ALIX binds a YPX3L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting

Michael R. Dores  
*School of Medicine, University of California - San Diego*

Buxin Chen  
*School of Medicine, University of California - San Diego*

Huilan Lin  
*School of Medicine, University of California - San Diego*

May M. Paing  
*Washington University School of Medicine in St. Louis*

William A. Montagne  
*School of Medicine, University of California - San Diego*

See next page for additional authors

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Authors
Michael R. Dores, Buxin Chen, Huilan Lin, May M. Paing, William A. Montagne, Timo Meerlo, and JoAnn Trejo
ALIX binds a YPX3L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting

Michael R. Dores,1 Buxin Chen,1 Huilan Lin,1 Unice J.K. Soh,1 May M. Paing,3 William A. Montagne,1 Timo Meerloo,2 and JoAnn Trejo1

1Department of Pharmacology, and 2Department of Cellular and Molecular Medicine, School of Medicine, University of California San Diego, La Jolla, CA 92093
3Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110

The sorting of signaling receptors to lysosomes is an essential regulatory process in mammalian cells. During degradation, receptors are modified with ubiquitin and sorted by endosomal sorting complex required for transport (ESCRT)-0, -I, -II, and -III complexes into intraluminal vesicles (ILVs) of multivesicular bodies (MVBs). However, it remains unclear whether a single universal mechanism mediates MVB sorting of all receptors. We previously showed that protease-activated receptor 1 (PAR1), a G protein–coupled receptor (GPCR) for thrombin, is internalized after activation and sorted to lysosomes independent of ubiquitination and the ubiquitin-binding ESCRT components hepatocyte growth factor–regulated tyrosine kinase substrate and Tsg101. In this paper, we report that PAR1 sorted to ILVs of MVBs through an ESCRT-III–dependent pathway independent of ubiquitination. We further demonstrate that ALIX, a charged MVB protein 4–ESCRT-III interacting protein, bound to a YPX3L motif of PAR1 via its central V domain to mediate lysosomal degradation. This study reveals a novel MVB/lysosomal sorting pathway for signaling receptors that bypasses the requirement for ubiquitination and ubiquitin-binding ESCRTs and may be applicable to a subset of GPCRs containing YPX3L motifs.

Introduction

G protein–coupled receptors (GPCRs) are the largest family of signaling receptors expressed in mammalian cells and mediate vast physiological responses. The temporal and spatial fidelity of GPCR signaling is critical for appropriate cellular responses. Moreover, dysregulated GPCR signaling has been implicated in numerous human diseases including neurodegeneration and cancer progression (Hanyaloglu and von Zastrow, 2008; Marchese et al., 2008). In addition to desensitization, GPCR trafficking is important for the precise regulation of signaling responses. This is particularly true for protease-activated receptor 1 (PAR1), a G protein–coupled receptor (GPCR) for thrombin, is internalized after activation and sorted to lysosomes independent of ubiquitination and the ubiquitin-binding ESCRT components hepatocyte growth factor–regulated tyrosine kinase substrate and Tsg101. In this paper, we report that PAR1 sorted to ILVs of MVBs through an ESCRT-III–dependent pathway independent of ubiquitination. We further demonstrate that ALIX, a charged MVB protein 4–ESCRT-III interacting protein, bound to a YPX3L motif of PAR1 via its central V domain to mediate lysosomal degradation. This study reveals a novel MVB/lysosomal sorting pathway for signaling receptors that bypasses the requirement for ubiquitination and ubiquitin-binding ESCRTs and may be applicable to a subset of GPCRs containing YPX3L motifs.

Once activated, PAR1 is internalized and sorted directly to lysosomes and degraded, a process important for termination of G protein signaling (Trejo et al., 1998; Booden et al., 2004). The mechanism by which activated PAR1 is trafficked to lysosomes is not known.

The sorting of transmembrane proteins such as EGF receptor (EGFR) from the plasma membrane to lysosomes has been extensively studied and is mediated by the endosomal sorting complex required for transport (ESCRT). The ESCRT machinery is comprised of distinct complexes that function coordinately to sort ubiquitinated receptors to intraluminal vesicles (ILVs) of multivesicular bodies (MVBs; Hurley and Hanson, 2010). Hepatocyte growth factor–regulated tyrosine kinase substrate (HRS), a component of ESCRT-0, recruits ubiquitinated receptors and Tsg101, a ubiquitin-binding subunit of ESCRT-I (Lu et al., 2003). ESCRT-I and -II function in receptor sorting to
ILVs and ILV formation (Wollert and Hurley, 2010). ESCRT-III polymerizes on endosomal membranes and is the main driver of ILV scission. The AAA-ATPase vacuolar protein sorting 4 (Vps4) disassembles and recycles ESCRT-III components and is essential for ESCRT function. In addition to receptor sorting at the MVB, ESCRT mediates viral budding and cytokinesis through processes that require ESCRT-I and -III and ALIX, an ESCRT-III–interacting protein, but not ESCRT-0 or -II (Strack et al., 2003; Carlton et al., 2008). Whether there are differences in ESCRT requirements for the sorting of signaling receptors at the MVB in mammalian cells remains unclear.

Most GPCRs require posttranslational modification with ubiquitin and ESCRTs for sorting from endosomes to lysosomes. The chemokine receptor CXCR4 is ubiquitinated after activation and sorted from endosomes to lysosomes through a pathway that requires HRS and Vps4 (Marchese et al., 2003). PAR2, a GPCR related to PAR1, also undergoes agonist-induced ubiquitination and is sorted to lysosomes through an HRS-dependent pathway (Hasdemir et al., 2007). However, not all GPCRs require direct ubiquitination for MVBS sorting and lysosomal degradation, as exemplified by the δ-opioid receptor (DOR). A ubiquitination-deficient DOR mutant is efficiently sorted to ILVs of MVBS similar to wild-type (WT) receptor (Henry et al., 2011). However, degradation of DOR requires HRS and Vps4 but not Tsg101 (Hislop et al., 2004), indicating that receptor sorting can occur independent of ubiquitination and requires some but not all components of the ubiquitin-binding ESCRT machinery. Thus, it remains to be determined whether a signaling receptor can bypass the requirement for both ubiquitination and ubiquitin-binding components of the ESCRT machinery and sort to MVBS/lysosomes.

We previously showed that activated PAR1 is efficiently sorted from endosomes to lysosomes and degraded independent of ubiquitination (Wolfe et al., 2007). In contrast to DOR, however, neither HRS nor Tsg101 is essential for lysosomal degradation of PAR1 (Gullapalli et al., 2006). Thus, it is not known whether activated PAR1 ultimately sorts to ILVs of MVBS and requires any components of the ESCRT machinery for lysosomal degradation. Here, we report that activated PAR1 sorts to ILVs of MVBS through a pathway that requires ALIX and ESCRT-III function but not receptor ubiquitination. Moreover, our findings indicate that ALIX binds to a YPX3L motif of PAR1 and recruits ESCRT-III to mediate MVBS/lysosomal sorting.

### Results

**Activated PAR1 sorts to ILVs of MVBS independent of ubiquitination**

Activated PAR1 WT is internalized, sorted efficiently to lysosomes, and rapidly degraded with a half-life of 30 min (Trejo and Coughlin, 1999). In previous work, we showed that a PAR1 lysine-less 0K mutant defective in ubiquitination is degraded comparably with WT receptor after activation (Wolfe et al., 2007), suggesting that PAR1 degradation occurs independent of ubiquitination. To investigate the mechanism of ubiquitin-independent degradation of PAR1, we first determined whether agonist promoted lysosomal sorting of the PAR1 0K mutant by examining its colocalization with the lysosomal-associated membrane protein 1 (LAMP1) in HeLa cells using immunofluorescence confocal microscopy. In the absence of agonist stimulation, PAR1 WT and 0K mutant cycle constitutively between the plasma membrane and early endosomes (Paing et al., 2006; Wolfe et al., 2007). Consistent with these studies, we found that PAR1 WT and 0K mutant localized predominantly to the cell surface and early endosomes and showed no significant colocalization with LAMP1 under control conditions (Figs. 1 A and S1 A). However, after 60 min of agonist stimulation, PAR1 WT colocalized extensively with LAMP1-positive vesicles (Fig. 1 A), as previously reported (Trejo et al., 2000). Activation of PAR1 0K mutant with agonist for 60 min also resulted in substantial receptor colocalization with LAMP1 (Fig. 1 A). The colocalization of activated PAR1 WT and 0K mutant with LAMP1 was verified by determining Pearson’s correlation coefficient for PAR1 and LAMP1 colocalization for control versus agonist-stimulated conditions and was significant, as determined by Student’s t test (***, P < 0.001; n = 6).
receptor, suggesting that ubiquitination is not essential for PAR1 lysosomal sorting.

A critical step in lysosomal sorting and degradation of most proteins in mammalian cells requires incorporation into ILVs of the MVB. To determine whether PAR1 directly enters ILVs of MVBs independent of receptor ubiquitination, we performed immunohistochemistry on ultrathin cryosections of HeLa cells expressing PAR1 WT or ubiquitin-deficient 0K mutant. To examine endocytic sorting of the cell surface cohort of PAR1 WT and 0K mutant after agonist activation, receptors were prelabeled with anti-FLAG antibody at 4°C and then treated with agonist for 20 min. After agonist stimulation, activated PAR1 WT (12-nm gold particles) sorted to an endosomal compartment that colabeled with the late endosome/MVB marker CD63 (6-nm gold particles; Fig. 1 B), suggesting that activated PAR1 traffics to MVBs. Remarkably, the ubiquitination-deficient PAR1 0K mutant also localized to ILVs of CD63-positive MVBs after agonist activation (Fig. 1 C), indicating that PAR1 traffics to ILVs of MVBs independent of receptor ubiquitination. To ensure that antibody prelabeling did not alter the internalization and sorting of PAR1, we examined the colocalization of PAR1 WT and 0K mutant with LAMP1 by immunofluorescence confocal microscopy in parallel. As expected, PAR1 WT and 0K mutant localized to the cell surface and early endosomes and did not colocalize with LAMP1 after incubation for 20 min in the absence of agonist (Fig. S1, A and C). In contrast, however, stimulation of PAR1 WT and 0K mutant with agonist for 20 min caused significant colocalization with LAMP1 (Fig. S1, B and C), consistent with that observed in cells not prelabeled with antibody (Fig. 1 A). Together, these data demonstrate that ubiquitination of activated PAR1 is not required for entry into ILVs of MVBs and suggest that a novel pathway exists for the sorting of nonubiquitinated receptors to the lysosome.

**ESCRT-III and Vps4 mediate PAR1 lysosomal degradation**

To define the ubiquitin-independent lysosomal degradation pathway of PAR1, we examined the function of ESCRT-III components, which do not contain any known ubiquitin-binding domains (Hurley and Hanson, 2010). Charged MVB protein 4 (CHMP4) is the most abundant subunit of ESCRT-III, which serves as the critical mediator of ILV scission, and exists as three isoforms: CHMP4A, B, and C (Wollert and Hurley, 2010). We first determined the expression of CHMP4 isoforms in HeLa cells using RT-PCR and CHMP4 immunofluorescence (specific primers (Fig. 2 A, lanes 6–8)). RT-PCR assay of HeLa cell RNA revealed the presence of all three isoforms, including CHMP4A, B, and C (Fig. 2 A, lanes 1 and 2). To assess CHMP4 function in PAR1 degradation, siRNAs that specifically target each of the three CHMP4 isoforms expressed in HeLa cells were used. HeLa cells transfected with CHMP4 isoform-specific siRNA SMARTpools exhibited a substantial decrease in CHMP4A, B, and C mRNA transcripts, as revealed by RT-PCR (Fig. 2 A, lanes 3–5). Consistent with diminished mRNA transcripts, CHMP4-specific siRNAs also caused significant depletion of endogenous CHMP4A and CHMP4B protein, as detected by immunoblotting (Fig. 2 B). Because there are no anti-CHMP4C antibodies commercially available, we were unable to examine endogenous CHMP4C protein expression in siRNA-transfected cells.

To assess the function of CHMP4 in PAR1 degradation, HeLa cells expressing FLAG-PAR1 were transfected with either nonspecific or CHMP4-specific siRNAs. Cells were then incubated with agonist for 60 min, and PAR1 degradation was assessed. In nonspecific siRNA-transfected cells, an ~50% decrease in the amount of PAR1 protein was detected after 60 min of agonist incubation compared with unstimulated control cells (Fig. 2 B, lanes 1 and 2). The extent of PAR1 degradation was similar in nonspecific and CHMP4A siRNA–transfected cells after agonist stimulation, suggesting that CHMP4A is not essential for PAR1 degradation (Fig. 2 B, lanes 1–4). In contrast, agonist-induced degradation of PAR1 was significantly inhibited in cells transfected with either CHMP4B or CHMP4C siRNA SMARTpools (Fig. 2 B, lanes 5–8), indicating that both CHMP4B and C isoforms are necessary for PAR1 degradation. Cells transfected with single siRNAs targeting CHMP4B resulted in loss of endogenous CHMP4B expression and also blocked agonist-promoted PAR1 degradation (Fig. S2). These results suggest that CHMP4B and C are important for PAR1 lysosomal sorting.
suggest that CHMP4 mediates agonist-induced PAR1 lysosomal degradation.

The AAA-ATPase Vps4 catalyzes ESCRT-III disassembly and recycling and is critical for ESCRT function (Hurley and Hanson, 2010). To examine the role of Vps4 in agonist-induced PAR1 degradation, we used a catalytically inactive Vps4 E228Q variant, which functions as a dominant-negative mutant (Babst et al., 1998; Bishop and Woodman, 2000). HeLa cells coexpressing PAR1 and either Vps4 WT or E228Q mutant fused to GFP were incubated with agonist, and receptor degradation was assessed. Interestingly, agonist-promoted PAR1 degradation was significantly inhibited in cells coexpressing Vps4 E228Q mutant compared with Vps4 WT–expressing cells (Fig. 3 A), indicating that Vps4 mediates MVB sorting of PAR1. Agonist-promoted PAR2 degradation was similarly inhibited in Vps4 E228Q mutant–expressing cells compared with control cells expressing WT Vps4 (Fig. 3 B). PAR1 and PAR2 degradation induced by agonist was also inhibited in cells transfected with siRNAs targeting both Vps4A and B isoforms (Fig. S3, A and B). These results suggest that Vps4 is required for both ubiquitin- and nonubiquitin-mediated lysosomal sorting of GPCRs.

Next, we evaluated Vps4 function in MVB/lysosomal sorting of activated PAR1 and PAR2 by immunofluorescence confocal microscopy. Cells coexpressing FLAG-PAR1 or FLAG-PAR2 and either Vps4 WT or E228Q mutant fused to GFP were incubated with anti-FLAG antibody at 4°C to ensure only the cell surface receptors would be labeled and then stimulated with agonist. In the absence of agonist, PAR1 and PAR2 localized predominantly to the cell surface, whereas agonist-stimulated PAR1 and PAR2 redistributed from the cell surface to endosomes (Fig. 3, C and D). As expected, coexpression of Vps4 E228Q mutant resulted in accumulation of internalized PAR2 in enlarged vesicles, defined as a class E phenotype that results from disruption of ESCRT-III recycling (Fig. 3 D; Babst et al., 1998; Bishop and Woodman, 2000). In contrast, PAR1-containing endosomes were unperturbed in Vps4 E228Q mutant–expressing cells and appeared similar to those in adjacent untransfected cells and cells expressing Vps4 WT (Fig. 3 C). Aberrant localization of CD63 observed in cells coexpressing PAR1 and Vps4 E228Q mutant indicates disruption of the MVB compartment (Fig. 3 C). Thus, in contrast to ubiquitinated cargo, PAR1 fails to accumulate at the MVB when the pathway is disrupted, suggesting that MVB sorting of PAR1 is distinctly regulated.

To confirm ESCRT-III function in PAR1 lysosomal degradation, we examined whether activated PAR1 forms a complex with CHMP4. We first assessed the coassociation of endogenous CHMP4B with activated PAR1 in HeLa cells by coimmunoprecipitation. HeLa cells expressing FLAG-PAR1 WT were incubated with agonist for various times, PAR1 was immunoprecipitated, and association of CHMP4B was examined. In the absence of agonist, PAR1 and CHMP4B interaction was undetectable (Fig. 4 A). However, an interaction between PAR1 and endogenous CHMP4B was detected after 10 min of agonist stimulation, and the proteins remained associated for ~20 min (Fig. 4 A). These findings coincide with the time course for agonist-induced PAR1 localization to ILVs of MVBs and suggest that CHMP4B is a component of the ESCRT-III complex that binds to activated PAR1. Next, we examined PAR1 interaction with CHMP4C in HeLa cells transfected with an HA-tagged CHMP4C, as CHMP4C antibodies are not available. HeLa cells coexpressing FLAG-PAR1 or FLAG-PAR2 were transfected with either Vps4-GFP or Vps4 E228Q–GFP. Cells were stimulated with 100 μM SFLLRN or 100 μM SLIGKV, as indicated, and lysed, and equivalent amounts of lysates were immunoprecipitated (IP), and PAR degradation was assessed by immunoblotting (IB). Cell lysates were immunoblotted with anti-GFP and -actin antibodies. The data (mean ± SD) are expressed as the fraction of PAR1 remaining compared with untreated control (Ctrl) and were analyzed by a two-way analysis of variance (**P < 0.01; ***P < 0.001; n = 3). (C and D) HeLa cells expressing FLAG-PAR1 or FLAG-PAR2 and either GFP-tagged Vps4 or E228Q mutant were prelabeled with anti-FLAG antibody for 1 h at 4°C, washed, and stimulated with 100 μM SFLLRN or 100 μM SLIGKV at 37°C, as indicated. Cells were fixed, permeabilized, and immunostained for PAR1 and CD63. Cells coexpressing PAR1 or PAR2 and either Vps4 WT or E228Q mutant are indicated by the arrowheads. The insets are magnifications of the boxed areas. Bars, 10 μm.
with CHMP4 proteins. Similar to WT PAR1, both CHMP4B and CHMP4C coimmunoprecipitated with activated PAR1 0K mutant after 10 min of agonist stimulation (Fig. 4 C), indicating that CHMP4B and CHMP4C are components of the PAR1 ubiquitin–independent sorting complex. In contrast, FLAG-tagged β2-adrenergic receptor (β2AR), a classical GPCR, failed to coimmunoprecipitate with HA-CHMP4B or HA-CHMP4C in PAR1-expressing cells stimulated with the PAR1-specific agonist for 10 min (Fig. 4 D). Together, these findings demonstrate that ESCRT-III binds specifically to activated PAR1 and regulates MVB/lysosomal degradation.

The ESCRT-III-associated protein ALIX is required for PAR1 degradation

To further define the PAR1 ubiquitin–independent lysosomal sorting pathway, we investigated the function of ALIX, a CHMP4-interacting protein that recruits ESCRT-III to membranes (Fisher et al., 2007). We first examined whether PAR1 interacted with endogenous ALIX in HeLa cells. Cells expressing FLAG-PAR1 were stimulated with agonist for various times, PAR1 was immunoprecipitated, and association of endogenous ALIX was assessed. In cells treated with agonist for 10 min, PAR1 association with endogenous ALIX was markedly increased compared with untreated control cells (Fig. 5 A, lanes 1 and 2), whereas PAR1 and ALIX interaction was diminished after 20 min of agonist exposure (Fig. 5 A, lanes 1–3). In contrast, PAR2 and ALIX interaction was negligible in control and agonist-treated cells (Fig. 5 A, lanes 4 and 5). PAR1 also associated with HA-ALIX in an agonist-dependent manner (Fig. S4 A), whereas HA-ALIX failed to bind to β2AR in cells treated with PAR1-specific agonist (Fig. S4 B) or to activated PAR2 (Fig. S4 C). Next, we examined the association of endogenous ALIX with ubiquitination-deficient PAR1 in HeLa cells. In contrast to control cells, activation of the PAR1 0K mutant for 10 min resulted in endogenous ALIX association (Fig. 5 B), suggesting that ubiquitination is not required for ALIX interaction with activated PAR1.

ALIX has been shown to link certain viral proteins to the ESCRT-III complex, a process important for viral budding (Usami et al., 2007). Therefore, we examined whether ALIX was required for agonist-induced PAR1 interaction with CHMP4. HeLa cells coexpressing FLAG-PAR1 and HA-CHMP4B transfected with nonspecific or ALIX-specific siRNAs were treated with agonist, PAR1 was immunoprecipitated, and CHMP4 association was assessed. In nonspecific siRNA control cells, a 10-min incubation with agonist resulted in robust PAR1 association with CHMP4 (Fig. 5 C, lanes 1–4). In striking contrast, activated PAR1 interaction with CHMP4 was virtually ablated in ALIX-deficient cells (Fig. 5 C, lanes 5–8). These findings suggest that ALIX links PAR1 to the ESCRT-III machinery to facilitate lysosomal sorting.

Next, we assessed ALIX function in agonist-induced PAR1 lysosomal degradation. HeLa cells expressing FLAG-PAR1 were transfected with ALIX-specific siRNAs and stimulated with agonist for various times, and PAR1 degradation was examined. In ALIX-deficient cells, agonist-promoted degradation of PAR1 was significantly inhibited compared with the extent of PAR1 degradation observed after 60 min of agonist stimulation in siRNA-treated control cells (Fig. 6 A). However, internalization of activated PAR1 occurred normally in ALIX-deficient cells (Fig. S4 D), indicating that ALIX mediates PAR1 sorting to a lysosomal degradative pathway after internalization. Depletion of endogenous ALIX expression also caused a significant defect in agonist-promoted degradation of endogenous PAR1-expressed human endothelial cells (Fig. 6 B). Moreover, agonist-induced degradation of ubiquitination-deficient PAR1 0K mutant was also impaired in ALIX-deficient cells, whereas activated PAR2 degradation...
ALIX binds to a highly conserved YPX₃L motif in PAR1 via its central V domain

ALIX contains an N-terminal Bro1 domain, a central V domain, and C-terminal proline-rich region. ALIX binds to YPX₃L motifs present in the late domain of viral Gag proteins via its central V domain (Fisher et al., 2007; Zhai et al., 2008) and is critical for ESCRT-III recruitment and viral budding (Usami et al., 2007). A search of the PAR1 cytoplasmic domains for such a sequence revealed a highly conserved motif within the second intracellular loop of the receptor (Fig. 7A). The PAR1 Y₂₀₆ᵀPMQSL²¹¹ motif conforms to the ALIX-binding motif YPX₃L (with n = 3) and is not present in PAR2 cytoplasmic domains (Fig. 7A). A search of several hundred class A GPCRs for such a motif revealed the presence of a highly conserved sequence that conforms to the YPX₃L motif residing in the second intracellular loop of seven other mammalian GPCRs (Table 1). Only one class A GPCR harbors a conserved YPX₃L motif within the cytoplasmic tail domain (Table 1). To examine the importance of the YPX₃L motif in PAR1 degradation, we mutated the critical Y206 to alanine, as analogous Y mutations in YPX₃L motifs of viral Gag proteins abrogated ALIX interaction and function (Strack et al., 2003). The PAR1 Y206A mutant expressed at the surface and internalized after activation comparable with WT PAR1 (Fig. S5, A and B), suggesting that the PAR1 Y206A mutant traffics to and from the cell surface normally.

Next, we examined whether the PAR1 YPX₃L motif was necessary for interaction with ALIX. HeLa cells coexpressing FLAG-PAR1 WT or Y206A mutant together with HA-ALIX were stimulated with agonist, and coassociation was examined. PAR1 WT robustly associated with ALIX after 10 min of agonist stimulation, whereas activated PAR1 Y206A mutant interaction with ALIX was negligible (Fig. 7B). PAR2, which lacks a YPX₃L motif, also failed to bind to HA-ALIX (Fig. S4C), suggesting that the YPX₃L motif is an important determinant for ALIX interaction. We also examined whether the ALIX V domain is required for association with PAR1. The ALIX central V domain binds directly to YPX₃L sequences of viral Gag proteins, and mutation of the critical phenylalanine to aspartic acid (F676D) was shown to disrupt this interaction (Zhai et al., 2008). In contrast to PAR1 interaction with WT ALIX, the HA-ALIX F676D mutant failed to bind to PAR1 after 10 min of agonist incubation (Fig. 7C). To confirm direct interaction between the PAR1 YPX₃L motif and the ALIX V domain, we performed in vitro binding assays using a biotinylated PAR1 ICL2 peptide containing the YPX₃L motif in pull-down experiments with purified GST-tagged ALIX. Strikingly, the ALIX V domain showed robust interaction with the PAR1 YPX3L containing ICL2 peptide (Fig. 7D), whereas neither the ALIX V domain harboring the F676D mutant nor the ALIX Bro1 domain bound to the PAR1 ICL2 peptide (Fig. 7D). Together, these findings strongly suggest that ALIX can bind directly to the YPX₃L motif of PAR1 via its central V domain.

Our experiments indicate that the PAR1 YPX₃L motif is critical for binding to ALIX. Therefore, we tested whether the PAR1 YPX₃L motif was necessary for agonist-promoted PAR1 degradation. Incubation with agonist for 60 min caused an ~50% loss of PAR1 protein (Fig. 8A). In contrast to WT

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*Figure 5.* ALIX associates with PAR1 and mediates interaction with CHMP4. (A and B) HeLa cells expressing either FLAG-tagged PAR1 WT, 0K mutant, or PAR2 were stimulated with 100 μM SFLRN or 100 μM SLIGKV for various times at 37°C. Equivalent amounts of cell lysates were immunoprecipitated with HA-CHMP4B and either 100 nM nonspecific (ns) or ALIX-specific immunoblotting. (C) HeLa cells expressing FLAG-PAR1 were cotransfected with HA-CHMP4B and either 100 nM nonspecific (ns) or ALIX-specific siRNAs. Equivalent amounts of cell lysates were immunoprecipitated and analyzed as described in Fig. 4B.
PAR1, agonist-stimulated degradation of the PAR1 Y206A mutant was significantly inhibited (Fig. 8 A). Next, we examined whether PAR1 Y206A mutant remained at the limiting membrane of MVBs or sorted to an internal compartment by evaluating its sensitivity to proteinase K digestion after activation and internalization. Proteinase K protection assays have been used previously to assess EGFR sorting to ILVs of MVBs (Malerød et al., 2007). HeLa cells expressing PAR1 WT or PAR1 Y206A mutant were incubated with 100 μM SFLLRN at 37°C. Equivalent amounts of lysates were analyzed for PAR1, endogenous ALIX (lanes 1–4), HA-AUX WT (lanes 5 and 6), or HA-AUX Bro1 Δ (lanes 7 and 8) expression by immunoblotting.

Figure 6. ALIX is required for agonist-induced PAR1 degradation. (A and C) HeLa cells expressing FLAG-tagged PAR1 (A), PAR1 0K mutant, or PAR2 (C) were transfected with 100 nM nonspecific (ns) or ALIX-specific siRNAs and stimulated with 100 μM SFLLRN or 100 μM SUCKY at 37°C, as indicated. PAR degradation was assessed by immunoblotting (IB). The data (mean ± SD) are expressed as the fraction of PAR1 remaining compared with untreated control (Ctrl) and were analyzed by a two-way analysis of variance (*, P < 0.05; n = 3). (B) Human endothelial cells transfected with 100 nM of nonspecific or ALIX-specific siRNAs stimulated with 100 μM TFLBRNKPDN at 37°C, as indicated, and immunoprecipitated (IP) with anti-PAR1 or IgG antibodies (Ab). Endogenous PAR1 was detected by immunoblotting. Cell lysates were immunoblotted with anti-ALIX and anti-actin antibodies. The data (mean ± SD) are expressed as a fraction of untreated control and were analyzed by a two-way analysis of variance (*, P < 0.05; n = 3). (D) HEK293 cells cotransfected with either 100 nM of nonspecific or two ALIX-specific siRNAs together with FLAG-PAR1 and either siRNA-resistant HA-ALIX WT or HA-ALIX Bro1 Δ were incubated with or without 100 μM SFLLRN for 50 min at 37°C. Equivalent amounts of lysates were analyzed for PAR1, endogenous ALIX (lanes 1–4), HA-AUX WT (lanes 5 and 6), or HA-AUX Bro1 Δ (lanes 7 and 8) expression by immunoblotting.

Figure 7. A YPXL motif of PAR1 mediates binding to ALIX. (A) Alignment of PAR1 and PAR2 ICL2 sequences from various species. The conserved residues of the YPXL motif are shaded in gray. (B) HeLa cells coexpressing HA-ALIX WT and either FLAG-PAR1 WT or Y206A mutant were stimulated with 100 μM SFLLRN at 37°C, as indicated. Cell lysates were immunoprecipitated and examined as described in Fig. 4 B. IB, immunoblotting. The data (mean ± SD) represent the amount of immunoprecipitated ALIX normalized to the amount of immunoprecipitated PAR1 and were significant, as determined by Student’s t test (**, P < 0.01; n = 3). (C) HeLa cells transfected with Flag-PAR1 and either HA-ALIX WT or F676D mutant were stimulated with agonist and processed as previously described. Cell lysates were immunoblotted with anti-ALIX and -actin antibodies. The data (mean ± SD) were calculated, as previously described, and significant, as determined by Student’s t test (**, P < 0.01; n = 3). (D) Biotinylated PAR1 ICL2 peptide containing the YPXL motif was immobilized on streptavidin beads and incubated with GST, GST–ALIX V domain, GST–ALIX V F676D, or the GST–ALIX Bro1 domain. Pulldowns (PD) were analyzed for the presence of bound protein by immunoblotting. Input was analyzed by Coomassie staining.
were efficiently degraded after exposure to proteinase K in samples supplemented by Triton X-100, which allows proteinase K access to internal membranous compartments (Fig. 8 B, lanes 3, 6, 9, and 12). The early endosomal antigen protein 1 (EEA1) was also efficiently degraded in all proteinase K–treated samples, demonstrating effective plasma membrane permeabilization and proteinase K activity (Fig. 8 B). In addition, the PAR1 Y206A mutant internalized to EEA1-positive endosomes like WT receptor in cells treated with agonist for 10 min (Fig. 8 C), and both sorted from the EEA1-containing endosomes after 20 min (Fig. 8 C). The extent of colocalization with EEA1 at 10 min was quantified by determining the Pearson’s correlation coefficient for PAR1 WT (r = 0.50 ± 0.1; n = 6) and Y206A mutant (r = 0.43 ± 0.09; n = 6).

Table 1. Human GPCRs with conserved YPX\(_n\)L motifs

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<td>Melanocortin</td>
<td>MC(_4)</td>
<td>P32245</td>
<td>Cytoplasmic tail</td>
<td>318-YPLGGLCDLLSSRY-stop</td>
</tr>
</tbody>
</table>

A search of 397 class A GPCR cytoplasmic sequences for YPX\(_n\)L motifs was completed by sequence alignment using the GPCR database (Yrold et al., 2011). Of these, several GPCRs contained sequences conforming to the YPX\(_n\)L motif in the ICL2 (bold) and were aligned starting with the DRY/F motif. The list includes the GPCR family name, International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature, and NCBI accession numbers.

Figure 8. The YPX\(_3\)L motif of PAR1 is essential for MVB/lysosomal sorting. (A) HeLa cells expressing FLAG-PAR1 WT or Y206A mutant were incubated with 100 \(\mu\)m SFLLRN at 37°C, as indicated. PAR1 degradation was analyzed as described in Fig. 2 B. IB, immunoblotting. The data (mean ± SD) are expressed as a fraction of untreated control (Ctrl) and were analyzed by a two-way analysis of variance (*, P < 0.05; ***, P < 0.001; n = 3). (B) HeLa cells expressing FLAG-PAR1 WT or Y206A were untreated (control) or treated with 100 \(\mu\)m SFLLRN (SF) for 15 min at 37°C. Membranes were isolated and treated with proteinase K or proteinase K supplemented with 0.1% Triton X-100, and PAR1 or EEA1 was detected by immunoblotting. The data (mean ± SD) are expressed as the fraction of PAR1 remaining after proteinase K treatment compared with untreated control (−) from either the control or SFLLRN (+SF)-treated samples and were analyzed by Student’s t test (*, P < 0.05; n = 3). (C and D) HeLa cells expressing either FLAG-PAR1 WT or Y206A mutant were pretreated with anti-FLAG antibody at 4°C and incubated with 100 \(\mu\)m SFLLRN at 37°C, as indicated. Cells were immunostained for either EEA1 or LAMP1 and analyzed, as described in Fig. 1 A. Insets show magnifications of boxed areas. Bars, 10 \(\mu\)m. The data (mean ± SD) represent Pearson’s correlation coefficients that were calculated, as described in Fig. 1 A, and were analyzed by Student’s t test (**, P < 0.01; ***, P < 0.001; n = 6).
ubiquitin-dependent and -independent MVB sorting. The ubiquitin-mediated MVB sorting pathway of PAR2 requires the ESCRT-0, -I, and -II complexes before ESCRT-III-mediated incorporation into ILVs. In contrast, PAR1 MVB sorting is mediated by ALIX recruitment of the ESCRT-III complex, independent of ubiquitination ([Ub]).

Interestingly, however, PAR1 Y206A mutant showed minimal colocalization with LAMP1 after 20 min of agonist stimulation compared with WT PAR1 (Fig. 8 D), which was verified by Pearson’s correlation coefficient for PAR1 WT (r = 0.28 ± 0.08; n = 6) and Y206A (r = 0.08 ± 0.02; n = 6). Thus, the YPX3L motif is a critical determinant for PAR1 ubiquitin-independent MVB/lysosomal sorting (Fig. 9).

Discussion

In this study, we define a new ubiquitin-independent MVB/lysosomal sorting pathway for signaling receptors that requires ALIX and ESCRT-III function in mammalian cells. We demonstrate that activated PAR1 is sorted to ILVs of MVBs independent of receptor ubiquitination but requires ESCRT-III function. Our data further indicate that PAR1 degradation is regulated by ALIX, which binds directly to a YPX3L motif localized within the PAR1’s intracellular loop via its central V domain and mediates CHMP4/ESCRT-III recruitment to the receptor. Thus, PAR1 bypasses the requirement for ubiquitination and ubiquitin-binding ESCRTs but requires ALIX and ESCRT-III for sorting to ILVs of MVBs (Fig. 9).

We previously showed that lysosomal sorting of PAR1 occurs independent of ubiquitination and the ubiquitin-binding ESCRT components HRS and Tsg101 (Gullapalli et al., 2006; Wolfe et al., 2007), but whether PAR1 sorts to ILVs of MVBs remained unknown. Here, we show that a ubiquitination-deficient PAR1 mutant sorted to ILVs similar to WT receptor, indicating that ubiquitination is not essential for ILV sorting of PAR1. Our experiments further indicate that CHMP4, the major subunit of ESCRT-III and driver of ILV scission, is essential for agonist-induced PAR1 degradation. In contrast to other ESCRT complexes, ESCRT-III does not contain any known ubiquitin-binding subunits, suggesting that ubiquitin binding is not essential for ESCRT-III-mediated PAR1 degradation. Moreover, ubiquitination is not a prerequisite for cargo passage into ILVs, as ESCRT-III subunits recruit enzymes that appear to deubiquitinate cargo before ILV entry (Kyuuma et al., 2007). Thus, unlike most cargo sorted to ILVs of MVBs via the canonical ESCRT pathway, our findings indicate that PAR1 is uniquely sorted by ESCRT-III independent of ubiquitination. This pathway is also distinct from the entirely ESCRT-independent ILV sorting pathway previously described for the melanosome constituent Pmel and the proteolipid protein (Theos et al., 2006; Trajkovic et al., 2008; van Niel et al., 2011). Other cargo, such as the yeast Sna3 and mammalian DOR, are also not directly ubiquitinated, but certain ubiquitin-binding ESCRT components facilitate MVB/lysosomal sorting (Hislop et al., 2004; McNatt et al., 2007; Oestreich et al., 2007; Watson and Bonifacino, 2007).

Our findings also reveal a novel function for ALIX, a CHMP4–ESCRT-III interacting protein, in mediating MVB/lysosomal sorting of PAR1. ALIX directly binds to and recruits CHMP4–ESCRT-III to membranes via interaction with its Bro domain and is important for abscission during cytokinesis and budding of certain viruses (Strack et al., 2003; Morita et al., 2007). However, ALIX function in cargo sorting at MVBs in mammalian cells is not known. Bro1, the yeast homolog of ALIX, facilitates the sorting of ubiquitinated cargo at MVBs (Odorizzi et al., 2003). In yeast, Bro1 recruits the deubiquitinating enzyme Doa4 to the late endosome, which is essential for cargo deubiquitination and sorting at the MVB. However, in mammalian cells, ALIX does not appear to function in lysosomal sorting of ubiquitinated cargo, as depletion of ALIX by siRNA failed to affect degradation of EGFR (Cabezás et al., 2005), a receptor that requires direct ubiquitination and ESCRTs for degradation. We found that the association of ALIX with activated PAR1 coincided with PAR1 sorting to ILVs at the MVB. Moreover, degradation of endogenous and ectopically expressed PAR1 was significantly impaired in ALIX-deficient cells and was restored by expression of an siRNA-resistant ALIX WT variant, indicating that ALIX function is specific for this pathway. ALIX also mediated degradation of ubiquitination-deficient PAR1, but not the related PAR2, which requires ubiquitination for lysosomal sorting. In addition, ALIX is required for activated PAR1 recruitment of CHMP4 subunits, the critical components of ESCRT-III, and the ALIX Bro domain important for CHMP4 interaction is necessary for the rescue of agonist-promoted PAR1 degradation in cells deficient in endogenous ALIX expression. Thus, ALIX has the capacity to mediate cargo sorting at the MVB in mammalian cells through a pathway that requires ESCRT-III recruitment. ALIX-mediated MVB sorting of PAR1 also appears to be distinctly regulated, as PAR1 does not accumulate at MVBs when ILV sorting is blocked, compared with ubiquitinated cargo that use canonical ESCRT components. The mechanistic basis for this observation is not known but may involve trafficking of PAR1 from the MVB to a different compartment and/or recycling back to the cell surface.

This study is the first to demonstrate ALIX-mediated MVB/lysosomal sorting of a mammalian cell host protein via a YPX3L motif. The ALIX central V domain has been shown to
bind to YPX\(_n\)L motifs of retroviral Gag proteins (Fisher et al., 2007; Zhai et al., 2008). We found that ALIX directly interacts with a highly conserved YPX\(_n\)L motif within the PAR1’s intracellular loop via its V domain to facilitate MVB/lysosomal degradation. Previous studies showed that the HIV-1 and EIAV viruses recruit the ESCRT machinery through interaction with ALIX (Strack et al., 2003; Zhai et al., 2008) The HIV-1 structural Gag protein harbors a tetrapeptide motif that binds to Tsg101 (Garrus et al., 2001; Martin-Serrano et al., 2001) and a YPX\(_n\)L motif that interacts with the V domain of ALIX (Strack et al., 2003; Fisher et al., 2007) to mediate ESCRT-III recruitment. In contrast, EIAV Gag proteins only possess an ALIX-binding YPX\(_n\)L motif that is critical for ESCRT-III recruitment (Martin-Serrano et al., 2003) and viral budding (Tanzi et al., 2003). Similar to HIV-1 and EIAV (Strack et al., 2003), mutation of the critical tyrosine within the YPX\(_n\)L motif of PAR1 disrupted interaction with ALIX and impaired PAR1 MVB/lysosomal degradation. In vitro binding assays confirmed that the V domain of ALIX binds directly to the YPX\(_n\)L motif of PAR1. Thus, ALIX directly interacts with PAR1 and mediates MVB/lysosomal sorting via an ESCRT-III–dependent pathway.

In summary, this study provides the first molecular insight into a mechanism by which a signaling receptor can sort to ILVs of MVBs independent of ubiquitination and ubiquitin-binding ESCRTs. Unlike most ubiquitinated cargo, we show that PAR1 sorts to ILVs of MVBs through a distinct ubiquitin-independent pathway that requires ALIX and ESCRT-III function but not the canonical ubiquitin-binding ESCRT components. These findings unveil a new mechanism that enriches the diversity of MVB sorting pathways for signaling receptors. This work also suggests that ubiquitination of mammalian GPCRs is likely to have additional functions besides mediating receptor sorting at the MVB. ALIX has also been shown to bind to the cytoplasmic tail domains of the transferrin receptor (Géninard et al., 2004), the GPCR vasopressin V2R (Yi et al., 2007), and D1-like and D3 dopamine GPCRs (Zhan et al., 2008) and appears to regulate various aspects of receptor trafficking. However, these cargo lack the ALIX-binding YPX\(_n\)L motif, and, thus, mechanistically how ALIX functions in these pathways is not known. Remarkably, we discovered seven other mammalian GPCRs that contained conserved YPX\(_n\)L motifs within their second intracellular loop, raising the intriguing possibility that ALIX mediates MVB/lysosomal sorting of a subset of GPCRs in mammalian cells.

Materials and methods

**Antibodies and reagents**

PAR1 peptide agonists (SFLRIN and TFLRNPNDK) and PAR2 peptide agonist (SIIGKV) were synthesized and purified by reverse-phase high-pressure liquid chromatography at the Tufts University Core Facility. Polyclonal anti-FLAG and -HA antibodies were obtained from Rockland Immunocyticals. Anti-LAMP1 and -CD63 antibodies were obtained from Abcam (Cambridge, MA). The monoclonal anti-HA and -GFP antibodies were purchased from Covance. The M2 anti-FLAG and -actin antibodies were obtained from Sigma-Aldrich. The HA antibody conjugated to 6-His was purchased from Roche. 6-His-conjugated goat–anti rabbit and goat–anti mouse antibodies were purchased from Bio-Rad Laboratories. Alexa Fluor 488, 594, and 647 secondary antibodies were purchased from Invitrogen.

**Plasmids and cell lines**

The full-length human N-terminal FLAG-tagged PAR1 WT, FLAG-PAR1 0K mutant cDNAs cloned into the mammalian expression vector pBluescript SK (a kind gift from J. Martin-Serrano, King’s College, London, England, UK) were purchased as SMARTpools from Thermo Fisher Scientific. The single FLAG-PAR1 siRNAs used were 15’-GAAAGAGGAGCAGGGAGGAA-3’ and 15’-CGGAAAGAGGAAGUAAACGAA-3’. Non-specific siRNA (5’-GGCUCAGCUCGCAGCCAGCC-3’) was used as a negative control. The ALIX knockdown rescue experiment was performed by cotransfecting HEK293 cells with ALIX-specific siRNAs (5’-GUAAUCCAGUCGUAUUAGAA-3’ and 5’-UCGAGACGCCGUCGAGAUA-3’; Thermo Fisher Scientific) together with cDNAs containing HA-AUX WT or HA-AUX Bro domain mutant harboring silent mutations within the siRNA-targeted region using Lipofectamine 2000 reagent.

**Immun-EM**

Hela cells stably expressing FLAG-PAR1 or FLAG-PAR1 0K mutant were grown in 10-cm dishes (1.0 x 10\(^6\) cells/dish) for 48 h. Cells were washed and preincubated on ice for 1 h with anti-FLAG antibody to label only receptors at the cell surface. Cells were washed to remove unbound antibody and treated with agonist for 20 min at 37°C, fixed with 4% PFA in 0.1 M phosphate buffer, pH 7.4, overnight at room temperature, washed with 0.1 M glycine/phosphate buffer, and then embedded in 10% gelatin/phosphate buffer infused with 2.3 M sucrose/phosphate buffer overnight at 4°C. 1-mm\(^3\) cell blocks were mounted onto specimen holders and snap frozen in liquid nitrogen. Ultracytomeronomy was performed at −100°C on an ultramicrotome with an EM FCS cryoattachment (Ultratome UCT; Leica) using a diamond knife (DiATOME US). 80–90-nm sections were picked up with a 1:1 mixture of 2.3 M sucrose/phosphate buffer and then embedded in 10% gelatin/phosphate buffer infused with 2.3 M sucrose/phosphate buffer overnight at 4°C. 1-mm\(^3\) cell blocks were mounted onto specimen holders and snap frozen in liquid nitrogen. Ultracytomeronomy was performed at −100°C on an ultramicrotome with an EM FCS cryoattachment (Ultratome UCT; Leica) using a diamond knife (DiATOME US). 80–90-nm frozen sections were picked up with a 1:1 mixture of 2.3 M sucrose and 2% polyvinyl alcohol, as previously described (Lavoie et al., 2002), and transferred onto Formvar and carbon-coated copper grids. Immunolabeling was performed by placing grids on 2% gelatin at 37°C for 20 min and rinsed with 0.15 M glycine/phosphate buffer, and then embedded in 10% gelatin/phosphate buffer infused with 2.3 M sucrose/phosphate buffer overnight at 4°C. 1-mm\(^3\) cell blocks were mounted onto specimen holders and snap frozen in liquid nitrogen. Ultracytomeronomy was performed at −100°C on an ultramicrotome with an EM FCS cryoattachment (Ultratome UCT; Leica) using a diamond knife (DiATOME US). 80–90-nm frozen sections were picked up with a 1:1 mixture of 2.3 M sucrose and 2% polyvinyl alcohol, as previously described (Lavoie et al., 2002), and transferred onto Formvar and carbon-coated copper grids. Immunolabeling was performed by placing grids on 2% gelatin at 37°C for 20 min and rinsed with 0.15 M glycine/phosphate buffer, and then blocked using 1% cold water fish skin gelatin. Previously titrated primary antibodies were diluted in 1% BSA/PBS. Incubation with primary antibodies for 1 h at room temperature was followed by gold-conjugated goat anti–mouse IgG and IgM (Jackson Immunoresearch Laboratories, Inc.) or goat-conjugated goat anti–rabbit IgG, both diluted 1/25 in 1% BSA/PBS at room temperature for 30 min. Grids were viewed using a transmission electron microscope (1200-EX II, JEOL) and photographed using a digital camera (Gatan, Inc) at the University of California San Diego Electron Microscopy Facility.

**PAR degradation assay**

Hela cells stably expressing FLAG-PAR1, FLAG-PAR1 0K, or FLAG-PAR2 were plated in 12-well dishes (1.5 x 10\(^6\) cells/well) and grown overnight
at 37°C and transfected with either siRNAs or cDNA plasmids. After 48 h, cells were washed and treated with or without 100 μM SFLRN (PAR1-specific agonist) or 100 μM SU6506 (PAR2-specific agonist) for various times at 37°C. Cells were placed on ice, washed with PBS, and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM NaPF6, and 1% Triton X-100) supplemented with protease inhibitors. Cell lysates were collected and sonicated for 10 s at 10% amplitude (model 450 sonifier; Branson), and protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Equivalent amounts of lysates were used for analysis by immunoblotting. Endothelial cells were plated in 6-cm dishes (3.0 x 106 cells/plate), grown overnight, washed, and lysed in 2× sample buffer, resolved by SDS-PAGE, transferred to membranes, and probed with species-specific secondary antibodies conjugated to Alexa Fluors and stained with Coomassie to visualize proteins.

Immunofluorescence confocal microscopy
Hela cells stably expressing FLAG-PAR1 or FLAG-PAR2 were plated on coverslips in 12-well dishes and grown overnight. Cells were washed, stimulated with 100 μM SFLRN (PAR1-specific agonist) for varying times at 37°C, washed, and lysed with 0.5% NP-40 lysis buffer containing 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl. Cell lysates were sonicated and cleared by centrifugation, and equivalent amounts of lysates determined by BCA assay were precleared with protein A Sepharose CL-4B for 1 h at 4°C. Precleared lysates were then immunoprecipitated with M2 anti-FLAG antibody or IgG overnight at 4°C. Immunoprecipitates were washed, resuspended in 2x sample buffer, resolved by SDS-PAGE, transferred to membranes, and examined by immunoblotting. For PAR1 coimmunoprecipitation of endogenous CHMP4 and ALIX, Hela cells expressing FLAG-PAR1 WT or FLAG-PAR1 OK mutant were grown in six-well plates (4.0 x 105 cells/well). After 48 h, cells were treated with agonist and lysed in buffer, as previously stated. The amount of ALIX in immunoprecipitates was examined by immunoblotting.

Cell surface ELISA
Hela cells were plated at 5 x 105 cells/well in a 24-well culture dish, grown overnight, and transfected, as previously described. After 48 h, cells were either left untreated or treated with 100 μM SFLRN (PAR1-specific agonist) for varying times at 37°C, and the amount of receptor remaining at the cell surface was determined, as previously described [Soto and Trejo, 2010]. In brief, cells were fixed in 4% PFA, washed, and then incubated with secondary antibodies conjugated to Alexa Fluor or imaged by confocal microscopy. To specifically track activated PAR1 or PAR2 from the cell surface, cells were prelabeled with anti-FLAG antibody for 1 h at 4°C to ensure only surface FLAG-PAR1 or PAR2-bound antibody, stimulated with agonist for various times at 37°C, and then processed for microscopy. Fixed cells were mounted in FluorSave reagent (EMD). The fluorochromes used were Alexa Fluor 488, 594, and 647 and GFP. Images were acquired with a disk-spinning unit confocal system (Olympus) configured with a microscope (IX81; Olympus) fitted with a PlanApo 60x oil objective (1.4 NA; Olympus) and a digital camera (ORCA-ER; Hamamatsu Photonics). Fluorescent images of 0.28-μm-thick X–Y sections were acquired at room temperature using SlideBook software (version 4.2; Intelligent Imaging Innovations, Inc.). Pearson’s correlation coefficients for quantifying colocalization of PAR1 WT and mutants with either EEA1 or LAMP1 were calculated for six independent cells from multiple experiments using SlideBook software (version 4.2).

Protein sequence alignment
The following protein sequences were acquired from the National Center for Biotechnology Information protein database: Rattus norvegicus (available from GenBank under accession no. AAAA2274.1; Xenopus laevis [GenBank accession no. AA18498.1], Mus musculus [GenBank accession no. AAAA40438.1], Papio hamadryas [GenBank accession no. AAA840191.1], Cricetus longicaudatus [GenBank accession no. AAAB6474.1], Bos taurus [NCBI Protein database accession no. NP_001196656.1], Equus caballus [NCBI Protein database accession no. XP_005104007.2], Danio rerio [GenBank accession no. CAM13317.1], and Homo sapiens PAR1 [GenBank accession no. AAAA3743.1] and PAR2 [GenBank accession no. AAAP79012.1]. Sequences were aligned using ClustalX 2.1 software [Larkin et al., 2007].

In vitro binding assay
A biotinylated PAR1 ICL2 peptide representing the second intracellular loop sequence LAVPVMQGSSWTLG (the segment in italics denotes the YPX motif within the peptide fragment, which is required for binding to ALIX in the context of full-length PAR1) was synthesized and purified by the Tufts University Core Facility. Biotinylated PAR1 IC2 peptide was immobilized on streptavidin beads (Sigma-Aldrich). GST vector, GST–ALIX V, GST–ALIX V F676D, and GST–ALIX B1 1 DNA plasmids were transformed into BL21 (DE3) Escherichia coli cells (Agilent Technologies). E. coli were grown in ZYP-5052 media (1% tryptone, 0.5% yeast extract, 0.025 M (NH4)2SO4, 0.05 M KH2PO4, 0.05 M Na2HPO4, 0.5% glycerol, 0.05% glucose, and 0.2% β-lactose) and lysed, and GST fusion proteins were purified on glutathione resin and analyzed by SDS-PAGE and Coomassie to visualize proteins.

Protease K protection assay
The protease K protection assay was performed as previously described [Malerad et al., 2007], with minor modifications. Hela cells were plated in six-well culture dishes (4.0 x 105 cells/well), grown overnight at 37°C, and treated with or without agonist. Cells were placed on ice and incubated for 5 min with PBS, harvested, and gently permeabilized using 6.5 μg/ml digitonin. Membranes were collected by centrifugation and resuspended in buffer (100 mM K2HPO4/KH2PO4, 5 mM MgCl2, and 250 mM sucrose). Membranes were then divided into three aliquots either left untreated, treated with 2.5 ng/ml protease K, or treated with protease K supplemented with 0.1% Triton X-100 for 10 min at room temperature. After treatments, samples were diluted with 100 μl of 2x SDS sample buffer containing 20 mM PMSF and analyzed by immunoblotting.

RT-PCR
The first-strand cDNA was generated from mRNA extracted from either Hela using SuperScript II reverse transcription (Invitrogen) following the manufacturer’s instructions. The RT enzyme was omitted from the cDNA synthesis reaction in the control samples. The first-strand cDNA was amplified via PCR using primer pairs specific for CHMP4A [forward 5′-GGTGATCTTAGGGCTTACGC-3′ and reverse 5′-GAGGCGGTGTTCACTGAC-3′], CHMPB [forward 5′-CCCCATGACACACAGGACC-3′ and reverse 5′-GGCCAGTTCTCCAATCTCC-3′], CHMP4C [forward 5′-GAATTCCAGGCTAAGACACG-3′ and reverse 5′-CTGACGACGATCCTAGG-3′], and CHMP4D [forward 5′-CCCCATGACACACAGGACC-3′]. The PCR amplification products were resolved by 1.8% (wt/vol) agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistics
Data were analyzed using Prism software (version 4.0; GraphPad Software). Statistical analysis was determined, as indicated, by performing either Student’s t test or two-way analysis of variance.

Online supplemental material
Fig. S1 shows the localization of PAR1 WT and OK mutant at steady state and after stimulation with agonist for 60 min, relative to EEA1-positive.
early endosomes, and shows localization of surface-labeled PAR1 WT and OK mutant relative to LAMP1 at 0 min and after a 20-min incubation with or without agonist. Fig. S2 shows PAR1 degradation in cells treated with individual siRNAs targeting CHMP4B. Fig. S3 demonstrates that Vps4 siRNA SMARTpools inhibit agonist-induced degradation of PAR1 and PAR2. Fig. S4 shows HA-Alix coimmunoprecipitation with FLAG-PAR1 but not with the unrelated FLAG-ζadr adrenergic receptor or FLAG-PAR2 and demonstrates that Alix knockdown does not impair internalization of PAR1. Fig. S5 show that PAR1 Y206A is expressed at the cell surface and is internalized comparably with WT PAR1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201110031/DC1.

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Author contributions: M.R. Dores and B. Chen designed, performed, and analyzed the experiments and prepared the manuscript. H. Lin, U.J.K. Soh, M.M. Paing, W.A. Montagane, and T. Meeflo contributed experimental work. J. Trejo contributed to project design and manuscript preparation.

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