernatants was determined by the BCA (Pierce) method. Equal amounts of protein lysates were incubated overnight at 4°C with a monoclonal GFP antibody (1 µg of antibody per 400 µg of protein), followed by incubation (2 h, 4°C) with protein-A-Sepharose beads. The beads were recovered by centrifugation (10,000 × g, 1 min) and washing; three times in radioimmune precipitation assay buffer: PBS (1:1) without protease inhibitors and twice in PBS. The beads were suspended in Laemmli buffer containing 2-mercaptoethanol and boiled for 10 min, and proteins in the beads were separated by SDS-PAGE using either 4—12% gradient NuPage gels (Invitrogen) or 10% linear gels. The gels were transferred to polyvinylidene difluoride membranes, which were blocked in 5% milk and subsequently probed with a polyclonal GFP antibody (1:10,000; 1 h at room temperature) for detection of S1P1-GFP. Horseradish peroxidase-labeled goat anti-rabbit (1:5,000) antibodies were visualized by ECL chemiluminescence (Amersham Biosciences).

Agonist-induced down-regulation was measured in cells treated with 15 µg/ml CHX to prevent new receptor synthesis. Cells were exposed to either 10 µM SEW2871, 500 nM AFD-R, 500 nM S1P, or vehicle (0.1% fatty acid-free BSA) for the indicated times, and lysed as described above. S1P1-GFP down-regulation was analyzed by comparing the density of immunoprecipitated S1P1-GFP bands in agonist versus vehicle-treated cells, quantified by scanning densitometry using Kodak-1D Application software.

The effect of proteasomal inhibition, chloroquine, or the S1P1 competitive antagonist W146 (19) on AFD-R-stimulated S1P1-GFP down-regulation was studied by comparing intensity of S1P1-GFP bands in cells incubated with the respective agents (5 µM MG132, 10 µM lactacystin, 80 µM chloroquine, and 10 µM W146) for 30 min prior to and during incubation with AFD-R or vehicle for an additional 4 h.

Detection of Ubiquitinated S1P1-GFP—Ubiquitinated S1P1-GFP was probed for in the same lysates used for measuring S1P1-GFP down-regulation. Briefly, aliquots of boiled GFP immunoprecipitates were resolved by SDS-PAGE, and membranes were incubated overnight at 4°C with mouse monoclonal P4D1 antibody (1:100–1:1,800) and horseradish peroxidase-conjugated goat anti-mouse (1:5,000) secondary antibody.

AFD-R-stimulated S1P1 ubiquitination was additionally tested in HEK293 cells transiently transfected with N-terminal-tagged hemagglutinin (HA)-S1P1 (purchased from cDNA.org).
Cells were transfected in 6-well plates with either 3, 2, or 1 μg of receptor plasmid for 48 h using Lipofectamine 2000. Following transfection, cells were incubated with vehicle or 500 nM AFD-R for 1 h, and cellular lysates were obtained as described above. Receptor expression and AFD-R-stimulated receptor ubiquitination were determined by immunoblotting with an anti-HA (Bio-Rad) antibody at 1:1000 dilution.

**Acute Receptor Activation Experiments—Activation of ERK phosphorylation (pERK) by S1P, agonists was determined by incubating cells with either 10 μM SEW2871, 500 nM AFD-R, or 500 nM S1P for the indicated times. The conditions for determining activation of pERK were done as described previously (17). The potency (EC_{50}) and maximal responses of S1P, AFD-R, and SEW2871 in activating GTP binding were determined in S1P1-GFP membranes using GTPγS. Membrane preparation and conditions for binding were done as reported before (15), using 40-μg membranes per well. Data analysis was performed using GraphPad Prism (San Diego, CA).

**Imaging—**Single S1P1-GFP cells grown in 0.2% gelatin-coated coverslips were used to study ligand-induced localization with the late endosomal M6P-receptor marker. Incubation with agonists (500 nM AFD-R, 500 nM S1P, or 10 μM SEW2871) was terminated by removal of medium and washing with PBS. Cells were fixed in 3.7% paraformaldehyde in PBS for 10 min, permeabilized in PBS/0.1% Triton X-100 (PBST) for 30 min, and blocked for 30 min in PBST containing 1% BSA and 5% normal goat serum. Primary antibody incubation (1:1000) was performed in blocking buffer overnight at 4 °C. Secondary antibody (goat-anti-mouse Alexafluor-546) incubation was performed in blocking buffer for 30 min at room temperature. Coverslips were washed three times with PBS and mounted onto slides by using Gel Mount (Biomedica Corp.) mounting media. Cells were scanned with an Olympus BX61 scanning confocal fluorescence microscope. For detecting GFP, fluorescence was excited by using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected at 510–520 nm. For detecting Alexa Fluor 546, fluorescence was excited by using a helium-neon laser at a wavelength of 522 nm. In experiments using Lysotracker Red (Cambrex) dye (75 nM) was added to the medium 15 min before the end of agonist incubation. Cells were then washed and fixed with 3.7% paraformaldehyde in PBS for 10 min. For all experiments, images were processed with Adobe Photoshop 6.0.

**Chromatography and Mass Spectrometry—**AFD-R-stimulated ubiquitin recruitment to S1P1-GFP was determined using a scale-up GFP immunoprecipitation using identical conditions as above. Briefly, vehicle and AFD-R lysates isolated from five 150-μm plates per condition (vehicle or 500 nM AFD-R, 1-h incubation) were immunoprecipitated with anti-GFP (400 μg of protein to 1 μg of antibody), separated by SDS-PAGE (4–12% NuPage gels), and the gel was subsequently stained by Colloidal Blue. In-gel stained proteins derived from three separate gel fragments (bottom, 73–85 kDa; medium, 100–115 kDa; top, 150–170 kDa, see Fig. 8) were cut out from either vehicle or AFD-R lanes and sent for LC-MS/MS analysis. The gel fragments were chosen by imaging against a matched AFD-R-induced “ladder-like” ubiquitin receptor complex (run in an adjacent well of the same gel and derived from the same lysates). As controls, corresponding vehicle-only gel fragments were analyzed. The gel bands were excised and treated with 10 mM dithiothreitol to reduce disulfide linkages. Alkylation was performed with 55 mM iodoacetamide (Sigma-Aldrich) before digestion with trypsin (Promega) overnight at 37 °C using an estimated (1:30) enzyme to substrate ratio in 50 mM ammonium bicarbonate. The LC separation was performed on a linear ion mass spectrometer (LTQ, ThermoFisher Corp.). Data-dependent scanning was used to maximize the number of peptides sequenced in the highly complex mixture. This mode of operation uses preset criteria to select unique peptides on-the-fly for undergoing MS/MS. Over 10,000 MS/MS spectra were obtained during the run. These were searched using Mascot (Matrix Science, Ltd.) and Sequest (University of Washington, WA) search engine software using the NCBI (non-redundant data base). To improve searching efficiency, the taxonomic category was limited to mammalian proteins. Only peptides producing good quality fragmentation spectra and scoring higher than the threshold required for 95% confidence level for Mascot were used for protein identification.

**RESULTS**

We have shown previously (17) that stable HEK293 cells expressing C-terminal GFP-tagged human S1P1, differ in trafficking pattern when stimulated with FTY720-P (which induces receptor degradation), compared with S1P or SEW2871 (which induce receptor recycling). To further investigate agonist-induced receptor fate differences, we used the same HEK293-S1P1-GFP cell line (18) to compare three agonists (AFD-R, SEW2871, and S1P) for stimulating 1) receptor down-regulation, 2) acute receptor signaling (GTPγS binding and pERK activation), and 3) short- and long-term receptor trafficking. Additionally, we augmented biochemical and pharmacological data with a proteomics identification approach in exploring possible mechanism(s) of agonist-induced S1P1 fate.

Agonist-stimulated S1P1-GFP down-regulation was evaluated by immunoprecipitating the receptor with GFP antibodies (Fig. 1A). We could detect S1P1-GFP in transfected S1P1-GFP cells, but not in vector-GFP-expressing cells, as a band that migrated between 64 and 82 kDa, corresponding to the GFP-tagged human S1P1 (44-kDa S1P1 plus 27-kDa GFP). Heavy- and light-IgG chains were visible in both samples, and a GFP band (immediately above the light chain) was detected in vector-GFP-expressing cells.

Down-regulation was measured as the loss of receptor band to agonist stimulation in experiments in the presence of CHX. Treatment of S1P1-GFP cells with either AFD-R (500 nM), S1P (500 nM), SEW2871 (10 μM), or vehicle (0.1% fatty acid-free BSA) for 4 h led to the finding that AFD-R down-regulated approximately half (44 ± 2%) of total S1P1-GFP expression versus vehicle-treated cells (Fig. 1B, bottom). On the other hand,
there were no significant differences in S1P1-GFP expression in cells incubated for the same time with either SEW2871 or S1P. Because protein ubiquitination has been implicated in regulating GPCR trafficking and fate, we employed an antibody that recognizes ubiquitinated substrates (P4D1) and probed the same GFP immunoprecipitates used for determining agonist-induced receptor down-regulation. Fig. 1B shows that agonist incubation was associated with increases in the ubiquitination of high (\(\sim 115-180\) kDa) molecular mass protein(s), which we have referred to as the “ubiquitinated receptor complex.” Ubiquitinated receptor complex was dependent upon agonist stimulation in receptor-transfected cells only, because it was not found to be associated with either vehicle-treated S1P1-GFP cells or vector-GFP stimulated (using 500 nM S1P) cells. Interestingly, at the 4-h agonist incubation studied, the ubiquitinated receptor complex was found to differ in magnitude depending on agonist utilized, with AFD-R stimulating significantly (1.8- and 2.5-fold) higher ubiquitination relative to S1P and SEW2871, respectively.

A time course (0–6 h) of agonist-induced receptor down-regulation and associated ubiquitinated receptor complex is shown in Fig. 2. Again, depending on choice of agonist, two main differences in the measures were observed. First, only cells receiving AFD-R (500 nM) displayed significant receptor down-regulation relative to untreated cells. Down-regulation by AFD-R was apparent at 4 h (with a loss of approximately half of the total S1P1 expression), and continued at 6 h. In contrast, SEW2871 (10 \(\mu\)M) or S1P (500 nM) incubations did not significantly alter S1P1-GFP expression up to 6 h, relative to untreated control cells.

FIGURE 1. AFD-R promotes S1P1-GFP down-regulation and enhanced protein ubiquitination. Equal amounts of 293-vector-GFP or 293-S1P1-GFP cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with GFP antibodies to detect S1P1-GFP expression. A, S1P1-GFP expression was detected as a band running between 64 and 82 kDa (lane 2), whereas vector-GFP cells without insert did not express receptor (lane 1). B, vector GFP or S1P1-GFP cells were incubated with S1P1 agonists: AFD-R (500 nM), S1P (500 nM), SEW2871 (10 \(\mu\)M), or vehicle (0.1% fatty acid-free BSA) for 4 h in the presence of CHX. Lower panel, GFP immunoprecipitation-immunoblotting experiments from equivalent protein amounts revealed AFD-R-mediated S1P1-GFP down-regulation versus vehicle (Veh) treatment (n = 3). Upper panel, membranes were subsequently probed with P4D1 antibody for detection of agonist-induced ubiquitination. Note that P4D1 only recognized protein ubiquitination in ligand-stimulated, S1P1-GFP-expressing cells. The positions of molecular mass markers are indicated on the right (in kilodaltons). C, densitometric analysis of S1P1-GFP expression (left graph) and agonist-induced ubiquitinated-receptor complex (right graph) in cells treated with S1P1 agonists for 4 h. *, \(p < 0.05\) relative to vehicle alone (left graph); **, \(p < 0.05\) versus AFD-R treatment (right graph). Bars represent the mean ± S.E. of three independent experiments.

FIGURE 2. Time course of agonist-stimulated ubiquitin receptor complex and receptor down-regulation. 293-S1P1-GFP cells were incubated for the indicated times with either 500 nM AFD-R, 500 nM S1P, or 10 \(\mu\)M SEW2871 in the presence of CHX. A, equal amounts of protein lysates were immunoprecipitated (IP) with a GFP antibody, and the membranes were immunoblotted (IB) with either anti-GFP (lower panels) to detect receptor-GFP expression or P4D1 (upper panels) for determination of agonist-induced ubiquitinated receptor complex. B, densitometry analysis of receptor-GFP and ubiquitinated receptor complex following either AFD-R, SEW2871, or S1P stimulation. Time courses were plotted as the fraction of each agonist maximum, relative to unstimulated (time 0) cells. The graphs are from a representative experiment that was repeated twice with identical conditions.
A. Mapping Pathways Downstream of S1P

AFD-R (nM)  S1P (nM)  SEW2871 (µM)

IP: GFP

0 0.3 5 30 100 300 1000

B. AAL-S

P4D1

AFD-R (nM)  AAL-R  AAL-S

IP: GFP

0 0.1 1 10 100 1000

C. W146

IP: GFP

0 0.1 1 10 100

FIGURE 3. Ubiquitination of cellular substrates by distinct agonists displays vast differences in potency. 293-S1P1-GFP cells were incubated for 1 h with different concentrations of AFD-R, S1P, or SEW2871 in presence of CHX-A, equal amounts of protein lysates were immunoprecipitated (IP) with a GFP antibody and the membranes were immunoblotted (IB) with either anti-GFP (lower panels) to detect receptor-GFP expression or P4D1 (upper panels) for determination of agonist-induced ubiquitinated receptor complex. Representative blots shown from two independent experiments. B, densitometry analysis of ubiquitinated receptor complex following either AFD-R (■), SEW2871 (▲), or S1P (▼) stimulation. Time courses were plotted as fraction of AFD-R maximum, relative to unstimulated (vehicle) cells.

The specificity of AFD-R-induced ubiquitinated receptor complex and S1P1-GFP down-regulation in 293-S1P1-GFP cells. 293-S1P1-GFP cells were incubated with either 0.1% fatty acid-free BSA (Veh) or drugs (500 nM AFD-R, the active S1P agonist pro-drug AAL-R or its inactive stereoisomer AAL-S) for 4 h, and equal protein lysates immunoprecipitated (IP) and immunoblotted (IB) with GFP antibodies (lower panels) and P4D1 (upper panels) for detection of agonist-induced receptor down-regulation and ubiquitinated receptor complex, respectively. A, note that only AFD-R and AAL-R stimulated receptor down-regulation and induce ubiquitinated receptor complex, relative to vehicle, whereas the inactive compound (AAL-S) did not. W146, a selective S1P1 antagonist, reversed the AFD-R-mediated actions on down-regulation and ubiquitin receptor complex. Results of a representative experiment (4–12% gel) are shown (n = 3). B, AFD-R-induced S1P1-GFP down-regulation and ubiquitinated receptor complex are dependent on concentration of AFD-R (n = 3). C, the potency of AFD-R-stimulated S1P1 down-regulation was determined by plotting the intensity of S1P1-GFP versus agonist concentration (run in a 10% gel) as a percentage of S1P1-GFP density in unstimulated cells. —, refers to vehicle.

P. J. Gonzalez-Cabrera (2006), personal communication.
blockade of AFD-R-stimulated S1P1-GFP down-regulation and abolished detection of the ubiquitinated receptor complex.

AFD-R mediated S1P1-GFP down-regulation was also found to be concentration-dependent (Fig. 4B). The potency for inducing receptor down-regulation (AFD-R incubation was 4 h) was determined to be 7.1 nM. For these experiments, AFD-R-stimulated ubiquitin receptor complex was found to be ~10-fold more potent than receptor down-regulation, consistent with results obtained at 1-h AFD-R incubation (Fig. 3).

The discrepancy in agonist action in stimulating ubiquitin receptor complex and receptor down-regulation could be explained in part by differences in agonist intrinsic activities. To explore this possibility, we compared agonists for activating proximal receptor pathways such as pERK activation and GTPγS binding. Fig. 5A shows the kinetics of SEW2871-, AFD-R-, and S1P-activated pERK. Agonists stimulated ERK phosphorylation in a time-dependent manner, and, despite subtle differences in onset and time of maximal activation, neither agonist activated pERK beyond 30 min. Agonist-stimulated GTPγS binding was found to be concentration-dependent (Fig. 5B), and although there were differences in potency among agonists (SEW2871, 0.42 μM; AFD-R, 42 nM; and S1P, 0.9 nM), all agonists were found to be similarly efficacious (SEW2871, 90%; AFD-R, 100; and S1P, 90%; percent own maximal responses).

We next studied agonist-stimulated trafficking of S1P1-GFP by confocal microscopy, using two cytoplasmic vesicle markers: the mannose 6-phosphate (M6P) receptor, which associates with late endosomes and lysosomes, and Lysotracker® Red, a pH-sensitive dye, which labels lysosomes. Two time points were chosen, a 1-h protocol, which corresponded to maximal agonist-induced ubiquitin receptor complex, and 4 h, where agonist differences in down-regulation were first noticed. Fig. 6A shows that at 1 h, all agonists internalized S1P1-GFP from membrane to cytoplasmic vesicles, which were found to be colocalized in part with M6P-positive vesicles. On the contrary, no colocalization was observed between S1P1-GFP vesicles and Lysotracker® Red-stained vesicles at the 1-h treatment, irrespective of agonist utilized (Fig. 6B). The effect of 4 h agonist incubation on receptor-lysosome colocalization is shown in Fig. 6C. Here, S1P1-GFP vesicles internalized by AFD-R were found to completely colocalize with vesicles stained by Lysotracker® Red, whereas SEW2871 incubation did not stimulate appreciable GFP-Lysotracker® Red vesicle colocalization. A mixed population of Lysotracker® Red-positive and -negative S1P1-GFP vesicles was observed in S1P-treated cells at the 4-h incubation.

Trafficking of GPCRs within cells requires fusion of internalized receptor vesicles with those of acidic compartments (endosomal and lysosomal vesicles). We used the weak base, chloroquine, to determine the requirement of membrane fusion in AFD-R-stimulated ubiquitin receptor complex formation, and induced receptor down-regulation. In addition, because ubiquitination of proteins is known to lead to their degradation by the proteasome, we assessed the effect of two proteasomal inhibitors (MG132 and lactacystin) on stability of AFD-R-induced S1P1-GFP ubiquitin receptor complex and receptor down-regulation. Fig. 7 shows that 4 h after AFD-R stimulation, chloroquine inhibited the formation of receptor-ubiquitin complex and abolished AFD-R-mediated receptor down-regulation. Inhibition of AFD-R ubiquitin receptor complex by chloroquine was seen as early as 1 h (not shown). Incubation with either of the proteasomal inhibitors was shown to increase the molecular weight of the ubiquitinated receptor complex (relative to its migration versus AFD-R alone), while resulting in partial, but not significant inhibition of AFD-R-induced receptor down-regulation (by 35 ± 24% with MG132) and (by 26 ± 25% with lactacystin).

There is precedent for ligand-dependent GPCR down-regulation via recruitment of ubiquitin chains to lysine receptor residues; thus we looked for evidence of a ligand-induced receptor ubiquitination complex. Because the P4D1 antibody was not useful in our hands for immunoprecipitation, we explored this question using an LC-MS/MS proteomics scale-up method, and chose a 1-h AFD-R/vehicle incubation protocol (determined in previous experiments to be optimal for agonist P4D1 signaling). The results from LC-MS/MS analysis are summarized in Table 1. In all three fragments analyzed, there was detection of several peptides from human S1P1 receptor and several peptides from GFP, in both the treated and untreated groups. The peptides detected from human S1P1 spanned proximal, transmembrane, and distal regions of the
FIGURE 6. S1P₁-GFP trafficking following 1- or 4-h incubation with agonists. Cells expressing S1P₁-GFP were incubated with either 500 nM AFD-R, 500 nM S1P, or 10 μM SEW2871 for 1 h or 4 h. A, receptor-GFP (green) distribution was visualized by GFP fluorescence and distribution within late endosomes was detected using an antibody to M6P-Receptor (red). Note that, in unstimulated cells, S1P₁-GFP fluorescence is primarily localized to the plasma membrane, whereas agonist incubation results in internalization of receptor into cytoplasmic vesicles, some of which are found to colocalize with M6P-receptor vesicles (merged images, yellow). B, receptor-lysosome interaction was determined using the pH-sensitive marker Lysotracker® Red (LT-Red) as shown under “Experimental Procedures.” Note the lack of colocalization between agonist-internalized GFP vesicles and LT-Red vesicles at 1-h agonist incubation. C, at 4-h AFD-R incubation, there was complete colocalization of S1P₁-GFP vesicles with LT-Red vesicles, whereas internalized S1P₁-GFP by SEW2871 and S1P did not show complete LT-Red colocalization. Shown are representative images of one of three independent experiments.
receptor, and, as expected of tryptic fragmentation, all contained C-terminal KR residues.

The main difference between vehicle and AFD-R treatments was the identification of tryptic peptides corresponding to ubiquitin, found only in AFD-R treated cells. In fact, all three gel fragments isolated from the AFD-R-treated sample contained ubiquitin peptides (nearly 50% of ubiquitin content) relative to none detected in vehicle.

The proteomics data corroborated the immunoblotting data and strongly suggests that ubiquitin is recruited to S1P1-GFP upon agonist treatment.

The scale-up proteomics method provided additional information about S1P1-GFP expression. We noticed that in both vehicle- and AFD-R-treated groups, S1P1-GFP was detected as a single running band (monomer S1P1-GFP running at ~71 kDa), and as a higher molecular mass multimer (likely a dimer, ~150 kDa). Besides proteomics, the putative receptor dimer was also resolved in the scaled-up preparation by immunoprecipitation-immunoblotting experiments (Fig. 8).

Additional supporting evidence of ligand-induced S1P1 ubiquitination is shown in experiments in which HA-tagged S1P1 (HA fused to the N terminus) was transiently transfected into 293-cells. In supplemental Fig. S1, HA-tagged S1P1 was found to undergo AFD-R-stimulated HA-S1P1 ubiquitination, relative to vehicle-treated transfectants, as shown by the presence of a high molecular weight smear when immunoblotted with the HA antibody.

**DISCUSSION**

There is increasing evidence linking agonist-promoted ubiquitination of GPCRs with receptor sorting and degradation. Examples of ubiquitinated GPCRs include the μ and δ opioid receptors (23), the CXCR4 chemokine receptor (7), the β2-adrenergic receptor (9), the V2 vasopressin receptor (10), the sst3 somatostatin receptor (24), and the protease-activated receptor-2 (8). Although these studies have demonstrated the involvement of the ubiquitin-proteasome pathway in regulat-
Mapping Pathways Downstream of S1P₁

ing trafficking and fate of receptors, little is known regarding agonist efficacy of receptor ubiquitination and signaling and fate.

In the present study we used biochemical studies and proteomics to differentiate between two types of agonists with respect to S1P₁ fate. Using stable HEK293-S1P₁-GFP cells, we propose that there are supraphysiological agonists, such as AFD-R, which promote S1P₁ down-regulation, and physiological-like agonists, such as S1P and SEW2871, which do not promote significant S1P₁ down-regulation when incubated continuously (up to 6 h; Figs. 1 and 2) in the presence of CHX.

Combining proteomics and immunoprecipitation studies, we determined that following internalization, S1P₁ agonists commonly stimulate ubiquitination of the receptor, which persists throughout trafficking to late endosomes. The main finding was the strong association between the supraphysiological agonist-induced receptor down-regulation and lysosomal targeting and its intrinsic ability to stimulate ubiquitin-receptor immunocomplexes at relatively low concentrations. In fact, the threshold concentration to achieve optimal S1P₁ ubiquitination (determined in the time course to be 1 h for all agonists, Fig. 2), was found to be significantly left-shifted (by 3 and 4 logs) in AFD-R-treated cells versus S1P and SEW2871, respectively (Fig. 3); thus, making AFD-R a high potency full agonist in recruiting ubiquitin S1P₁-GFP relative to S1P or SEW2871. We found that receptor down-regulation occurred at 10-fold higher AFD-R concentration than optimal receptor ubiquitination, suggesting that a certain level of ubiquitin-receptor recruitment may be necessary for receptor degradation in lysosomes (i.e. beyond that stimulated by SEW2871 or S1P herein). Furthermore, it is interesting that receptor ubiquitination by AFD-R was readily detectable at a concentration as low as 0.5 nm, similar to the reported affinity of its chiral analog, FTY720-P, in activating recombinant systems (13), and within the reported EC₅₀ value for AFD-R in vivo actions on lymphocyte sequestration and CD69 thymocyte maturation, reported to be 0.7 nm (25).

Specificity of AFD-R functions on ubiquitin recruitment and down-regulation came from studies with the inactive (S)-isomer of the agonist AAL (26), which had no effect on receptor ubiquitination or down-regulation (Fig. 4A). Further, the S1P₁-selective antagonist W146 (19) was able to reverse the actions of AFD-R on both measures. In addition, we were able to reproduce AFD-R-dependent receptor ubiquitination in transiently transfected HEK293-cells with HA-tagged receptors (see supplemental Fig. S1), ruling out an artifact induced by the S1P₁-GFP fusion construct.

These differences in agonist-mediated S1P₁ fate and/or extent of receptor ubiquitination were found to be independent of agonist intrinsic activity (Fig. 5), because all agonists reached comparable maximal activation profiles in GTP binding and similar kinetics in activating ERK phosphorylation. The potency values calculated in the present study for activating agonist GTP binding and pERK kinetics are within reported values for drug actions in other recombinant systems and primary human umbilical vein endothelial cells (13, 15, 17). This suggested that agonist-stimulated receptor fate decisions seem likely to take place distal to receptor activation. In fact, incuba-
biquitination, and our studies indicate that the amount of polyubiquitinated S1P₁ can be modulated depending on the choice of agonist.

The proteomic analysis of the ubiquitin ladder stimulated by AFD-R revealed the identity of ubiquitinated S1P₁-GFP, as compared with vehicle control. Interestingly, we were able to resolve monomer and multimer versions of S1P₁ in the scale-up proteomic preparation. We attribute detection of the higher molecular weight S1P₁-GFP version to the scale-up method, rather than a gel-running artifact, because the ligand-induced “ubiquitin ladder” pattern ran unchanged from previous results in which only monomers were detected, and the multimer S1P₁-GFP version was also found to be ubiquitinated by AFD-R (see Fig. 8). Detection of multimeric GPCR versions by similar immunoprecipitation methods is not uncommon, as in the case of the β₁-adrenergic (9), V₂ vasopressin (28), and recently shown for S1P₁ (29).

There seems to be GPCR species differences in the utilization of the ubiquitin-proteasome pathway for receptor trafficking. For instance, β₁ adrenergic receptors are entirely resistant to ubiquitination (30), whereas the β₂ receptor is dependent on the ubiquitin-proteasome for internalization and trafficking (9). In some cases, such as the V₂ vasopressin receptor, blocking the proteasome is a requirement for detection of ligand-induced receptor ubiquitination (10). The use of proteasomal inhibitors in this study suggested that preserving ubiquitinated receptor (as seen by the shift in electrophoretic mobility of the ubiquitinated smear in Western blots), has a modest effect on AFD-R-induced receptor down-regulation. Because MG132 increased dose-dependently the accumulation of polyubiquitinated receptor in the absence of ligand (not shown), it is likely that the proteasome may also be involved in basal receptor turnover, as is the case for opioid μ and δ subtypes (23).

Our model suggests the involvement of a ligand-dependent ubiquitin-stimulated lysosomal targeting pathway that degrades S1P₁. Accumulation of polyubiquitinated receptor by proteasome inhibition alone also suggests a ligand-independent pathway that may be responsible for maintaining balance between synthesis and degradation at equilibrium.

Taken together, these results strongly suggest that ligand-induced S1P₁ ubiquitination serves as a sorting signal for lysosomal receptor degradation and that ubiquitin recruitment to S1P₁ can be down-modulated using in vivo active physiologically-like agonists, such as SEW2871. We have shown recently that a selective competitive S1P₁ antagonist (19) reversed SEW2871-mediated lymphopenia in vivo without having a measurable effect on lymphocyte recirculation when tested alone. In addition, two photon studies indicated that S1P₁ antagonism was able to restore SEW2871-arrested lymphocyte movement in lymph node medulla. These results, which have now been reproduced by an independent group using a chemically distinct S1P₁-competitive antagonist (31), collectively disfavor functional antagonism as the mechanism of S1P₁-mediated lymphopenia. Compelling evidence exists for FTY720-P actions on S1P₁-rich endothelium (as opposed to lymphocytes that express few cell surface S1P₁ receptors), and a study by Singer et al. (32) demonstrated that mice treated with FTY720-P up-regulate endothelial junctional proteins (CD31, β-catenin, and ZO-1) and S1P₁ receptor expression in lymph node, presumably leading to an increased endothelial barrier integrity. Consistent with an endothelial target for S1P₁ agonists, S1P₁ antagonist studies have demonstrated a prominent role for S1P₁-S1P₁ tone in the maintenance of lung vascular endothelial integrity (19, 31), suggesting that minimalist S1P₁-like agonists that do not significantly down-regulate S1P₁ may be better suited in long term studies to preserve lung endothelial receptor reserve. Finally, both SEW2871 (15, 17) and S1P (13) define the minimal signaling requirements for inducing and maintaining reversible S1P₁-induced lymphopenia, yet neither lead to significant S1P₁ down-regulation. These data suggest that AFD-R and SEW2871/S1P alter receptor fate and ubiquitination to different extents and that S1P₁ degradation is not an essential shared downstream outcome of agonist action; therefore, it is not essential for induction of lymphopenia and therapeutic efficacy.

Acknowledgments—We thank S. R. Saldana, A. P. Semana, and J. O. Verango for expert technical assistance and Drs. S. A. Traeger and G. Siuzdak from The Scripps Research Institute Mass Spectrometry Core Facility for LC-MS/MS analysis.

REFERENCES

1. Prossnitz, E. R. (2004) Life Sci. 75, 893–899
2. Luttrell, L. M. (2006) Methods Mol. Biol. 332, 3–49
3. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) J. Biol. Chem. 275, 17201–17210
4. Wojcikiewicz, R. J. (2004) Trends Pharmacol. Sci. 25, 35–41
5. Marchese, A., and Benovic, J. L. (2004) Methods Mol. Biol. 259, 299–305
6. Ciechanover, A. (2006) Exp. Biol. Med. 231, 1197–1211
7. Marchese, A., and Benovic, J. L. (2001) J. Biol. Chem. 276, 45509–45512
8. Jacob, C., Cottrell, G. S., Gehringer, D., Schmidlin, F., Grady, E. F., and Bunnett, N. W. (2005) J. Biol. Chem. 280, 16076–16087
9. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) Science 294, 1307–1313
10. Martin, N. P., Lefkowitz, R. J., and Shenoy, S. K. (2003) J. Biol. Chem. 278, 45954–45959
11. Chun, J., and Rosen, H. (2006) Curr. Pharm. Des. 12, 161–171
12. MacLennan, A. J., Benner, S. J., Andringa, A., Chaves, A. H., Rosing, J. L., Vesey, R., Karpman, A. M., Cronier, S. A., Lee, N., Erway, L. C., and Miller, M. L. (2006) Hear. Res. 220, 38–48
13. Mandalia, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., and Rosen, H. (2002) Science 296, 346–349
14. Matloubian, M., Lo, C. G., Cinnamon, G., Lesneski, M. J., Xu, Y., Brinkman, V., Allende, M. L., Proia, R. L., and Cyster, J. G. (2004) Nature 427, 355–360
15. Sanna, M. G., Liao, J., Jo, E., Alfonso, C., A., Ahn, M. Y., Peterson, M. S., Webb, B., Lefebvre, S., Chun, J., Gray, N., and Rosen, H. (2004) J. Biol. Chem. 279, 13839–13848
16. Forrest, M., Sun, S. Y., Hajdu, R., Bergstrom, J., Card, D., Doherty, G., Hale, J., Keohane, C., Meyers, C., Milligan, J., Mills, S., Nomura, N., Rosen, H., Rosenbach, M., Shei, G. J., Singer, I. I., Tian, M., West, S., White, V., Xie, J., Proia, R. L., and Mandalia, S. (2004) J. Pharmacol. Exp. Ther. 309, 758–768
17. Jo, E., Sanna, M. G., Gonzalez-Cabrera, P. J., Thangada, S., Tigi, G., Osborne, D. A., Hla, T., Parrill, A. L., and Rosen, H. (2005) J. Biol. Chem. 290, 703–715
18. Liu, C. H., Thangada, S., Lee, M. I., Van Brocklyn, J. R., Spiegel, S., and Hla, T. (1999) Mol. Biol. Cell 10, 1179–1190
19. Sanna, M. G., Wang, S. K., Gonzalez-Cabrera, P. J., Don, A., Marsolais, D.,
Mapping Pathways Downstream of S1P₁

20. Paugh, S. W., Payne, S. G., Barbour, S. E., Milstien, S., and Spiegel, S. (2003) FEBS Lett. 554, 189–193
21. Dugast, M., Toussaint, H., Dousset, C., and Benaroch, P. (2005) J. Biol. Chem. 280, 19656–19664
22. Lemieux, B., Percival, M. D., and Falgueyret, J. P. (2004) Anal. Biochem. 327, 247–251
23. Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001) J. Biol. Chem. 276, 12345–12365
24. Tulipano, G., Stumm, R., Pfeiffer, M., Kreienkamp, H. J., Hollt, V., and Schulz, S. (2004) J. Biol. Chem. 279, 21374–21382
25. Alfonso, C., McHeyzer-Williams, M. G., and Rosen, H. (2006) Eur. J. Immunol. 36, 149–159
26. Rosen, H., Alfonso, C., Surh, C. D., and McHeyzer-Williams, M. G. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10907–10912
27. Urbe, S. (2005) Essays Biochem. 41, 81–98
28. Bouley, R., Lin, H. Y., Raychowdhury, M. K., Marshansky, V., Brown, D., and Ausiello, D. A. (2005) Am. J. Physiol. 288, C1390–C1401
29. Zaslavsky, A., Singh, L. S., Tan, H., Ding, H., Liang, Z., and Xu, Y. (2006) Biochim. Biophys. Acta 1761, 1200–1212
30. Liang, W., and Fishman, P. H. (2004) J. Biol. Chem. 279, 46882–46889
31. Foss, F. W., Jr., Snyder, A. H., Davis, M. D., Rouse, M., Okusa, M. D., Lynch, K. R., and Macdonald, T. L. (2007) Bioorg. Med. Chem. 15, 663–677
32. Singer, I. I., Tian, M., Wickham, L. A., Lin, J., Matheravidathu, S. S., Forrest, M. J., Mandala, S., and Quackenbush, E. J. (2005) J. Immunol. 175, 7151–7161