Hepatocyte Growth Factor-regulated Tyrosine Kinase Substrate (HRS) Mediates Post-endocytic Trafficking of Protease-activated Receptor 2 and Calcitonin Receptor-like Receptor*  

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The E3 ligase c-Cbl ubiquitinates protease-activated receptor 2 (PAR2), which is required for post-endocytic sorting of PAR2 to lysosomes, where degradation arrests signaling. The mechanisms of post-endocytic sorting of ubiquitinated receptors are incompletely understood. Here, we investigated the role of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), in post-endocytic sorting and signaling of PAR2. In HEK-PAR2 cells, PAR2-activating peptide (PAR2-AP) induced PAR2 trafficking from the cell surface to early endosomes containing endogenous HRS, and then to lysosomes. HRS overexpression or knockdown with small interfering RNA caused formation of enlarged HRS-positive endosomes, where activated PAR2 and c-Cbl accumulated, and PAR2 failed to traffic to lysosomes. Overexpression of HRS prevented PAR2-AP-induced degradation of PAR2 as determined by Western blotting. Overexpression of HRS mutant lacking an ubiquitin-binding motif similarly caused retention of PAR2 in enlarged endosomes. Moreover, HRS overexpression or knockdown caused retention of ubiquitin-resistant PAR2Δ14K/R in enlarged HRS-containing endosomes, preventing recycling and resensitization of PAR2Δ14K/R. HRS overexpression or knockdown similarly prevented lysosomal trafficking and recycling of calcitonin receptor-like receptor, a non-ubiquitinated receptor that traffics to lysosomes after sustained activation and recycles after transient activation. Thus, HRS plays a critically important role in the post-endocytic sorting of single receptors, PAR2 and CLR, to both degradative and recycling pathways. This sorting role for HRS is independent of its ubiquitin-interacting motif, and it can regulate trafficking of both ubiquitinated and non-ubiquitinated PAR2 and non-ubiquitinated CLR. The ultimate sorting decision to degradative or recycling pathways appears to occur downstream from HRS.

Many G protein-coupled receptors (GPCRs) 2 are rapidly endocytosed after agonist binding, but the pathway of post-endocytic trafficking depends on the receptor and the nature of the stimulus. Some GPCRs are sorted to lysosomes or proteasomes, where degradation irreversibly inactivates internalized receptors and prevents uncontrolled signaling during chronic stimulation (1–4). Other GPCRs recycle to the plasma membrane, which mediates resensitization of signal transduction (5–8). Our understanding of the mechanisms underlying the critical sorting of GPCRs to these divergent pathways, degradative or recycling, is incomplete and controversial. Ubiquitination of certain receptors, exemplified by protease-activated receptor 2 (PAR2) and the chemokine (C-X-C motif) receptor 4 (CXCR4), serves as a signal for sorting receptors into a lysosomal or proteasomal degradation pathway (4, 9–12). However, other receptors, including the δ-opioid receptor and calcitonin receptor-like receptor (CLR) (8, 13, 14), traffic to the degradative pathway by ubiquitin-independent mechanisms. It is important to elucidate the sorting machinery that diverts a receptor, in an ubiquitin-dependent or independent manner, to lysosomes or the plasma membrane, as this trafficking regulates cellular responses to agonists.

Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is a 115-kDa endosomal protein that has attracted much interest in this context (14–18). HRS associates with early endosomes as part of a multiprotein complex that has a key role in sorting ubiquitinated proteins to lysosomes (19–24). It has an ubiquitin-interacting motif (UIM) and can bind directly to ubiquitinated receptors, resulting in their accumulation in internal vesicles of multivesicular bodies and their delivery to lysosomes (21, 25). Non-ubiquitinated proteins are not recognized by HRS and escape sorting to the lysosomal pathway, enabling them to recycle (21, 26). However, recent studies have identified additional roles of HRS in controlling trafficking on non-ubiquitinated GPCRs to degradative and recycling pathways. Thus, HRS mediates lysosomal trafficking of the non-ubiquitinated δ-opioid receptor, and mediates recycling of the ubiquitinated β2-adrenergic receptor and μ-opioid receptor (14, 17). Thus, the function of HRS in post-endocytic sorting depends on the GPCR in question and its ubiquitination.

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We examined the role of HRS in trafficking and signaling of PAR_2, a GPCR that is cleaved and activated by several serine proteases that are generated during injury and inflammation (27–29). These proteases cleave PAR_2 to expose a tethered ligand domain that binds to and activates the cleaved receptor (27, 28). Given the irreversible nature of proteolytic activation of PAR_2, and its proinflammatory and nociceptive actions (29), mechanisms that arrest PAR_2 signaling are of considerable interest. Permanent signal arrest requires PAR_2 degradation in lysosomes, but the post-endocytic sorting mechanisms that target the receptor to lysosomes are incompletely understood (30, 31). The E3 ubiquitin ligase c-Cbl monoubiquitinitates PAR_2 at multiple sites, which is necessary for lysosomal trafficking, because a non-ubiquitinated PAR_2 mutant, PAR_2ΔΔ14K/R, escapes degradation and recycles (4). Investigation of the role of HRS in post-endocytic sorting of PAR_2 to lysosomes and PAR_2ΔΔ14K/R to the plasma membrane provided a unique opportunity to define the role of HRS in the post-endocytic trafficking of a single receptor with divergent fates after internalization.

For comparison, we also examined the role of HRS in the post-endocytic sorting of the receptor for calcitonin gene-related peptide (CGRP), a heterodimer of calcitonin receptor-like receptor (CLR), a GPCR, and receptor activity-modifying protein 1 (RAMP1), a protein with a single transmembrane domain. An understanding of the control of CLR and RAMP1 is important because CGRP is a potent vasodilator and mediator of neurogenic inflammation and pain transmission (32). CLR and RAMP1 recycle after transient stimulation with CGRP, but during continuous stimulation traffic to lysosomes by processes that do not require receptor ubiquitination (8). These studies enabled us to evaluate the role of HRS in the divergent trafficking of a non-ubiquitinated heterodimeric receptor to lysosomes or the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies were from the following sources: mouse anti-HRS from Alexis Biochemicals, Switzerland; rabbit anti-FLAG, rabbit anti-HA11, rabbit anti-Myc, mouse anti-β-actin, and rabbit anti-γ-tubulin from Sigma; mouse anti-human transferrin receptor (TIR) from Zymed Laboratories; mouse anti-early endosomal antigen-1 (EEA1) from BD Transduction Laboratories, Lexington, KY; rabbit anti-early endosomal antigen-1 (EEA1) from Calbiochem, San Diego, CA; mouse anti-human lysosomal-associated glycosomal protein-1 (LAMP1) from Developmental Studies Hybridoma Bank, Iowa City, IA; rat high affinity anti-HA11 from Roche Applied Science; goat anti-mouse, -rat, or -rabbit IgG coupled to horseradish peroxidase, fluorescein isothiocyanate, or rhodamine red-X from Jackson ImmunoResearch, West Grove, PA; goat anti-mouse or rabbit IgG coupled to Alexa Fluor® 680 from Invitrogen, and coupled to IRDye™ 800 from Rockland Immunocchemicals, Gilbertsville, PA.

cDNAs and siRNA—The pcDNA5/FRT plasmid was from Invitrogen. cDNA encoding c-Cbl with N-terminal HA11 was a gift from Dr. C. Thien (University of Western Australia) (33). Myc-tagged HRS, Myc-tagged HRS lacking the UIM (HRSΔUIM), and Myc-tagged HRSΔVHS (all in pcDNA3) were gifts from Dr. M. von Zastrow, Dr. J. N. Hislop, and Dr. A. C. Hanyaloglu (University of California, San Francisco, CA). cDNAs for PAR_2, PAR_2ΔΔ14K/R, CLR, and RAMP1 have been described (4, 8). Knockdown of HRS using siRNA was achieved by transfection of duplex RNA oligonucleotides (Qiagen, Valencia, CA) corresponding to part of the coding region of human HRS (CGACAGAAGCCACAGTGC, HRS-siRNA1), as described (14, 34). To further confirm HRS knockdown specificity, a distinct siRNA directed against a second target sequence in the human HRS transcript (GCACGTCTTCCAGAATTCAA, HRS-siRNA2) was also used (35). In control experiments, cells were transfected with nonsense duplex RNA oligonucleotide (ATTCTG-CGAAACGTGTGCAG).

**Transfected Cells and Cell Lines**—Human embryonic kidney 293 (HEK) and Henrietta Lacks (HeLa) cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum in 95% air, 5% CO_2 at 37 °C. The generation and maintenance of HEK-FLP cells (Invitrogen) stably expressing PAR_2 with an N-terminal FLAG epitope and a C-terminal HA11 or T7 epitope, or the ubiquitin-defective PAR_2 mutant (PAR_2ΔΔ14K/R) with an N-terminal FLAG epitope and a C-terminal HA11 epitope (HEK-PAR_2), or CLR with an N-terminal HA11 epitope and RAMP1 with an N-terminal Myc epitope (HEK-CLR-RAMP1) have been described (4, 8, 36). In some experiments, HEK cells were transiently transfected using Lipo-fectamine™ 2000 according to the manufacturer's guidelines (Invitrogen). Cells were plated 48 h prior to the experiments. Endogenous HRS was knocked-down in HeLa cells using siRNA. Cells were plated in 6-well plates at 80% confluence. After 16 h, cells were transfected with duplex RNA strands (HRS-siRNA1, HRS-siRNA2, or control siRNA) using Lipofectamine™ 2000. After 24 h, cells were transfected again with cDNA for PAR_2-HA11 or CLR-HA11 and RAMP1. Cells were studied 48 h after the second transfection.

**Activation of PAR_2 or CLR and Drug Treatments**—PAR_2 activating peptide (corresponding to the tethered ligand of rat/mouse PAR_2, SLIGRL-NH_2, CPC Scientific, San Jose, CA) and rat α-CGRP (Bachem, Torrance, CA) were used to activate PAR_2 and CLR/RAMP1, respectively. HEK-PAR_2 or HEK-CLR-RAMP1 cells were washed three times with PBS and placed in Dulbecco's modified Eagle's medium, 0.1% bovine serum albumin. Cells were stimulated with PAR_2-AP (100 μM, various times) or CGRP (100 nM, various times). To inhibit new protein synthesis (during degradation assays and during calcium experiments), cells were incubated with cycloheximide (140 μM). To interfere with Golgi vesicular transport of PAR_2 (during calcium experiments), cells were incubated with brefeldin A (10 μg/ml) (Sigma). Inhibitors were preincubated with cells 1 h prior to stimulation. Controls included the appropriate vehicle.

**Immunochemistry and Confocal Microscopy**—HEK or HeLa cells were plated at ~3 × 10^5 per 35-mm dish onto coverslips coated with poly-D-lysine (100 μg/ml). Cells were washed in 100 mM PBS, pH 7.4, and fixed in PBS containing 4% paraformaldehyde, pH 7.4 (20 min, 4 °C). Cells were
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washed with PBS containing 0.1% saponin and 1% heat-inactivated normal goat serum for 30 min. Proteins were localized using the primary antibodies: HRS (1:200), HA11 (rabbit, 1:200), HA11 (rat, 1:200), EEA1 (mouse, 1:500), EEA1 (rabbit, 1:1,000), LAMP1 (1:1,000), Myc (1:500), and TIR (1:1,000) (overnight, 4 °C). Cells were washed for 30 min with PBS containing 0.1% saponin and 1% normal goat serum, and incubated with secondary antibodies conjugated to fluorescein isothiocyanate or rhodamine red-X (1:200, 2 h at room temperature). To examine trafficking of receptors from the plasma membrane, thereby avoiding confounding effects of receptors present in internal pools, we labeled receptors at the cell surface with antibodies to extracellular epitope tags. Cells expressing FLAG-PAR₂ or HA11-CLR-RAMP1 were incubated with rabbit anti-FLAG (to detect PAR₂, 1:100) or rabbit anti-HA11 (to detect CLR, 1:100) for 1 h at 37 °C. Cells were washed with PBS and stimulated with PAR₂-AP or CGRP. Cells were fixed at specified times, washed, and incubated with fluorescent secondary antibodies. These antibody-tagged receptors traffic similarly to non-tagged receptors (4, 8). Cells were observed with a Zeiss laser-scanning confocal microscope (LSM Meta 510) using a Plan-Apochromat ×63 oil immersion objective with a 1.4 numerical aperture. Images were collected and simultaneously processed (colored and merged) with the Zeiss (LSM 510) software.

Image Analysis—Data were analyzed using Zeiss LSM 510 software. Targeting of recycling receptors to the plasma membrane was analyzed by drawing regions of interest on the outside and the inside of the plasma membrane, which allowed determination of the percentage of total cellular fluorescence at the plasma membrane, as previously described (37). Colocalization of two proteins in organelles was analyzed by drawing regions of interest around the outside of a cell in the merged image and measuring the overlap coefficient, with a coefficient of 0 indicating no colocalization and of 1 indicating complete co-localization. >20 cells were analyzed for each experiment.

SDS-PAGE and Western Blotting—Cells were plated at 1 × 10⁶ cells per 35-mm dish coated with poly-D-lysine. Cells were lysed in 50 mM Tris/HCl, pH 7.4, 1% SDS, boiled and centrifuged. Lysates (10 µg of protein) were separated by SDS-PAGE (8% acrylamide gels). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-FL, Millipore, Billerica, MA) and blocked for 1 h at room temperature (Odyssey Blocking Buffer, LiCOR, Lincoln, NE). Membranes were incubated with antibodies to rabbit HA11 (1:5,000), HRS (1:1,000), and β-actin (1:20,000, overnight at 4 °C (Odyssey Blocking Buffer). Membranes were washed for 30 min (1× PBS, 0.1% Tween 20) and incubated with secondary antibodies conjugated to Alexa Fluor® 680 or IRDye™ 800 (1:20,000) (1 h at room temperature), and blots were analyzed with the Odyssey Infrared Imaging System (LiCOR). To quantify PAR₂ degradation, PAR₂ signals were compared with β-actin signals.

Measurement of [Ca²⁺]:—PAR₂ signaling was evaluated by measuring [Ca²⁺]. Cells were plated at 1.2 × 10⁶ cells per 35-mm dish onto coverslips coated with poly-D-lysine. Cells were washed, incubated in Hank’s balanced salt solution containing Ca²⁺ and Mg²⁺, 0.1% bovine serum albumin, 20 mM HEPES, pH 7.4, with 2.5 µM fura-2 acetoxyethyl ester (Invitrogen) for 20 min at 37 °C and washed. Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission in a F-2500 spectrophotometer (Hitachi Instruments, Irvine, CA). The ratio of the fluorescence at the two excitation wavelengths, which is proportional to [Ca²⁺]i, was calculated. To assess desensitization and resensitization, cells were exposed to PAR₂-AP (100 µM) or vehicle (control) for 1 h at 37 °C, washed 3 times, and then challenged a second time with PAR₂-AP (100 µM) at 0 or 4 h after washing. The response to the second challenge was measured.
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**RESULTS**

**PAR\(_2\) Traffics to Early Endosomes Containing HRS**—As a first step toward examining the role of HRS in post-endocytic trafficking of PAR\(_2\), we localized endogenous HRS and overexpressed Myc-HRS in HEK cells by immunofluorescence, and determined whether these proteins were in endosomes. We found that in cells not overexpressing HRS, endogenous immunoreactive HRS was detected in multiple small vesicles containing EEA1, which are thus early endosomes (Fig. 1A). In cells expressing Myc-HRS, antibodies to HRS and Myc both labeled the same vesicles (Fig. 1B). As previously reported, overexpressed HRS was present in enlarged early endosomes (Fig. 1B) (17). Thus, the HRS antibody specifically recognizes HRS, and HRS is present in early endosomes.

To assess the role of HRS in the trafficking of PAR\(_2\), we localized endogenous HRS in HEK-PAR\(_2\) cells. We observed that in unstimulated cells, PAR\(_2\) was detected at the plasma membrane and in a perinuclear location, which we have previously identified as the Golgi apparatus (30, 31), whereas HRS was restricted to endosomes (21) (Fig. 1A). In stimulated cells expressing Myc-HRS, antibodies to HRS and Myc both labeled the same vesicles (Fig. 1B). As previously reported, overexpressed HRS was present in enlarged early endosomes (Fig. 1B) (17). Thus, the HRS antibody specifically recognizes HRS, and HRS is present in early endosomes.

**HRS Overexpression Causes Accumulation of PAR\(_2\) and c-Cbl in Enlarged Endosomes**—The overexpression of HRS disrupts function similarly to HRS knockdown, inducing formation of enlarged endosomes (14, 17). Therefore, we examined the effects of overexpression of HRS on the trafficking of PAR\(_2\). HEK cells stably expressing

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**FIGURE 2. Effects of HRS overexpression on trafficking of PAR\(_2\) and c-Cbl.** A, HEK-PAR\(_2\) cells were transiently transfected with Myc-HRS and challenged with PAR\(_2\)-AP (0, 30, or 120 min). Proteins were localized by indirect immunofluorescence using HA11 (PAR\(_2\)) and HRS antibodies. In unstimulated cells, PAR\(_2\) was located at the plasma membrane (arrowheads) and in enlarged HRS-containing endosomes (arrows), which probably represents constitutively trafficked PAR\(_2\). In stimulated cells, PAR\(_2\) was depleted from the plasma membrane and detected in HRS-containing endosomes (arrows). B, HEK-PAR\(_2\) cells were transiently transfected with HA11-c-Cbl and Myc-HRS and challenged with PAR\(_2\)-AP (0, 30, or 120 min). Proteins were localized by indirect immunofluorescence using HA11 (c-Cbl) and Myc (HRS) antibodies. In unstimulated cells, c-Cbl was cytosolic and HRS was present in intracellular vesicles (arrows). In stimulated cells, c-Cbl redistributed to enlarged HRS-containing endosomes (arrow). C, HEK-PAR\(_2\) cells were transiently transfected with control vector (pcDNA5) or Myc-HRS, and cell-surface PAR\(_2\) was labeled with an antibody to the extracellular epitope (Flag). Cells were challenged with PAR\(_2\)-AP (0 or 120 min), and proteins were labeled by indirect immunofluorescence using fluorescent secondary antibody to PAR\(_2\), c-Cbl, and myc antibody. In unstimulated cells expressing control vector or Myc-HRS, PAR\(_2\) was present at the plasma membrane (arrowheads). HRS overexpression caused accumulation of PAR\(_2\) in enlarged HRS-containing endosomes (arrows). In stimulated cells, PAR\(_2\) was depleted from the plasma membrane and detected in enlarged HRS-containing endosomes (arrows). D, HEK-PAR\(_2\) cells were transiently transfected with control vector (pcDNA5) or Myc-HRS, and cell-surface PAR\(_2\) labeled with an antibody to the extracellular epitope (Flag). Cells were challenged with PAR\(_2\)-AP (0 or 30 min), and proteins were localized by indirect immunofluorescence using fluorescent secondary antibody (PAR\(_2\)) and EEA1 antibody. In unstimulated cells expressing control vector, PAR\(_2\) was present at the plasma membrane (arrowheads) and EEA1 was in intracellular vesicles (arrow). In stimulated cells, PAR\(_2\) was detected in EEA1-containing endosomes. E, HEK-PAR\(_2\) cells were transiently transfected with Myc-HRS, and HRS (myc) and TFR were labeled by indirect immunofluorescence. TFR, which traffics constitutively, did not accumulate in the enlarged HRS-containing endosomes (arrows). Scale bars, 10 \(\mu\)m.
PAR2 were transiently transfected with HRS, and PAR2 and HRS were localized by immunofluorescence. In unstimulated cells, PAR2 was localized to the plasma membrane and also present in enlarged endosomes containing HRS (Fig. 2A). PAR2-AP (30 and 120 min) caused depletion of PAR2 from the plasma membrane and accumulation in enlarged HRS-containing endosomes (Fig. 2A).

The E3 ubiquitin ligase c-Cbl ubiquitinates PAR2, and PAR2 agonists induce trafficking of c-Cbl from the cytosol to endosomes containing PAR2 (4). Therefore, we examined the effect of HRS overexpression on trafficking of c-Cbl. In unstimulated HEK-PAR2 cells transiently expressing c-Cbl and HRS, c-Cbl PAR2 from the cell surface to EEA1-containing endosomes, but causes retention of PAR2 in these endosomes for prolonged periods. In contrast, overexpression of HRS did not affect the subcellular localization of the TIR (Fig. 2E), suggesting that the accumulation of PAR2 in enlarged endosomes is not due to a general disruption of the endocytic pathway.

HRS Overexpression Prevents Lysosomal Targeting and Degradation of PAR2—Overexpression of HRS caused retention of PAR2 in endosomes at times (120 min) when the receptor is usually in lysosomes (4), suggesting that HRS mediates lysosomal trafficking of this receptor. To examine this possibility in more detail, and to investigate the effect of HRS expression on trafficking of PAR2 from the plasma membrane to endosomes, we labeled cell-surface PAR2 using an antibody to an extracellular epitope tag (FLAG), and localized EEA1 and endogenous HRS and overexpressed HRS by immunofluorescence. In unstimulated HEK-PAR2 cells transiently expressing c-Cbl and HRS, c-Cbl was cytosolic and HRS was present in enlarged endosomes (Fig. 2B).

PAR2-AP caused trafficking of c-Cbl to HRS-containing endosomes (Fig. 2B). Thus, activated PAR2 together with c-Cbl traffic to HRS-containing vesicles.

The observation that HRS overexpression causes accumulation of unstimulated PAR2 in endosomes suggests constitutive endocytosis of this receptor. To examine this possibility in more detail, and to investigate the effect of HRS expression on trafficking of PAR2 from the plasma membrane to endosomes, we labeled cell-surface PAR2 using an antibody to an extracellular epitope tag (FLAG), and localized EEA1 and endogenous HRS and overexpressed HRS by immunofluorescence. In unstimulated HEK-PAR2 cells transiently expressing c-Cbl and HRS, c-Cbl was cytosolic and HRS was present in enlarged endosomes (Fig. 2B). PAR2-AP caused trafficking of c-Cbl to HRS-containing endosomes (Fig. 2B). Thus, activated PAR2 together with c-Cbl traffic to HRS-containing vesicles.
PAR2 cells transiently transfected with control vector or HRS. In cells expressing control vector, PAR2-AP (120 min) caused PAR2 trafficking to vesicles that colocalized with LAMP1 but not EEA1 (Fig. 3A). Thus, antibody-tagged PAR2 traffics to lysosomes similarly to untagged PAR2 (4). In contrast, HRS overexpression prevented the trafficking of PAR2 to lysosomes and caused accumulation of PAR2 in EEA1-containing vesicles (Fig. 3B). Quantitative analysis of these data revealed that the co-localization of PAR2 and EEA1 was significantly increased in cells transfected with HRS compared with control vector (Fig. 3C). Conversely, the localization of PAR2 and LAMP1 was significantly diminished in cells overexpressing HRS compared with control vector (Fig. 3C).

To assess the effect of HRS overexpression on the degradation of PAR2, we transfected HEK-PAR2 cells with control vector or HRS. Cycloheximide-treated cells were stimulated with PAR2-AP (0–3 h) and levels of PAR2 determined by Western blotting. We found that in cells expressing control vector, PAR2 was quickly degraded (64 ± 2% of control (100%), 3 h) (Fig. 3D). In contrast, in cells overexpressing HRS, degradation of PAR2 was prevented (96 ± 7% of control, 3 h). Thus, HRS overexpression prevents the lysosomal targeting and degradation of PAR2 and causes its retention in EEA1-containing vesicles.

Interaction of HRS and PAR2 does not require the HRS ubiquitin-interacting motif or PAR2 ubiquitination. HRS possesses several protein-protein interacting motifs, including an UIM (21). PAR2 is ubiquitinated as a prerequisite to degradation (4). To determine whether the UIM of HRS is required for interaction with PAR2, we expressed a mutant of HRS that lacks the UIM (HRSΔUIM) in HEK-PAR2 cells. In unstimulated cells, PAR2 was at the plasma membrane and in enlarged HRSΔUIM-containing vesicles (Fig. 4A). PAR2-AP (30–120 min) induced PAR2 depletion from the plasma membrane and accumulation in enlarged vesicles containing HRSΔUIM (Fig. 4A). Quantitative analysis of these data indicated an identical degree of colocalization of PAR2 with HRS or HRSΔUIM at 120 min after stimulation with PAR2-AP (Fig. 4B). To determine whether ubiquitination of PAR2 was necessary for this interaction, we studied an ubiquitin-defective mutant of PAR2 (PAR2Δ14K/R) (4). We observed that in unstimulated cells, PAR2Δ14K/R was at the cell surface and in enlarged HRS-containing vesicles (Fig. 4C). PAR2-AP (30 min) induced depletion of PAR2Δ14K/R from the plasma membrane and accumulation in enlarged HRS-containing vesicles (Fig. 4C). Thus, the colocalization of PAR2 and HRS is not dependent on the ubiquitin-interacting motif of HRS or on the ubiquitination of PAR2.

HRS overexpression prevents the ubiquitin-defective PAR2 from recycling. The ubiquitin-defective mutant PAR2Δ14K/R escapes from the normal degradative pathway and recycles to the plasma membrane (4). To examine the effect of HRS overexpression on PAR2Δ14K/R recycling, we transfected HEK-PAR2Δ14K/R cells with control vector or HRS, and examined endocytosis and recycling of PAR2Δ14K/R labeled at the cell surface with an antibody to the extracellular FLAG epitope. In unstimulated cells expressing control vector, PAR2Δ14K/R was at the plasma membrane (Fig. 5A). PAR2-AP (30 min) induced PAR2Δ14K/R depletion from the plasma membrane and trafficking to HRS-containing endosomes. After agonist washout and recovery for 4 h, PAR2Δ14K/R recycled to the plasma membrane (Fig. 5A). In unstimulated HRS-overexpressing cells, PAR2Δ14K/R was at the plasma membrane and in enlarged HRS-containing endosomes (Fig. 5B), suggesting constitutive endocytosis. PAR2-AP (30 min) induced PAR2Δ14K/R depletion from the plasma membrane and accumulation in HRS-containing endosomes. At 30 min after stimulation with PAR2-AP, PAR2Δ14K/R and wild-type PAR2 colocalized with HRS to the same extent (Fig. 5C). After agonist removal, PAR2Δ14K/R did not recycle and was instead retained in vesicles containing EE1 and HRS (Fig. 5B). Thus, HRS is required for post-endocytic recycling of an ubiquitin-defective PAR2 mutant.

HRS Overexpression Prevents Resensitization of PAR2Δ14K/R Ca2+ Signaling—Recycling mediates resensitization of PAR2Δ14K/R (4). To examine the effect of HRS overexpression on the resensitization of PAR2Δ14K/R Ca2+ signaling, HEK-
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**A. HEK-PARΔ14K/R (Flag,HA11)+pcDNA5**

antibody-tagged receptor

**B. HEK-PARΔ14K/R (Flag,HA11)+Hrs (myc)**

antibody-tagged receptor

**C. HEK-PAR2 (Flag,HA11) or HEK-PARΔ14K/R (Flag,HA11)**

AP (100 μM, 30 min)

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**FIGURE 5. Effect of HRS overexpression on recycling of PAR2Δ14K/R.** HEK-PAR2Δ14K/R cells were transiently transfected with control vector (pcDNA5, A) or Myc-HRS (B), and cell-surface PAR2 labeled with an antibody to the extracellular FLAG epitope. Cells were stimulated with PAR2-AP (0 or 30 min), washed, and placed in PAR2-AP-free medium for 4 h. Proteins were localized by indirect immunofluorescence using fluorescent secondary antibody (PAR2) and HRS and EEA1 antibodies. **A,** in unstimulated cells expressing control vector, PAR2Δ14K/R was present at the cell surface and HRS was present in intracellular vesicles. In stimulated cells, PAR2Δ14K/R was detected in HRS-containing vesicles (arrows). In recovered cells, PAR2Δ14K/R was recycled to the cell surface (arrowheads), and a minor proportion was present in HRS- and EEA1-containing endosomes (arrows). **B,** in unstimulated cells expressing Myc-HRS, PAR2Δ14K/R was at the cell surface (arrowheads) and in enlarged HRS-containing intracellular vesicles (arrows). In stimulated cells, PAR2Δ14K/R was detected in HRS-containing vesicles (arrows). In recovered cells, PAR2Δ14K/R did not return to the cell surface and was retained in HRS and EEA1-containing endosomes (arrows). **C,** quantification of the effect of deleting all intracellular lysine residues of PAR2 on colocalization of PAR2 and HRS. The overlap coefficient between PAR2 and HRS was determined in HEK-PAR2 and HEK-PAR2Δ14K/R cells both transiently transfected with pcDNA5, pcDNA5, and pcDNA5, quantification of the effect of deleting all intracellular lysine residues of PAR2 on colocalization of PAR2 and HRS. The overlap coefficient between PAR2 and HRS was determined in HEK-PAR2 and HEK-PAR2Δ14K/R cells both transiently transfected with pcDNA5, pcDNA5, and pcDNA5, respectively.

PAR2Δ14K/R cells were transfected with control vector or HRS. To prevent synthesis of new receptors or mobilization of receptors from the Golgi apparatus, which also contribute to PAR2 resensitization (30), cells were treated with cycloheximide and brefeldin A. Cells were treated with PAR2-AP (1 h) or vehicle (control), washed and recovered in PAR2-AP-free medium for 0–4 h, and then challenged with PAR2-AP (100 μM). When challenged without recovery, PAR2-AP-induced Ca2+ signaling was minimal (vector control, 23 ± 1% of vehicle; HRS, 22 ± 2% of vehicle), indicating desensitization (Fig. 6, A and B). However, after 4 h recovery, cells expressing control vector had resensitized (72 ± 6% of vehicle). In contrast, cells over-expressing HRS showed diminished resensitization (48 ± 5% of vehicle). Thus, HRS is required for the resensitization of a recycling PAR2Δ14K/R mutant.

**HRS Regulates Recycling and Lysosomal Trafficking of CLR, a Non-ubiquitinated GPCR**—Our results with PAR2 indicate that HRS participates in lysosomal trafficking and degradation of the wild-type receptor, and in recycling and resensitization of a non-ubiquitinated mutant, PAR2Δ14K/R. We have recently shown that CLR and RAMP1 recycle after transient activation with CGRP, but traffic to lysosomes for degradation after sustained activation, and that these processes do not require ubiquitination of CLR or RAMP1 (8). We therefore examined the role of HRS in this divergent trafficking of CLR and RAMP1.

To assess whether HRS plays a role in CLR and RAMP1 trafficking, we localized endogenous HRS and CLR in CLR-RAMP1-HEK cells. In unstimulated cells, CLR was detected mainly at the plasma membrane and was not associated with HRS (Fig. 7A). CGRP (100 nM, 30 min) induced endocytosis of CLR, which co-localized with endogenous HRS (Fig. 7A). Thus, CLR internalizes and colocalizes with HRS in early endosomes, where HRS could regulate its post-endosomal trafficking.

To evaluate whether HRS mediates post-endocytic trafficking of CLR to recycling pathways, we examined the effects of overexpressing HRS on CLR recycling after transient stimulation with CGRP. Cell-surface CLR was labeled by incubating cells with an antibody to the extracellular HA11 epitope of CLR, which we have shown does not affect CLR trafficking (8). We found that in cells not overexpressing HRS, or in cells expressing the control vector, CLR was initially at the cell surface, and CGRP (30 min) induced internalization of antibody-labeled receptor, which recycled at 4 h after washing and incubating cells in CGRP-free medium (Fig. 7, B and C). In cells overexpressing HRS, CLR was initially detected at the cell surface and in some HRS-containing endosomes, suggesting constitutive endocytosis (Fig. 7C). CGRP induced depletion of CLR from the plasma...
Recycling of CLR but Not PAR2
Thus, disrupting HRS blocks lysosomal trafficking of CLR. Detection of CLR in LAMP1-containing lysosomes 4 h (Fig. 7). Thus, consistent with our findings of HRS overexpression, PAR2 was still retained in HRS, whereas HRSVHs in preventing recycling of PAR2Δ14K/R, whereas HRSVHs was significantly less effective than wild-type HRS in preventing recycling of CLR (Fig. 8C). Thus, the VHS domain of HRS is required for recycling of CLR but not PAR2Δ14K/R.

Knockdown of HRS Inhibits Recycling and Lysosomal Trafficking of PAR2 and CLR—Knock-down of endogenous HRS by transfection of siRNA disrupts function similar to HRS overexpression (14, 17). To determine whether our findings were an indirect consequence of HRS overexpression, we examined the effects of depleting cellular HRS on post-endocytic trafficking of PAR2 and CLR. We chose HeLa cells for these experiments because a substantial reduction in cellular HRS can be achieved in this cell line by transfection with HRS-specific siRNA but not control (non-silencing) siRNA (14, 34, 35). When HeLa cells were transfected with siRNA to two distinct regions of HRS (HRS-siRNA1 or HRS-siRNA2), there was a >70% reduction in HRS levels detected in Western blots of whole cell lysates when compared with untransfected cells or cells transfected with control siRNA (Fig. 9A). The effects of HRS-siRNA1 and HRS-siRNA2 on receptor trafficking were identical. HRS-siRNA strongly inhibited recycling of transiently activated PAR2Δ14K/R and CLR. Thus, after stimulation with agonist for 30 min and 4 h recovery, PAR2Δ14K/R and CLR were recycled to the plasma membrane in cells expressing control siRNA, but were retained in enlarged EEA1-positive endosomes in cells expressing HRS-siRNA (Fig. 9B and C).

Quantification of plasma membrane targeting after 4 h of recovery revealed that HRS-siRNA inhibited recycling of both PAR2Δ14K/R and CLR (Fig. 9D).

To evaluate whether HRS knockdown affects trafficking of PAR2 and CLR to degradative pathways, we examined the effects of overexpressing HRS on CLR trafficking to lysosomes after sustained stimulation with CGRP. In cells not overexpressing HRS, or in cells expressing the control vector, CGRP (4 h) induced trafficking of antibody-labeled CLR to vesicles containing LAMP1 but not EEA1 (Fig. 7D). In cells overexpressing HRS, CLR was retained in HRS-containing endosomes, and was not detected in LAMP1-containing lysosomes after 4 h (Fig. 7D). Thus, disrupting HRS blocks lysosomal trafficking of CLR.

The VHS Domain of HRS Is Required for HRS-dependent Recycling of CLR but Not PAR2Δ14K/R—When overexpressed, a truncation mutant of HRS lacking the N-terminal VHS domain is defective in its ability to inhibit recycling of the β2-adrenergic receptor, suggesting that the VHS domain is required for controlling recycling of this receptor (17). To examine whether the VHS domain of HRS is also required for HRS-dependent recycling of CLR and PAR2Δ14K/R, we expressed a mutant of HRS lacking the VHS domain (HRSΔVHS) in HEK-CLR and HEK-PAR2Δ14K/R cells. In unstimulated cells, PAR2Δ14K/R and CLR were at the plasma membrane and in some HRSΔVHS-containing vesicles (Fig. 8, A and B). After stimulation with agonist for 30 min, PAR2Δ14K/R and CLR were depleted from the plasma membrane and accumulated in enlarged vesicles containing HRSΔVHS (Fig. 8, A and B). After agonist removal and recovery for 4 h, CLR recycled to the plasma membrane, with a portion still retained in HRSΔVHS-containing vesicles (Fig. 8B). In contrast, PAR2Δ14K/R did not recycle and was completely retained in HRSΔVHS-containing vesicles (Fig. 8A). Quantification of the plasma membrane targeting after 4 h of recovery revealed that overexpressed HRSΔVHS was as potent as wild-type HRS in preventing recycling of PAR2Δ14K/R, whereas HRSΔVHS was significantly less effective than wild-type HRS in preventing recycling of CLR (Fig. 8C). Thus, the VHS domain of HRS is required for recycling of CLR but not PAR2Δ14K/R.

DISCUSSION
We have identified two distinct functions of HRS in post-endocytic sorting of a single receptor, PAR2. First, HRS medi-
HRS Trafficking of PAR2 and CLR

A. HEK-CLR (HA11) + RAMP1

CLR (HA11) Hrs merge

0 min

CGRP (100 nM, 30 min), wash

B. HEK-CLR (HA11) + RAMP1; antibody-tagged receptor

unstimulated

CGRP (100 nM, 30 min), wash

0 h recovery 4 h recovery

D. HEK-CLR (HA11) + RAMP1; antibody-tagged receptor

CLR (HA11) LAMP1 merge

CGRP (100 nM, 4 h)

FIGURE 7. Effects of HRS on recycling and lysosomal trafficking of CLR. A, HEK-CLR-RAMP1 cells were incubated with CGRP (0 or 30 min), and proteins were localized by immunofluorescence using HA11 (CLR) and HRS antibodies. In unstimulated cells, CLR was present at the cell surface (arrowheads) and HRS was present in intracellular vesicles (arrows). In stimulated cells, CLR was depleted from the plasma membrane, and was detected in HRS-containing endosomes (arrows). B and C, HEK-CLR-RAMP1 cells were transiently transfected with control vector (pcDNA5) or Myc-HRS, and cell-surface CLR was labeled with an antibody to the extracellular epitope (HA11). Cells were stimulated with CGRP (0 or 30 min), washed, and placed in CGRP-free medium for 0–4 h. Proteins were localized using fluorescent secondary antibody (CLR) and HRS and EEA1 antibodies. B, in unstimulated cells expressing control vector, CLR was present at the cell surface (arrowheads). In stimulated cells, CLR was depleted from the plasma membrane and detected in intracellular vesicles (arrows). In recovered cells, CLR was recycled to the cell surface (arrowheads). C, in unstimulated cells expressing Myc-HRS, CLR was present at the cell surface (arrowheads) and in HRS-containing vesicles (arrows). In stimulated cells expressing Myc-HRS, CLR was depleted from the plasma membrane and detected in HRS-containing vesicles (arrows). In recovered cells expressing Myc-HRS, CLR was still retained in HRS-containing vesicles (arrows). In contrast, in cells expressing control vector, CLR trafficked back to the cell surface following stimulation and recovery (arrowheads). D, HEK-CLR-RAMP1 cells were transiently transfected with control vector (pcDNA5) or Myc-HRS, and cell-surface CLR was labeled with an antibody to the extracellular epitope (HA11). Cells were stimulated with CGRP (4 h), and proteins were localized using fluorescent secondary antibody (CLR) and HRS and LAMP1 antibodies. In stimulated cells expressing control vector, CLR colocalized with LAMP1 in lysosomes (arrows). In cells expressing Myc-HRS, CLR did not colocalize with LAMP1 but did colocalize with HRS (arrows). Scale bars, 10 μm.

ates agonist-induced trafficking of ubiquitinated PAR2 to lysosomes, where degradation occurs. Second, HRS mediates recycling and resensitization of non-ubiquitinated PAR2. We have also shown that HRS has a similar role in trafficking CLR, which is not ubiquitinated after activation. HRS mediates lysosomal trafficking of CLR after sustained activation, and recycling after transient activation. The role of HRS in the sorting of a single receptor into recycling and degradative pathways has not been demonstrated before. This study on PAR2 and CLR trafficking therefore provides a unique opportunity to dissect the role of HRS in these divergent sorting pathways and evaluates the role of receptor ubiquitination in this process.

HRS-dependent Trafficking of Ubiquitinated PAR2 and Its Ubiquitin Ligase c-Cbl—Although c-Cbl-mediated ubiquitination of PAR2 is required for sorting of the receptor from early endosomes to lysosomes, the mechanisms of this ubiquitin-dependent sorting are unknown. We observed that disruption of HRS function by overexpression prevented agonist-induced lysosomal trafficking and subsequent degradation of PAR2. This inhibition was due to the accumulation of PAR2 in enlarged HRS-containing endosomes. Thus, we have established a critical role for HRS in the lysosomal trafficking and subsequent degradation of PAR2. These findings are consistent with studies demonstrating that HRS mediates lysosomal targeting of other ubiquitinated GPCRs, such as CXCR4 (16). HRS recognizes ubiquitinated proteins through its UIM, and thereby functions as an adaptor between ubiquitin cargo and the endosomal sorting machinery that is responsible for multivesicular body formation and receptor sorting to lysosomes (21, 39). The overexpression of HRS disrupts inward vesiculation during multivesicular body formation and receptor sorting to lysosomes, indicating a role for HRS in both of these processes (40). Ubiquitinated receptors, such as epidermal growth factor receptor, interact with HRS in the multivesicular body and are targeted to lysosomes, whereas non-ubiquitinated receptors, such as TFR, may diffuse into this region but are not retained within it and recycle to the plasma membrane (20).

The E3 ligase c-Cbl is responsible for the ubiquitination of PAR2 and translocates to the plasma membrane after activation of the receptor and traffics with the receptor to early endosomes before returning to the cytosol (4). We observed that after disrupting HRS function and activating PAR2, c-Cbl traf-
To investigate if ubiquitination of the receptor or the UIM activation, it escapes normal lysosomal targeting and recycles pressed at the plasma membrane, signals normally, yet upon secondary antibody (an).

Indeed, c-Cbl, which is also responsible for the ubiquitination of HRS in the sorting of PAR2. Indeed, c-Cbl, which is also responsible for the ubiquitination of哌2, enhances HRS ubiquitination, thereby altering the trafficking of the receptor, which could influence the function of HRS in the sorting of PAR2. Indeed, c-Cbl, which is also responsible for the ubiquitination of the epidermal growth factor receptor, enhances HRS ubiquitination, thereby altering the composition of the endosomal sorting machinery and its ability to target this receptor for lysosomal degradation (2).

**Ubiquitin-independent Interaction between HRS and PAR2**—The ubiquitin-defective PAR2 mutant PAR2Δ14K/R is expressed at the plasma membrane, signals normally, yet upon activation, it escapes normal lysosomal targeting and recycles (4). To investigate if ubiquitination of the receptor or the UIM and then either recycles (after transient stimulation with CGRP) or traffics to lysosomes (after sustained stimulation).

Overexpression of HRS blocked both recycling and lysosomal trafficking of the receptor, which could influence the function of HRS in the sorting of PAR2. Indeed, c-Cbl, which is also responsible for the ubiquitination of the epidermal growth factor receptor, enhances HRS ubiquitination, thereby altering the composition of the endosomal sorting machinery and its ability to target this receptor for lysosomal degradation (18).

**Role of HRS in Post-endocytic Sorting of CLR, a Naturally Non-ubiquitinated GPCR**—By examining the post-endocytic sorting of CLR, we were able to define the contribution of HRS to recycling and lysosomal trafficking of a non-ubiquitinated GPCR (8). We observed that activated CLR traffics to endosomes containing endogenous HRS, of HRS were necessary for the interaction between PAR2 and HRS, we studied the trafficking of PAR2 and PAR2Δ14K/R in the presence of overexpressed HRSΔUIM and HRS, respectively. In both cases, the receptor accumulated in enlarged HRS-containing endosomes. Thus, HRS-mediated post-endocytic trafficking of PAR2 does not require receptor ubiquitination or the UIM of HRS. These findings are consistent with the report that HRS-mediated lysosomal trafficking of δ-opioid receptor is independent of receptor ubiquitination (14). New receptor synthesis and transport of Golgi stores to the plasma membrane mediate resensitization of wild-type PAR2 (30). PAR2Δ14K/R can resensitize by recycling (4), which provided an opportunity to determine the role of HRS in this process. We observed that overexpression of HRS prevented recycling and resensitization of activated PAR2Δ14K/R. In support of our results, HRS also mediates recycling of both the β2-adrenergic receptor and the μ-opioid receptor, which is critical for resensitization of signaling (17).

**HRS Trafficking of PAR2 and CLR**

- **FIGURE 8. Effects of HRSΔVHS overexpression on recycling of PAR2Δ14K/R and CLR.** A, HEK-PAR2Δ14K/R cells were transiently transfected with Myc-HRSΔVHS and cell-surface PAR2Δ14K/R labeled with an antibody to the extracellular FLAG epitope. Cells were stimulated with PAR2-AP (0 or 30 min), washed, and placed in PAR2-AP-free medium for 4 h. Proteins were localized by indirect immunofluorescence using fluorescent secondary antibody (PAR2) and HRS antibody. In unstimulated cells, PAR2Δ14K/R was at the cell surface (arrowsheads) and HRSΔVHS was in intraluminal vesicles. In stimulated cells, PAR2Δ14K/R was detected in HRSΔVHS-containing vesicles (arrows). In recovered cells, PAR2Δ14K/R remained in HRSΔVHS-containing vesicles (arrows) and did not recycle to the cell surface. B, HEK-CLR-RAMP1 cells were transiently transfected with Myc-HRSΔVHS and cell-surface CLR labeled with an antibody to the extracellular HA11 epitope. Cells were stimulated with CGRP (0 or 30 min), washed, and placed in CGRP-free medium for 4 h. Proteins were localized by indirect immunofluorescence using fluorescent secondary antibody (CLR) and HRS antibody. In unstimulated cells, CLR was at the cell surface (arrowheads) and HRSΔVHS was in intraluminal vesicles. In stimulated cells, CLR was detected in HRSΔVHS-containing vesicles (arrows). In recovered cells, CLR was recycled to the cell surface (arrowheads), and a proportion was present in HRSΔVHS-containing vesicles (arrows). Scale bars, 10 μm. C, quantification of the effects of Myc-HRS and Myc-HRSΔVHS on the recycling of PAR2Δ14K/R and CLR. The percentage of total fluorescence at the plasma membrane after 4 h of recovery was determined. n, 20 to 30 cells each. * p < 0.05.
HRS Trafficking of PAR2 and CLR

FIGURE 9. Effects of siRNA-mediated knockdown of endogenous HRS on lysosomal trafficking and recycling of PAR2, PAR2Δ14K/R, and CLR in HeLa cells. Cells were transfected with control (CON) or HRS duplex siRNA. After 24 h, they were retransfected with cDNAs for PAR2, PAR2Δ14K/R, or CLR and stimulated with PAR2-AP for 120 min. Cells were fixed and stained with antibodies to the extracellular epitope (PAR2) and EEA1 or LAMP1 as indicated. A-H, Western blot showing the efficiency of the knockdown of endogenous HRS protein levels in HeLa cells. B-H, HeLa expressing PAR2Δ14K/R (Flag, HA11); antibody-tagged receptor PAR2Δ14K/R (Flag, HA11) (B) or PAR2Δ14K/R (Flag, HA11) + RAMP1 (C, D). Cells were stimulated with PAR2-AP for 120 min, washed, and placed in PAR2-AP-free medium for 4 h. In cells transfected with CON siRNA, PAR2Δ14K/R was recycled to the cell surface (arrowheads). In cells transfected with HRS-siRNA, PAR2 did not recycle and instead colocalized with EEA1 in enlarged endosomes (arrows). C. HeLa expressing CLR (HA11) + RAMP1 were incubated with an antibody to the extracellular epitope (HA11) to label cell-surface CLR. Cells were stimulated with CGRP for 30 min, washed, and placed in CGRP-free medium for 4 h. In cells transfected with CON siRNA, CLR did not recycle and instead colocalized with EEA1 in enlarged endosomes (arrows). D. Cells were stimulated with PAR2-AP for 30 min, washed, and placed in PAR2-AP-free medium for 4 h. In cells transfected with CON siRNA, PAR2Δ14K/R was recycled to the cell surface (arrowheads). In cells transfected with HRS-siRNA, PAR2Δ14K/R did not recycle and instead colocalized with EEA1 in enlarged endosomes (arrows). E. Quantification of the effects of HRS knockdown on the recycling of PAR2Δ14K/R and CLR. The percentage of total fluorescence at the plasma membrane after 4 h of recovery was determined in CON-siRNA and HRS-siRNA transfected cells. n, 20 cells. *p < 0.05. F, in HeLa cells expressing PAR2Δ14K/R (Flag, HA11), cell-surface PAR2Δ14K/R was labeled as described in B. Cells were stimulated with PAR2-AP for 120 min, and proteins were localized using fluorescent secondary antibody (PAR2Δ14K/R) and EEA1 or LAMP1 antibodies. In stimulated cells transfected with CON-siRNA, PAR2Δ14K/R colocalized with LAMP1 in lysosomes (arrows). In cells transfected with HRS-siRNA, PAR2Δ14K/R colocalized in enlarged endosomes (arrows). G, in HeLa cells expressing CLR (HA11) + RAMP1, cell-surface CLR was labeled as described in C. Cells were stimulated with CGRP for 4 h, and proteins were localized using fluorescent secondary antibody (CLR) and EEA1 or LAMP1 antibodies. In stimulated cells transfected with CON-siRNA, CLR colocalized with LAMP1 in lysosomes (arrows). In cells transfected with CON-siRNA, CLR colocalized with EEA1 in enlarged endosomes (arrows). H, Scale bars, 10 μm.
for the function of HRS in PAR2Δ14K/R recycling. The VHS domain of HRS may thus have an important role in linking some but not all GPCRs to HRS, which requires further investigation.

HRS Knockdown Has the Same Inhibitory Effects on Post-endocytic Trafficking of PAR2 and CLR as HRS Overexpression—Depletion of endogenous HRS using siRNA produces an enlargement of EEA1-positive endosomes identical to overexpression (14, 16, 17, 24, 41). We noted that the enlargement of EEA1-positive endosomes was more pronounced in HEK cells compared with HeLa cells, which is consistent with previous studies (16, 17). The effects of HRS knockdown and HRS overexpression have been previously reported to have inhibitory effects on trafficking of other receptors, such as the β2-adrenergic receptor, μ-opioid receptor, and epidermal growth factor receptor (14, 17, 34, 44).

In summary, the present results demonstrate for the first time the critically important role of HRS in the divergent post-endocytic sorting of individual GPCRs to both degradative and recycling pathways, which have opposite functional effects on cell signaling. HRS is required for lysosomal trafficking and degradation of PAR2 and CLR, which would prevent sustained signaling of these receptors that could otherwise result in uncontrolled pain and inflammation. HRS also mediates recycling and resensitization of PAR2Δ14K/R and transiently activated CLR. These sorting roles of HRS are independent of its ubiquitin-interacting motif, and HRS can regulate trafficking of ubiquitinated PAR2 and non-ubiquitinated PAR2 and CLR. Thus, the ubiquitin-dependent sorting decision that leads to degradative or recycling pathways appears to be downstream from HRS and requires further investigation.

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