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Protease-activated Receptor-4 Signaling and Trafficking Is Regulated by the Clathrin Adaptor Protein Complex-2 Independent of β-Arrestins*

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Protease-activated receptor-4 (PAR4) is a G protein-coupled receptor (GPCR) for thrombin and is proteolytically activated, similar to the prototypical PAR1. Due to the irreversible activation of PAR1, receptor trafficking is intimately linked to signal regulation. However, unlike PAR1, the mechanisms that control PAR4 trafficking are not known. Here, we sought to define the mechanisms that control PAR4 trafficking and signaling. In HeLa cells depleted of clathrin by siRNA, activated PAR4 failed to internalize. Consistent with clathrin-mediated endocytosis, expression of a dynamin dominant-negative K44A mutant also blocked activated PAR4 internalization. However, unlike most GPCRs, PAR4 internalization occurred independently of β-arrestins and the receptor's C-tail domain. Rather, we discovered a highly conserved tyrosine-based motif in the third intracellular loop of PAR4 and found that the clathrin adaptor protein complex-2 (AP-2) is important for internalization. Depletion of AP-2 inhibited PAR4 internalization induced by agonist. In addition, mutation of the critical residues of the tyrosine-based motif disrupted agonist-induced PAR4 internalization. Using Dami megakaryocytic cells, we confirmed that AP-2 is required for agonist-induced internalization of endogenous PAR4. Moreover, inhibition of activated PAR4 internalization enhanced ERK1/2 signaling, whereas Akt signaling was markedly diminished. These findings indicate that activated PAR4 internalization requires AP-2 and a tyrosine-based motif and occurs independent of β-arrestins, unlike most classical GPCRs. Moreover, these findings are the first to show that internalization of activated PAR4 is linked to proper ERK1/2 and Akt activation.

Thrombin is a coagulant protease that plays an essential role in hemostasis and thrombosis in response to tissue injury (1). Thrombin is locally generated at sites of vascular injury, where it initiates responses in various cell types including platelets, endothelial, and smooth muscle cells. Thrombin elicits cellular responses through members of the protease-activated receptor (PAR)[5] family of G protein-coupled receptors (GPCRs). Unlike classic GPCRs, which are activated by a diffusible ligand, PARs are activated by the proteolytic cleavage of the receptor N-terminal domain (2, 3). The newly exposed N terminus, termed the "tethered ligand," then binds intramolecularly to the receptor's second extracellular loop, inducing a conformational change that facilitates coupling to heterotrimeric G proteins and downstream signaling. Studies have shown that a six-amino acid synthetic peptide mimic of a PAR's tethered ligand is sufficient to activate the receptor and evoke cellular responses similar to those resulting from proteolytic cleavage of the receptor (4). Because of the irreversible mechanism of PAR1 activation with the generation of a tethered ligand that cannot diffuse away, the mechanisms that regulate PAR1 trafficking are important for maintaining proper signaling and appropriate cellular responses (5).

After activation, most GPCRs are rapidly internalized from the cell surface via clathrin-mediated endocytosis (6). This process is often initiated by the recruitment of β-arrestin, a clathrin adaptor protein that recognizes both the activated and phosphorylated form of GPCRs (7, 8). β-arrestin and other clathrin adaptor proteins coordinate the formation of clathrin-coated pits at the plasma membrane. The GTPase dynamin facilitates scission of clathrin-coated pits, which bud off from the plasma membrane and fuse with early endosomes. Internalized GPCRs are then either recycled back to the cell surface or sorted to lysosomes and degraded (9).

The majority of proteolytically activated PAR1 and PAR2 are precluded from recycling, unlike most other GPCRs. Rather, the ultimate fate of an activated PAR is degradation at the lysosome, and PAR1 and PAR2 trafficking has been linked to either signal propagation or termination (5, 10, 11). Several studies have characterized the regulatory mechanisms that control signaling and trafficking of the prototypical PAR1. After activation, PAR1 undergoes rapid phosphorylation and

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[5] The abbreviations used are: PAR, protease-activated receptor; AP-2, adaptor protein complex-2; EEA1, early endosomal antigen-1; GPCR, G protein-coupled receptor; LAMP1, lysosomal-associated membrane protein-1; MEF, mouse embryonic fibroblast; β_2AR, β2-adrenergic receptor; ANOVA, analysis of variance; ICL, intracellular loop.

internalization through clathrin-coated pits. However, PAR1 internalization does not require β-arrestins (12) but instead requires phosphorylation and a cytoplasmic tail (C-tail) tyrosine-based motif that is recognized by the clathrin adaptor protein complex-2 (AP-2) (13, 14). Internalized PAR1 is then sorted from early endosomes to lysosomes, where the receptor is degraded (15–17). The processes that mediate PAR1 internalization and intracellular sorting are tightly regulated, and disruption of these processes has been shown to result in dysregulated signaling (5). Thus, PAR1 trafficking is important for proper signaling and appropriate cellular responses. In contrast to PAR1, the mechanisms that regulate PAR4 trafficking and signaling remain to be determined.

PAR4 is often coexpressed with PAR1 and is also an important receptor for thrombin-induced cellular responses. The activation of human platelets by thrombin is mediated by both PAR1 and PAR4 (18). PAR4 has a much lower affinity for thrombin than PAR1, and as a result PAR4 was initially hypothesized to be a "back-up" receptor. However, recent studies have demonstrated that PAR1 and PAR4 play distinct roles in platelet activation. PAR1 regulates the early stages of platelet activation, whereas PAR4 function appears to be more important for the later stages (19). The signaling kinetics exhibited by the two receptors support this model (20), wherein PAR1 signaling is rapid and transient compared with PAR4, which has a slower onset but a prolonged duration.

Thrombin signaling mediated by PARs is linked to cardiovascular pathophysiologies (21). Thus, modulating thrombin signaling via PAR1 is attractive for drug development. In fact, the Food and Drug Administration recently approved Zontivity® (also known as vorapaxar) as the First-in-Class PAR1 antagonist, indicated for reducing the risk of heart attack, stroke, and cardiovascular death in patients with a history of adverse cardiac events (22, 23). However, patients exhibit varying levels of responsiveness to this PAR1 antagonist. Moreover, recent work has shown that platelet PAR4 reactivity varies greatly between different patients (24–26). Specifically, platelets from African American subjects exhibit increased responsiveness to PAR4-specific agonists when compared with other populations. Thus, targeting PAR4 could prove beneficial in treating patients for whom PAR1-directed therapy is not effective.

In this study we sought to characterize the intracellular trafficking of activated PAR4 and to determine whether trafficking is linked to PAR4 signaling. In contrast to most classic GPCRs, we demonstrate that, like PAR1, activated PAR4 undergoes clathrin-mediated endocytosis independent of β-arrestins. Internalized PAR4 is initially sorted to early endosomes and then to lysosomes, similar to PAR1. However, unlike PAR1, we found that the C-tail region of PAR4 is not required for internalization. Rather, we identified a highly conserved tyrosine-based sorting motif in the third intracellular loop (ICL3) of PAR4 that regulates agonist-induced internalization. The clathrin adaptor AP-2 binds to tyrosine-based motifs, and siRNA-mediated depletion of AP-2 blocked activated PAR4 internalization in HeLa cells. Moreover, internalization of activated PAR4 expressed endogenously in Dami megakaryocytic cells was similarly regulated by AP-2. Additionally, inhibition of endogenous PAR4 internalization in Dami cells by AP-2 knockdown resulted in enhanced and prolonged extracellular signal-regulated kinase-1/2 (ERK1/2) signaling, consistent with defects in signal termination. In contrast, Akt activation in response to PAR4 stimulation was significantly attenuated, suggesting that internalization is important for coupling to this pathway. This study is the first to demonstrate that activated PAR4 internalization is distinctly regulated by AP-2 via a tyrosine-based motif and that intracellular trafficking is intimately linked to the regulation of the magnitude and duration of PAR4-induced ERK1/2 and Akt signaling.

Results

Agonist-induced Internalization of PAR4 Occurs through a Clathrin- and Dynamin-dependent Pathway—The mechanisms that regulate PAR4 intracellular trafficking are not known. To investigate whether PAR4 is internalized through a dynamin-mediated pathway, we initially expressed PAR4 in HeLa cells, a cell model system that has been used extensively to characterize clathrin-mediated endocytosis of GPCRs, including PAR1 and the β2-adrenergic receptor (β_2AR) (16, 27–29). Expression of a dominant-negative K44A mutant of dynamin, which is defective in its GTPase activity, inhibits clathrin-mediated endocytosis by preventing scission of coated pits from the plasma membrane (30). HeLa cells co-transfected with FLAG-tagged PAR4 together with dynamin wild type or K44A mutant fused to GFP were stimulated with PAR4-specific peptide agonist AYPGKF and imaged by immunofluorescence confocal microscopy. The AYPKGF peptide was used to activate PAR4, as thrombin cleaves off the N-terminal FLAG epitope required for detection of cell surface receptor. In cells expressing wild type dynamin, PAR4 localized to the cell surface in the absence of agonist and exhibited substantial internalization after 60 min of stimulation with PAR4 agonist peptide (Fig. 1*A*). PAR4 also localized to the cell surface in cells expressing the dynamin K44A mutant (Fig. 1*B*). However, agonist-induced internalization of PAR4 was not detectable in cells expressing dynamin K44A mutant (Fig. 1*B*, *arrowhead*), whereas PAR4 internalization occurred as expected in untransfected adjacent cells that were not expressing the dynamin K44A mutant (Fig. 1*B*). Thus, expression of the dynamin K44A mutant inhibits PAR4 internalization, suggesting that PAR4 undergoes dynamin-dependent endocytosis.

Dynamin-dependent GPCR internalization can occur through detachment of clathrin-coated pits or via release of caveolae from the plasma membrane (6). To determine the role of clathrin, we used siRNA targeting the clathrin heavy chain to deplete HeLa cells of endogenous clathrin. The expression of clathrin was markedly reduced in cells transiently transfected with siRNA targeting clathrin compared with nonspecific siRNA transfected control cells (Fig. 1*E*). In cells transfected with nonspecific siRNA, PAR4 localized to the cell surface in the absence of agonist and exhibited substantial internalization after stimulation with AYPGKF (Fig. 1*C*). PAR4 also localized to the plasma membrane in cells transfected with clathrin siRNA (Fig. 1*D*). However, PAR4 failed to undergo agonist-induced internalization in clathrin-depleted cells (Fig. 1*D*). Together these findings suggest that activated PAR4 is internalized through a clathrin- and dynamin-dependent pathway.

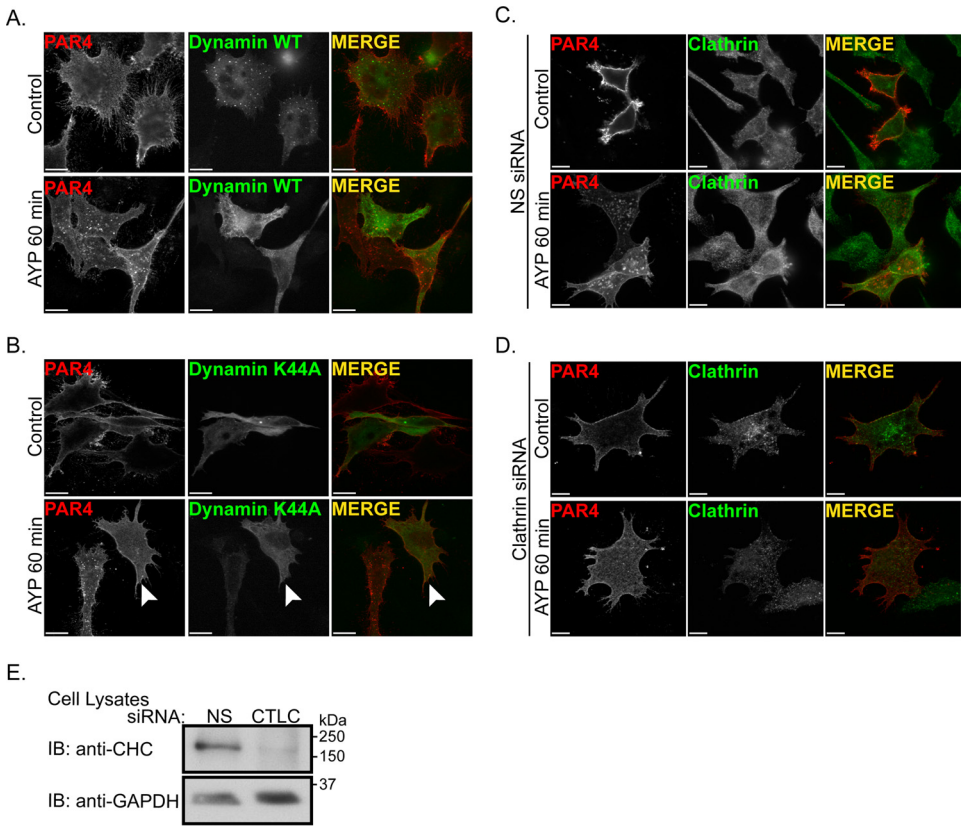

FIGURE 1. **Internalization of activated PAR4 occurs through a dynamin- and clathrin-dependent pathway**. *A* and *B*, HeLa cells transiently transfected with FLAG-PAR4 and either dynamin WT fused to GFP (*A*) or the dynamin dominant-negative K44A mutant fused to GFP (*B*) were pre-labeled with anti-FLAG antibody and stimulated with 500 μM AYPGKF for 60 min at 37 °C. Cells were fixed, permeabilized, immunostained, and imaged by confocal microscopy. *Arrowheads*, dynamin-GFP K44A mutant expressing cells. The images are representative of three independent experiments (*scale bar*, 10 μm). *C* and *D*, HeLa cells were transiently transfected with FLAG-PAR4 and either nonspecific (*NS*) or clathrin heavy chain (*CHC*)-specific siRNA. Cells were incubated with 500 μM AYPGKF for 60 min at 37 °C, processed, and imaged by confocal microscopy. Images are representative of three independent experiments (*scale bar*, 10 μm). *E*, cell lysates were collected in parallel, resolved by SDS-PAGE, and immunoblotted (*IB*) as indicated.

Internalized PAR4 Is Trafficked through Early Endosomes and Sorted to Lysosomes—Previous studies showed that activated PAR1 and the related PAR2 are internalized and sorted predominantly to lysosomes for degradation (17, 31); however, the intracellular trafficking route of PAR4 has not been determined. To examine if activated and internalized PAR4 is sorted to early endosomes, HeLa cells expressing FLAG-tagged PAR4 were treated with agonist for various times at 37 °C to stimulate internalization of PAR4. Trafficking to early endosomes was then measured by colocalization with early endosomal antigen-1 (EEA1), a marker of early endosomes, using immunofluorescence confocal microscopy. We observed significant accumulation of intracellular PAR4 at EEA1-positive puncta as early as 15 min after agonist stimulation compared with control cells (Fig. 2*A*, *top panels*), and a peak in colocalization occurred at 30 min of agonist stimulation (Fig. 2*A*, *middle panels*). The amount of intracellular PAR4 colocalized with EEA1 appeared to diminish after 60 and 90 min of agonist incubation (Fig. 2*A*, *bottom panels*). The extent of PAR4 colocalization with EEA1 was quantified by determining Pearson's correlation coefficient for PAR4 and EEA1 at different time points and is consistent with the peak in colocalization between PAR4 and EEA1 at 30 min (Fig. 2, *A* and *B*). A similar extent of colocalization between EEA1 and the δ-opioid and CXCR4 receptors was previously reported (32). These data indicate that internalized PAR4 is sorted into early endosomal compartments between 15 and 30 min after agonist stimulation.

To determine whether activated PAR4 is sorted to late endosomes/lysosomes, we used confocal microscopy to assess receptor colocalization with lysosomal-associated membrane protein-1 (LAMP1), a specific marker of late endosomes/lysosomes. In unstimulated HeLa cells, PAR4 largely resided on the cell surface. After agonist stimulation for 15 or 30 min, PAR4 internalized to intracellular puncta and showed minimal colocalization with LAMP1 (Fig. 3*A*, *top panels*). These findings are consistent with PAR4 sorting to early endosomes within 30 min of agonist stimulation (Fig. 2). In contrast, a significant accumulation of intracellular PAR4 was detected at LAMP1-positive late endosomes/lysosomes after 60 or 90 min of agonist stimulation (Fig. 3*A*, *bottom panels*). These findings were confirmed by calculating Pearson's correlation coefficients for PAR4 colocalization with LAMP1 at 60 min ($r = 0.1957 \pm 0.03360$) and 90 min ($r = 0.2813 \pm 0.02051$) (Fig. 3*B*). The β_2AR displayed comparable colocation with LAMP1 after prolonged agonist stimulation (33). These studies suggest that activated and internalized PAR4 sorts to early endosomes and then to late endosomes/lysosomes.

Internalization of PAR4 Occurs through a β-Arrestin-independent Pathway—After agonist activation, most GPCRs interact with β-arrestins, which facilitates rapid internalization

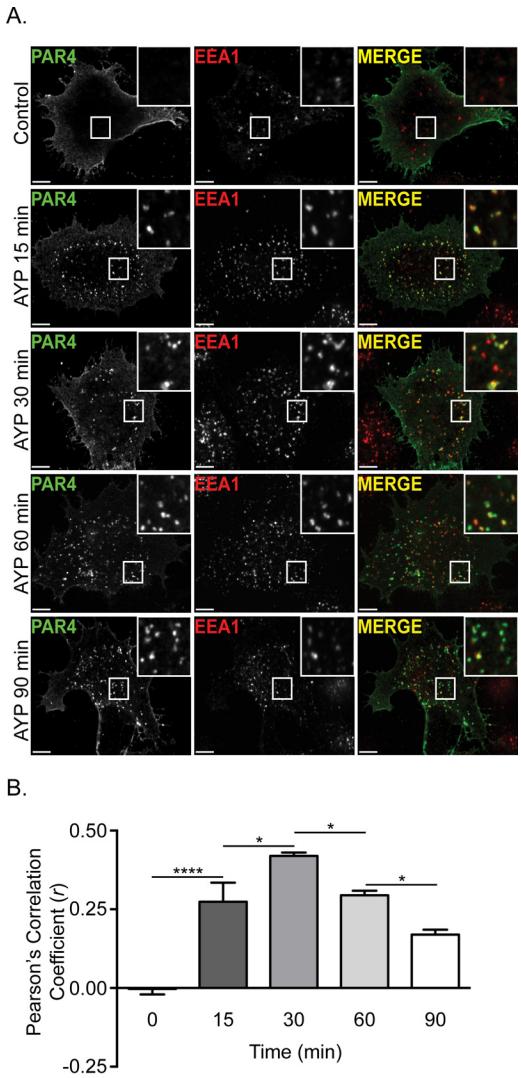

FIGURE 2. **Activated PAR4 is internalized and sorted to early endosomes.** *A*, HeLa cells were transfected with FLAG-PAR4, prelabeled with anti-FLAG antibody, stimulated with 500 μM AYPGKF for various times at 37 °C, processed, co-stained with anti-EEA1 antibody, and imaged by confocal microscopy. The images are representative of three independent experiments (*scale bar*, 10 μm). *Insets*, magnifications of *boxed areas*. *B*, Pearson's correlation coefficient was determined to quantify the degree of correlation in signal intensity between PAR4 and EEA1 at each pixel. Data (mean ± S.E.; $n \geq 9$ cells for each time point) shown were collected from three independent experiments, and statistical significance was determined by one-way ANOVA (*, $p < 0.05$; ****, $p < 0.0001$).

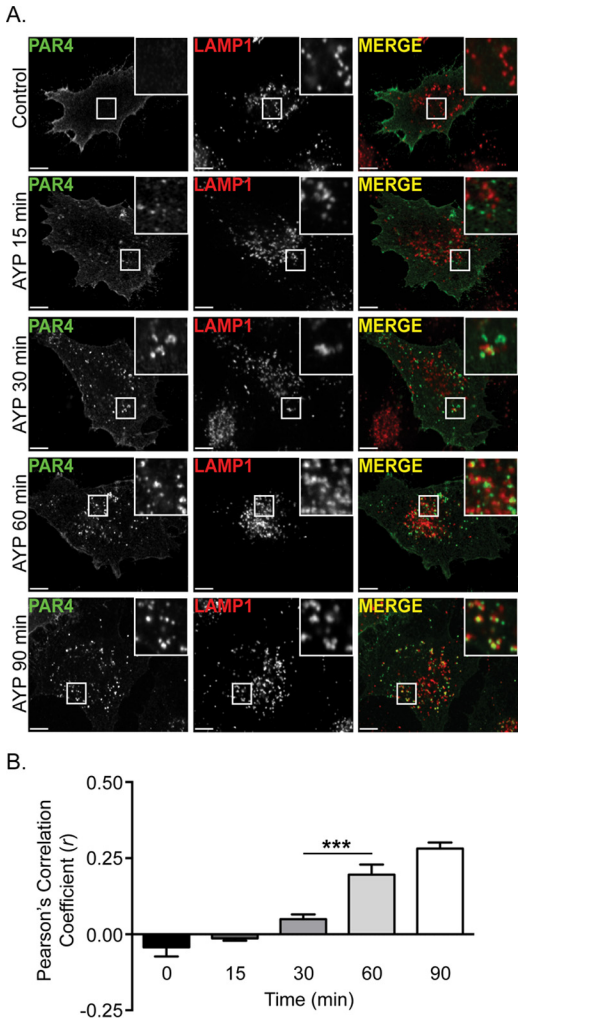

FIGURE 3. **Activated internalized PAR4 is sorted to late endosomes/lysosomes.** *A*, HeLa cells transfected with FLAG-PAR4 were prelabeled with anti-FLAG antibody, stimulated with 500 μM AYPGKF for various times at 37 °C, processed, co-stained with anti-LAMP1 antibody, and imaged by confocal microscopy. The images are representative of three independent experiments (*scale bar*, 10 μm). *Insets*, magnifications of *boxed areas*. *B*, Pearson's correlation coefficient was determined to quantify the degree of correlation in signal intensity between PAR4 and LAMP1 at each pixel. Data (mean ± S.E.; $n \geq 9$ cells for each time point) shown were collected from three independent experiments, and statistical significance was determined by one-way ANOVA (***, $p < 0.001$).

through clathrin-coated pits (34). However, some GPCRs, such as PAR1, do not require β-arrestins for clathrin-mediated internalization (6, 12). To examine the role of β-arrestins in PAR4 internalization, we expressed PAR4 in mouse embryonic fibroblasts (MEFs) derived from β-arrestin 1,2 double knock-out mice and wild type littermate control cells (35) and assessed agonist-induced internalization by immunofluorescence confocal microscopy. The loss of β-arrestin-1 and -2 expression was first confirmed in wild type and β-arrestin knock-out MEFs by immunoblotting (Fig. 4*A*). In these experiments the cell surface PAR4 cohort was prelabeled with anti-FLAG antibody. In wild type MEFs expressing both β-arrestin isoforms, we observed substantial internalization of PAR4 from the cell surface to intracellular vesicles after 60 min of agonist stimulation (Fig. 4*B*, *top panels*). Interestingly, PAR4 internalization was similarly induced by agonist in cells lacking expression of both β-arrestin isoforms (Fig. 4*B*, *bottom panels*). To exclude the possibility that PAR4 internalization in β-arrestin-1 and 2 MEFs was due to some anomaly, we examined isoproterenol-induced internalization of β_2AR in parallel. β_2AR is a classic GPCR that requires β-arrestins for agonist-induced internalization (35). FLAG-tagged β_2AR was transiently expressed in wild type and β-arrestin-1,2 null MEFs, and internalization was examined by immunofluorescence confocal microscopy as described above. In wild type MEFs, isoproterenol induced substantial internalization of β_2AR (Fig. 4*C*, *top panels*), whereas activated β_2AR internalization was completely abolished in β-arrestin-1 and -2 double knock-out MEFs (Fig. 4*C*, *lower panels*). These findings are consistent with previously published studies demonstrating β-arrestin-dependent internal-

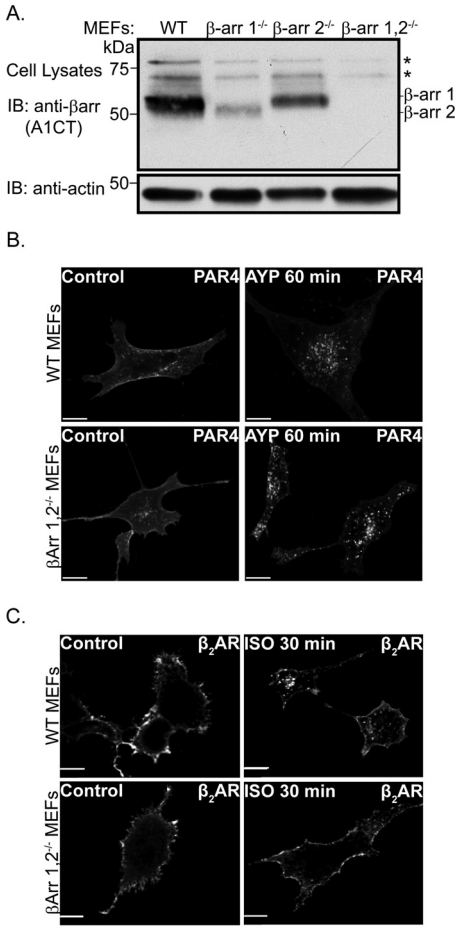

FIGURE 4. **Internalization of activated PAR4 occurs independent of β-arrestins.** *A*, cell lysates from WT and β-arrestin 1/2 double knock-out (*β-arr 1,2*$^{-/-}$) MEFs were immunoblotted (*IB*) as indicated. WT or β-arrestin 1/2 double knock-out MEFs were transiently transfected with FLAG-PAR4 (*B*) or FLAG-β_2AR (*C*), prelabeled with anti-FLAG antibody, and stimulated with 500 μM AYPGKF for 60 min at 37 °C (*B*) or 10 nM isoproterenol for 30 min at 37°*C* (*C*). After agonist stimulation, cells were processed and imaged by confocal microscopy. The images are representative of several cells from three independent experiments.

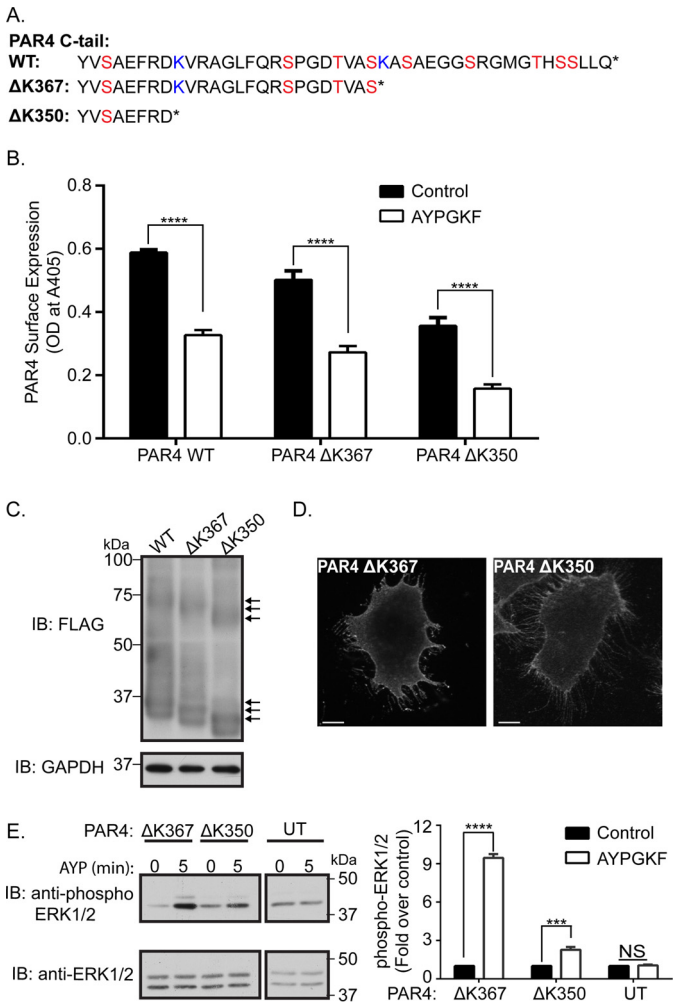

FIGURE 5. **The PAR4 C-tail is not required for internalization.** *A*, PAR4 amino acid sequence of full-length receptor and ΔK367 and ΔK350 C-tail truncation mutants. *B*, HeLa cells transfected with FLAG-PAR4 WT, ΔK367, or ΔK350 mutants were prelabeled with anti-FLAG antibody and stimulated with 500 μM AYPGKF for 60 min at 37 °C. Cell surface ELISA was then used to quantify the amount of PAR4 remaining at the cell surface following stimulation. Data (mean ± S.E.) are representative of three independent experiments, and statistical significance was determined by two-way ANOVA (****, $p < 0.0001$). *OD*, optical density. *C*, cell lysates from HeLa cells transfected as above in *B* were immunoblotted (*IB*) as indicated. *Arrows*, mobility shifts representing differentially processed PAR4 species. *D*, HeLa cells transfected as above in *B* were labeled with anti-FLAG antibody, processed, and imaged by confocal microscopy. The images are representative of several cells from three independent experiments. *E*, untransfected (*UT*) HeLa cells and HeLa cells transfected as above in *B* were stimulated with 500 μM AYPGKF (*AYP*) for 5 min at 37 °C. Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Changes in phospho-ERK1/2 signals were quantified, normalized to total ERK1/2, and expressed as a fraction of the untreated control. Samples were resolved on the same gel and separated for labeling. Data (mean ± S.E.) are representative of three independent experiments, and statistical significance was determined by two-way ANOVA (***, $p < 0.001$; ****, $p < 0.0001$; *NS*, not significant).

ization of β_2AR (35) and further reveal for the first time a β-arrestin-independent internalization pathway for activated PAR4.

The PAR4 C-tail Is Not Necessary for Agonist-induced Internalization—Internalization of GPCRs is mediated by clathrin adaptor proteins, which recognize short linear sorting motifs or post-translational modifications including phosphorylation of serine or threonine residues or ubiquitination of lysine residues, which often reside in the C-tail domain of the receptor (9). The PAR4 C-tail contains nine serine and threonine residues that may serve as potential phosphorylation sites and two lysines that could be targeted for ubiquitination (Fig. 5*A*). To delineate the mechanism responsible for internalization of PAR4, we first examined the importance of the C-tail domain by truncation mutagenesis. HeLa cells were transiently transfected with PAR4 wild type and two C-tail truncation mutants, ΔK367 and ΔK350, that were lacking the distal 19 amino acids or 36 amino acids, respectively (Fig. 5*A*). Cells were then incubated in the absence or presence of the PAR4-specific agonist AYPGKF for 60 min at 37 °C, and the amount of receptor remaining on the cell surface was quantified. In wild-type PAR4-expressing cells, we observed ~45% loss of surface receptor within 60 min of agonist stimulation (Fig. 5*B*). Remarkably, the extent of agonist-induced internalization of both PAR4 C-tail truncation mutants, ΔK367 and ΔK350, was comparable to wild type receptor, exhibiting ~45 and ~55% loss of surface receptor, respectively (Fig. 5*B*). To ensure that PAR4 ΔK367 and ΔK350 mutants are not globally defective, receptor expression and signaling were further examined.

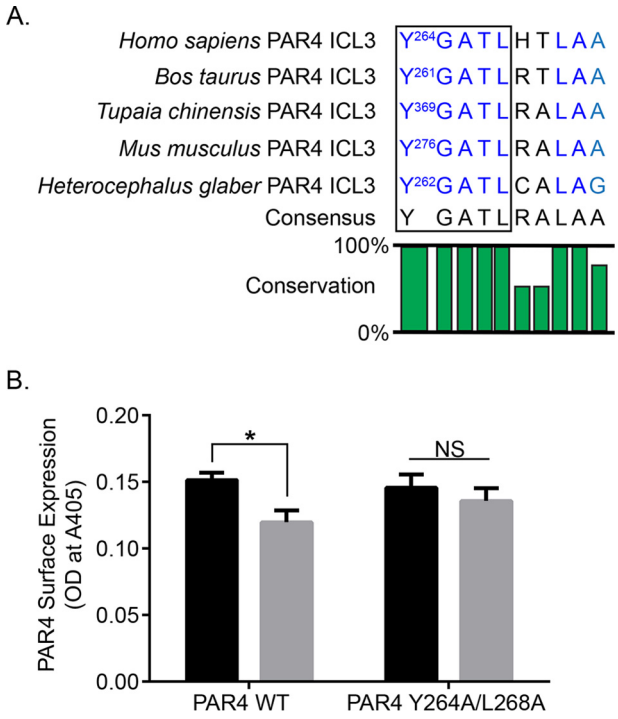

FIGURE 6. **PAR4 harbors a highly conserved tyrosine-based motif that is important for internalization.** *A*, alignment of human PAR4 intracellular loop 3 (*ICL3*) sequence with several mammalian orthologues. A highly conserved YX_3L motif is indicated by the *black box*. *B*, HeLa cells were transfected with FLAG-PAR4 WT or the tyrosine motif Y264A/L268A mutant, prelabeled with anti-FLAG antibody, and stimulated with 500 μM AYPGKF for 60 min at 37 °C. Cell surface ELISA was then used to quantify the amount of PAR4 remaining at the cell surface after stimulation. Data shown (mean ± S.E.) are representative of four independent experiments, and statistical significance was calculated by two-way ANOVA (*, $p < 0.05$; *NS*, not significant).

Analysis of cell lysates revealed a similar pattern of mature and immature bands, indicating that both PAR4 mutants are properly post-translationally processed (Fig. 5*C*). In addition, both PAR4 truncation mutants express at the cell surface and activate ERK1/2 in response to agonist stimulation (Fig. 5, *D* and *E*), although the PAR4 ΔK350 mutant exhibits lower surface expression and consequently less ERK1/2 signaling. These findings suggest that the PAR4 C-tail is not required for agonist-induced internalization and are consistent with a previous report showing that activated PAR4 fails to undergo phosphorylation (20). These findings indicate that phosphorylation is not a critical feature of PAR4 regulation. Together these results suggest that agonist-induced internalization of PAR4 via clathrin-coated pits occurs through a non-canonical pathway that is not dependent on β-arrestins or the C-tail domain.

PAR4 Internalization Requires AP-2—To identify other clathrin adaptor proteins besides β-arrestins that could mediate internalization of PAR4, we conducted a bioinformatics search for conserved sorting motifs in the cytoplasmic loops of PAR4 and found a highly conserved tyrosine-based motif within the third intracellular loop (ICL3) that appeared to be accessible for AP-2 binding (Fig. 6*A*). Tyrosine-based motifs are known binding sites for AP-2, a critical adaptor protein important for clathrin-mediated endocytosis (36). To investigate the function of this motif in PAR4 internalization, we generated a mutant PAR4 in which tyrosine (Tyr) 264 and leucine (Leu) 268 were converted to alanines (Ala) and designated Y264A/L268A. PAR4 Y264A/L268A mutant expressed at the cell surface like wild type (WT) receptor when transiently expressed in HeLa cells. We next compared agonist-induced internalization between PAR4 WT and the Y264A/L268A mutant. Internalization of PAR4 Y264A/L268A was markedly reduced and exhibited an only ~5% loss of surface receptor after 60 min of stimulation with AYPGKF, as compared with an ~20% loss of surface wild type receptor (Fig. 6*B*). These findings suggest that the putative AP-2 binding motif within the ICL3 domain of PAR4 is important for agonist-induced internalization.

To determine if AP-2 might regulate PAR4 internalization, we first determined whether PAR4 colocalizes with endogenous AP-2 in intact cells using immunofluorescence confocal microscopy. HeLa cells expressing FLAG-PAR4 were incubated with anti-FLAG antibody at 4 °C to ensure only the cell surface receptors bound antibody. Cells were then stimulated with AYPGKF for 12.5 min, fixed, and immunostained for PAR4 and the α-adaptin subunit of AP-2. PAR4-positive puncta co-stained for α-adaptin subunit as indicated by the overlapping puncta in the merge image (Fig. 7*A*). Line-scan analysis also revealed the extent of PAR4 and AP-2 colocalization at puncta (Fig. 7*A*). In contrast to wild type PAR4, agonist-stimulation did not result in substantial colocalization between PAR4 Y264A/L268A with AP-2 (Fig. 7*B*). To assess AP-2 function in PAR4 internalization, we used siRNA targeting the μ2-adaptin subunit to deplete HeLa cells of endogenous AP-2 complex (13, 37). The expression of μ2-adaptin was virtually abolished in cells transiently transfected with μ2 siRNA compared with cells transfected with nonspecific control siRNA (Fig. 7*C*). The cell surface cohort of FLAG-PAR4 was labeled with antibody in siRNA transfected HeLa cells and then incubated in the presence or absence of AYPGKF for 60 min at 37 °C. In control nonspecific siRNA-transfected cells, exposure to agonist caused a significant ~30% loss of PAR4 from the cell surface (Fig. 7*D*). In contrast, agonist failed to induce PAR4 internalization in cells depleted of the μ2-adaptin subunit (Fig. 7*D*). These data suggest that AP-2 serves as a critical clathrin adaptor protein that facilitates agonist-induced internalization of PAR4.

Endogenous PAR4 Internalization Is Regulated by AP-2—To determine if the regulation of endogenous PAR4 is also controlled by the clathrin adaptor AP-2, we utilized the human megakaryocytic Dami cell line, which endogenously expresses PAR4. We first tested whether AP-2 was required for mediating endogenous PAR4 internalization in Dami cells using μ2-adaptin-specific siRNA to deplete cells of AP-2 expression. In Dami cells transfected with siRNA targeting the μ2-adaptin subunit of AP-2, we observed a significant loss of μ2 adaptin expression compared with nonspecific siRNA transfected cells (Fig. 8*A*). Dami cells were grown on polylysine-coated glass coverslips, then surface PAR4 was prelabeled with an antibody specific for the N terminus of human PAR4, and agonist-induced internalization was examined by immunofluorescence confocal microscopy (38). In the absence of agonist, PAR4 localized predominantly to the cell surface (Fig. 8*B*, *upper panels*). Agonist-induced internalization of PAR4 and the resultant formation of puncta was significantly inhibited in μ2-adaptin-depleted cells

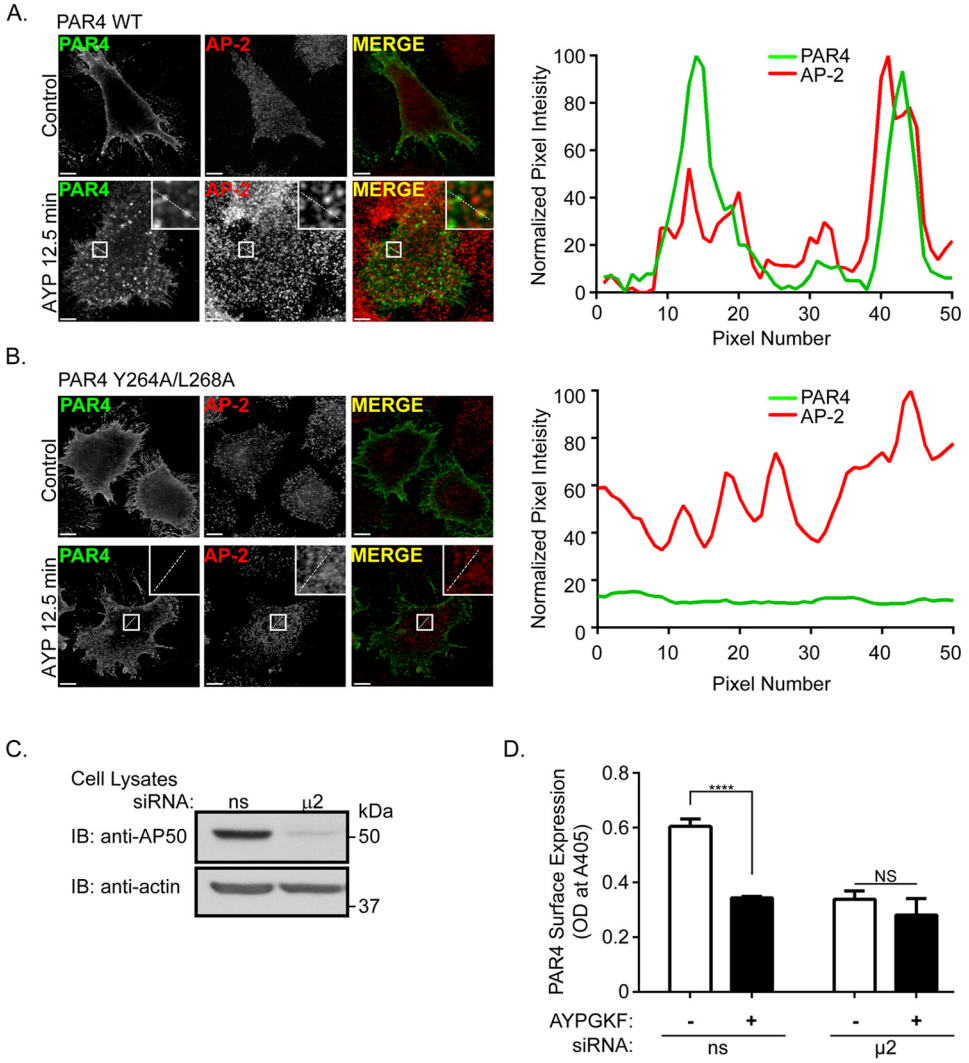

FIGURE 7. **AP-2 mediates internalization of activated PAR4.** *A* and *B*, HeLa cells transfected with FLAG-PAR4 WT (*A*) or the Y264A/L268A mutant (*B*) were prelabeled with anti-FLAG antibody, stimulated with 500 μM AYPGKF for 12.5 min at 37 °C, processed, co-stained with anti-AP-2 antibody, and imaged by confocal microscopy. The images are representative of three independent experiments (*scale bar*, 10 μm). *Insets*, magnifications of *boxed areas*. The fluorescence intensity line scans were generated from the regions denoted by the *white dashed line* in the agonist-stimulated images (*A* and *B*, *lower panels*). *C*, HeLa cells expressing FLAG-PAR4 were transfected with nonspecific (*ns*) siRNA or siRNA specific for the μ2 adaptin subunit of AP-2 and immunoblotted (*IB*) as indicated. *D*, HeLa cells transfected as described above in *C* were prelabeled with anti-FLAG antibody and stimulated with 500 μM AYPGKF for 60 min at 37 °C. ELISA was then used to quantify the amount of receptor at the cell surface. Data shown (mean ± S.E.) are representative of three independent experiments, and statistical significance was calculated by two-way ANOVA (****, $p < 0.0001$; *ns*, not significant).

compared with nonspecific siRNA-transfected control cells (Fig. 8*B*, *lower panels*). Quantification by automated image analysis revealed a significant blockade of agonist-induced internalization of PAR4 in cells depleted of μ2-adaptin (Fig. 8*C*). These data confirm that AP-2 plays an essential role in mediating internalization of endogenous PAR4.

PAR4 Signaling Is Differentially Regulated by AP-2 and Mutation of the Tyrosine Motif—To assess the role of dysregulated PAR4 trafficking caused by loss of AP-2 expression on receptor signaling, we examined ERK1/2 phosphorylation in Dami cells depleted of μ2-adaptin expression by siRNA knockdown. Activation of PAR4 elicited significantly enhanced and prolonged ERK1/2 signaling in cells depleted of μ2-adaptin relative to nonspecific siRNA-transfected control cells (Fig. 9*A*). In addition, agonist stimulation of the PAR4 Y264A/L268A mutant expressed in HeLa cells resulted in enhanced and prolonged ERK1/2 activation (Fig. 9*B*). PAR1-stimulated ERK1/2 activation is known to occur though $G\alpha_q$ protein signaling (39), suggesting that internalization is critical for terminating agonist-stimulated PAR4 signaling. Unlike ERK1/2, PAR4-induced Akt signaling has been shown to occur via a β-arrestin-dependent pathway independent of G proteins and is hypothesized to occur on endosomes (40–42). Thus, we determined if internalization of activated PAR4 was required for Akt signaling by comparing control and AP-2 depleted Dami cells. In contrast to ERK1/2, Akt signaling induced by activated PAR4 was markedly attenuated in μ2-adaptin knockdown cells compared with control cells transfected with nonspecific siRNA (Fig. 9*C*). These findings suggest that internalization of activated PAR4 is required for Akt signaling. Together, our study suggests that activated PAR4 is internalized through a clathrin- and dynamin-dependent pathway independent of β-arrestins. Rather than β-arrestins, PAR4 internalization is mediated by AP-2 and a tyrosine-based motif localized within ICL3 and

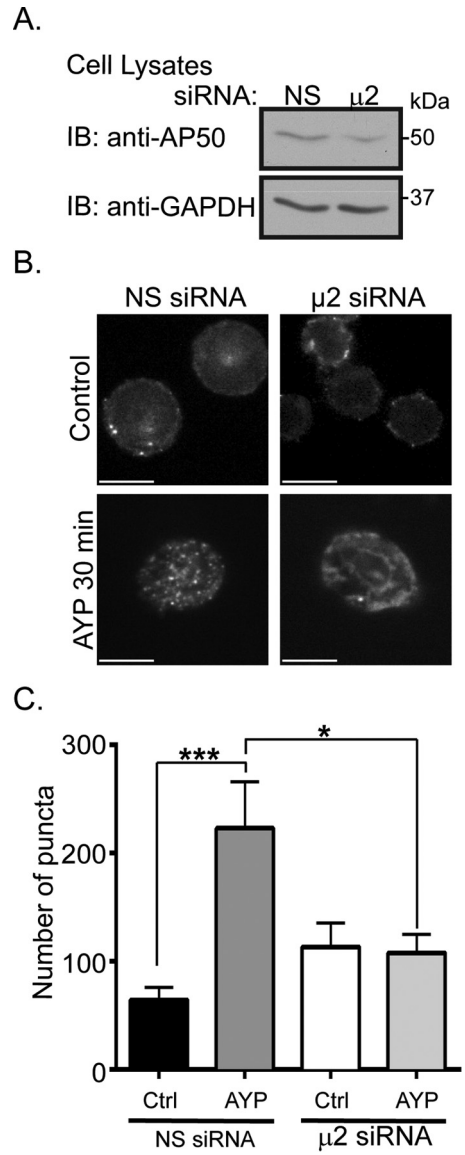

FIGURE 8. **AP-2 is required for internalization of endogenous PAR4 in Dami cells.** *A*, Dami cells were transfected with nonspecific (*ns*) siRNA or siRNA targeting the μ2 adaptin subunit of AP-2. Cell lysates were collected and immunoblotted (*IB*) as indicated. *B*, Dami cells transfected as above in *A* were prelabeled with anti-PAR4 antibody, treated with 500 μM AYPGKF (*AYP*) or left untreated (*Control*) for 30 min at 37 °C, processed, and imaged by confocal microscopy. The images are representative of three independent experiments (*scale bar*, 10 μm). *C*, PAR4 internalization was quantified using Slidebook 5.0 software's automated object counting. At least 10 cells were analyzed for each condition for each of three independent experiments. Data shown are combined from three independent experiments and were analyzed using a Student's *t* test (*, $p < 0.05$; ***, $p < 0.001$).

functions distinctly to regulate the magnitude and duration of ERK1/2 and Akt signaling (Fig. 10).

Discussion

In the present study we sought to characterize the intracellular trafficking route of activated PAR4 and to determine its function on receptor signaling. We found that PAR4 internalizes via a clathrin- and dynamin-dependent pathway and is then sorted from early endosomes to late endosomes/lysosomes. We also discovered that activated PAR4 internalization requires a highly conserved YX_3L motif localized within ICL3 and is mediated by the clathrin adaptor AP-2 rather than β-arrestins and the C-tail domain of the receptor. Intriguingly, activation of PAR4 in cells in which receptor internalization is blocked resulted in enhanced ERK1/2 signaling and attenuated Akt signaling. These studies are the first to identify the molecular determinants that mediate PAR4 internalization and demonstrate a link between PAR4 trafficking and signaling.

The majority of activated and phosphorylated GPCRs are recognized by β-arrestins, which facilitates interaction with clathrin, adaptor proteins, and internalization from the plasma membrane (7, 8, 43). However, not all GPCRs require β-arrestins for internalization including PAR1 (12). In this study we show that, like PAR1, β-arrestins are not required for agonist-induced PAR4 internalization in MEFs derived from β-arrestin1,2 knock-out mice. However, unlike PAR1, we found that the C-tail domain of PAR4 is not essential for agonist-promoted internalization. Consistent with this observation, a previous study showed that agonist stimulation of PAR4 fails to promote its phosphorylation, which is generally a requirement for interaction with β-arrestins (20). These findings suggest that neither phosphorylation nor β-arrestins are required for activated PAR4 internalization through clathrin-coated pits.

In addition to β-arrestins, other clathrin adaptor proteins can facilitate GPCR internalization (6, 9). Clathrin adaptor proteins recognize post-translational modifications or short linear peptide motifs that are typically localized within the cytoplasmic regions of the receptor. This is best characterized for PAR1 (44). The clathrin adaptor AP-2 mediates constitutive internalization of PAR1 wherein the μ2-adaptin subunit of AP-2 binds directly to a highly conserved C-tail tyrosine-based $YXXL$ motif (44). In addition, activated PAR1 is phosphorylated and ubiquitinated within the C-tail domain, which is required for AP-2- and epsin-1-mediated internalization of the receptor (45). However, in contrast to PAR1, activated PAR4 is not phosphorylated (20) and the C-tail domain is not required for internalization, suggesting that an alternative pathway may control internalization of activated PAR4.

Given that the C-tail of PAR4 is dispensable for internalization, we examined the possibility that signals for internalization may occur within the intracellular loops of the receptor. A sequence alignment of PAR4's intracellular loops revealed a highly conserved non-canonical tyrosine-based YX_3L motif within ICL3 of the receptor that was found to be required for agonist-promoted internalization. Similarly, a non-canonical tyrosine-based $YX_3\Phi$ motif (where X is any residue, and Φ is a hydrophobic residue) was identified in the thromboxane A2 TP-β isoform receptor C-tail region and shown to mediate receptor internalization (46). Although the C-tail region of most GPCRs serves as an important site for clathrin adaptor protein recognition, the seven transmembrane Wntless receptor contains a conserved $YXX\Phi$ motif within ICL3 that is required for internalization (47). However, it is not clear whether AP-2 is required for internalization of either the thromboxane TP-β or Wntless receptor. In contrast, we show that PAR4 and AP-2 co-localize and that AP-2 is required for agonist-induced internalization of both endogenous and ectopically expressed PAR4. Thus, unlike most classic GPCRs, PAR4 harbors a tyrosine-based $YX_3\Phi$ motif within ICL3 that is

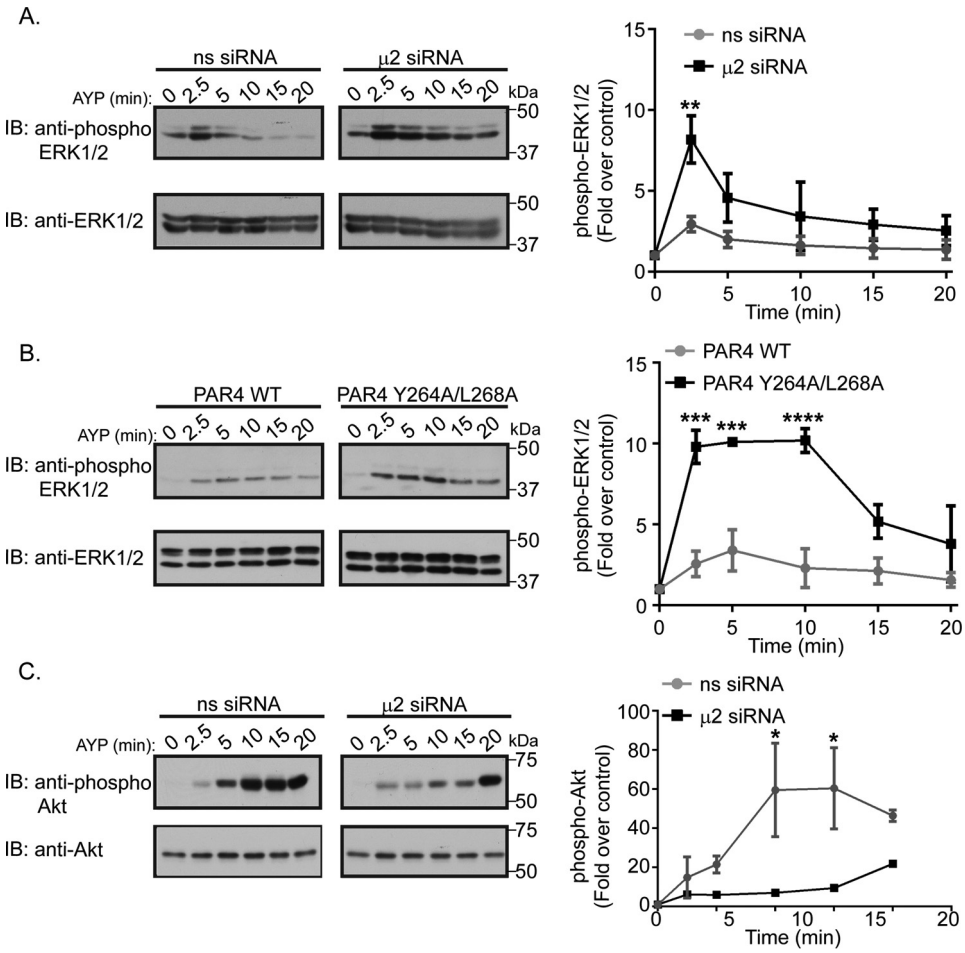

FIGURE 9. **PAR4 trafficking is linked to proper ERK1/2 and Akt signaling.** *A* and *C*, Dami cells transfected with nonspecific (*ns*) siRNA or siRNA targeting the μ2 adaptin subunit of AP-2 were stimulated with 500 μM AYPGKF (*AYP*) for various times at 37 °C. Cell lysates were resolved by SDS-PAGE and immunoblotted (*IB*) as indicated. Changes in phospho-ERK1/2 (*A*) and phospho-Akt (*C*) signals were quantified, normalized to total ERK1/2 and total Akt, respectively, and expressed as a fraction of the untreated controls. *B*, HeLa cells transfected with FLAG-PAR4 WT or Y264A/L268A mutant were stimulated with 500 μM AYPGKF for various times at 37 °C. Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Changes in phospho-ERK1/2 signals were quantified, normalized to total ERK1/2, and expressed as a fraction of the untreated controls. Samples were resolved on the same gel and separated for labeling. Data shown (mean ± S.E.) are representative of three independent experiments, and statistical significance was calculated by two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

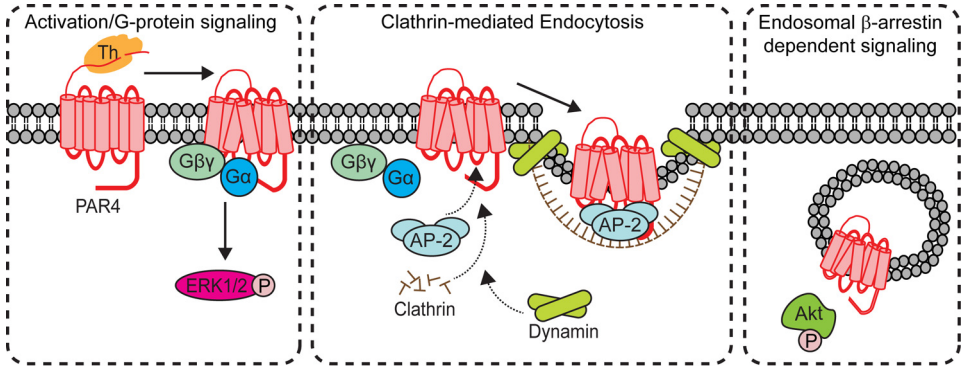

FIGURE 10. **Model of PAR4 trafficking and signaling.** PAR4 is a seven transmembrane GPCR that is cleaved and activated by thrombin. Thrombin cleavage generates an N terminus that binds intramolecularly to the receptor, facilitating coupling to heterotrimeric G proteins, which promotes ERK1/2 signaling. After activation, PAR4 is recruited to clathrin-coated pits and requires both an intact tyrosine-based motif and AP-2 for internalization. Once internalized, PAR4 is sorted to early endosomes and appears to stimulate Akt signaling.

required together with AP-2 for internalization through clathrin-coated pits. Interestingly, a second less conserved tyrosine-based motif also exists within ICL3 of PAR4 but may be inaccessible to AP-2 based on hydrophobicity analysis. However, it is unclear whether the second tyrosine-based motif has a function, and the mechanism by which AP-2 is recruited to activated PAR4 is not known.

In addition to characterizing PAR4 trafficking, we also examined the influence of dysregulated trafficking on receptor signaling in Dami cells endogenously expressing PAR4 (18) and in

HeLa cells ectopically expressing the PAR4 tyrosine-motif mutant. PAR4 couples to $G\alpha_q$ and $G\alpha_i$ subtypes and signals to ERK1/2 and Akt activation in multiple cell types (48, 49). We show that the magnitude and duration of agonist-stimulated ERK1/2 signaling was enhanced in Dami cells depleted of AP-2 and in HeLa cells expressing the PAR4 Y264A/L268A mutant relative to control cells. These findings suggest that PAR4 internalization may be important for attenuation of ERK1/2 signaling. There is also evidence that PAR4 is able to act in synergy with the purinergic receptor P2Y12 to elicit β-arrestin-dependent Akt signaling (40). In striking contrast to ERK1/2, we found that Akt signaling was markedly attenuated in Dami cells depleted of AP-2 compared with control cells. Thus, blocking PAR4 internalization may disrupt Akt signaling by preventing the formation of a P2Y12-dependent endosomal β-arrestin signaling complex in Dami cells. This would be consistent with recent evidence suggesting that β-arrestin-dependent signaling occurs on endosomes rather than at the plasma membrane (11). However, it remains to be determined if a presumptive PAR4-P2Y12 dimer signals to Akt activation via a β-arrestin-mediated pathway from endosomes. Intriguingly, PAR4-stimulated Akt signaling was not reliably detected in HeLa cells. This may result from low or absent P2Y12 expression, as HeLa cells are not normally responsive to ADP stimulation (50).

In summary, we have shown that PAR4 internalization is clathrin- and dynamin-dependent and that the internalized receptor is trafficked through the endosomal-lysosomal sorting pathway, similar to a prototypical GPCR. However, we also found that PAR4 trafficking is regulated by unique determinants. We discovered that PAR4 internalization requires an intact ICL3 localized tyrosine-based motif and AP-2 rather than β-arrestins and the C-tail domain. Moreover, disruption of PAR4 trafficking increased the magnitude and duration of ERK1/2 signaling while attenuating Akt signaling. These findings indicate that PAR4 trafficking is important for proper signaling and raise the intriguing possibility that like other GPCRs, PAR4 may elicit important signaling responses from endocytic vesicles.

Experimental Procedures

Reagents and Antibodies—The PAR4 activating peptide AYPGKF was synthesized as the carboxyl amide and purified by reverse-phase high pressure liquid chromatography by the Tufts University Core Facility (Boston, MA). Isoproterenol was purchased from Sigma. Anti-FLAG polyclonal rabbit antibody (#600-401-383) was purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse EEA1 (#610457) and anti-μ2 adaptin (anti-AP50) (#611350) antibodies were purchased from BD Biosciences. LAMP1 antibody was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA). Mouse monoclonal anti-α-adaptin (AP.6) antibody was generously provided by Dr. Linton Traub (University of Pittsburgh, Pittsburgh, PA). The mouse monoclonal anti-clathrin (X22) heavy chain antibody (GTX22731) and the anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) (GTX627408) were purchased from GeneTex (Irvine, CA). The mouse anti-β-actin antibody (AC-74) (A-5316) was purchased from Sigma. The mouse anti-PAR4 antibody was provided by Dr. Marvin Nieman (Case Western Reserve University, Cleveland, OH). The rabbit β-arrestin antibody (A1CT) was a generous gift from Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC). The rabbit polyclonal anti-Akt (#9272), rabbit monoclonal anti-phospho-Akt (Ser-473) (D9E) XP, rabbit polyclonal anti-p44/42 MAPK (#9102), and the mouse anti-phospho-p44/42 MAPK (#9106) were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488 (#A-11001) and Alexa Fluor 594 (A-11005) and goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 (A-11008) and Alexa Fluor 594 (A-11012) were purchased from ThermoFisher Scientific (Waltham, MA). FluorSave reagent was purchased from Calbiochem. Goat anti-mouse (#170-6516) and goat anti-rabbit (#170–6515) secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Bio-Rad. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Thermo Fisher Scientific.

Cell Culture and Transfections—HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (v/v). The Dami human megakaryocytic cell line was purchased from ATCC and maintained in RPMI media supplemented with 10% (v/v) fetal bovine serum. Mouse embryonic fibroblasts derived from WT or β-arrestin knock-out mice were described previously (12). HeLa cells were transiently transfected with cDNA plasmids using polyethyleneimine (Polysciences Inc., Warrington, PA) at a ratio of 6 μl PEI (1 mg/ml solution) to 1 μg of plasmid. Oligofectamine (Invitrogen) was used for siRNA transfections of HeLa cells, which were carried out according to the manufacturer's instructions. Dami cells were transfected with siRNA as previously described (51, 52). Briefly, cells were plated at a density of 5.0×10^5 cells/ml in reduced-serum media and transfected using Oligofectamine with siRNA oligonucleotides at a final concentration of 100 nM. A second transfection was carried out 24 h after the first, and cells were assayed after 72 h of transfection. Nonspecific siRNA 5′-CTACGTCCAGGAGCGCACC-3′, μ2 adaptin siRNA 5′-GTGGATGCCTTTCGGGTCA-3′, and clathrin heavy chain siRNA 5′-GCAATGAGCTGTTTGAAGA-3′ were previously described (45, 53) and obtained from Dharmacon (Lafayette, CO).

Plasmid cDNAs—Human PAR4 wild type containing an N-terminal FLAG epitope was cloned into the pBJ mammalian vector as previously described (18). Human FLAG-β_2AR cloned into pcDNA3.1 was generously provided by Dr. Mark von Zastrow (University of California, San Francisco, CA). Plasmids encoding wild type or K44A dynamin-GFP were described previously (27). All mutagenesis was performed using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA) per the manufacturer's protocol and confirmed by dideoxy sequencing. The PAR4 ΔK367 and ΔK350 C-tail truncation mutants were generated by site-directed mutagenesis of nucleotides a1101t and a1050t to convert lysine codons at position 367 and 350, respectively, to stop codons. The PAR4 tyrosine-based sorting motif mutant was generated using site-directed mutagenesis by mutating t792g and a793c to convert

tyrosine at position 264 to alanine followed by mutating c821g and t822c to convert leucine at position 268 to alanine and was designated PAR4 Y264A/L268A.

Immunoblotting—Cell lysates were collected in 2× Laemmli sample buffer containing 200 mM DTT. Samples were resolved by SDS-PAGE, transferred to PVDF membranes, immunoblotted with appropriate antibodies, and then developed by chemiluminescence. Immunoblots were quantified by densitometry using ImageJ software (NIH, Bethesda, MD).

Immunofluorescence Confocal Microscopy—Cells were plated at a density of 0.4×10^5 cells per well on fibronectin-coated glass coverslips placed in a 12-well dish and grown overnight. Cells were transfected, grown for 48 h, and then serum-starved in DMEM containing 1 mg/ml BSA and 10 mM HEPES for 1 h at 37 °C. Cells were incubated at 4 °C with anti-FLAG antibody to label the cohort of receptors at the cell surface, stimulated with agonist, then fixed in 4% paraformaldehyde, permeabilized with methanol, immunostained with the appropriate antibodies, and processed as described previously (16). Coverslips were mounted with FluorSave reagent. Confocal images of 0.28 μm *x-y* sections were collected sequentially using an Olympus IX81 DSU spinning confocal microscope fitted with a Plan Apo 60× oil objective and a Hamamatsu ORCA-ER digital camera using SlideBook 5.0 software (Intelligent Imaging Innovations). Pearson's correlation coefficients and line-scan analysis to assess colocalization were performed using SlideBook 5.0 software.

Internalization Assays—Cells were plated at a density of 0.5×10^5 cells per well on fibronectin-coated 24-well dishes and grown overnight for cell surface ELISA. Cells were transfected, grown for 48 h, and then serum-starved for 1 h in DMEM containing 1 mg/ml BSA and 10 mM HEPES at 37 °C. Cells were incubated at 4 °C with anti-FLAG antibody to label the cell surface cohort, stimulated with agonists, and then fixed in 4% paraformaldehyde. The amount of receptor remaining on the cell surface was then detected by incubation with HRP-conjugated secondary antibody, washed, and developed with ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) for at least 20 min at room temperature. The optical density of an aliquot was determined by absorbance at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

Signaling Assays—Dami cells were pelleted by centrifugation at $200 \times g$ for 5 min then washed twice with PBS. Cells were resuspended in RPMI containing 1 mg/ml BSA then counted and diluted to 5.0×10^5 cells/ml. HeLa cells were plated at a density of 0.4×10^5 cells per well in 24-well plates and grown overnight. Cells were transfected with PEI, grown for 48 h, and then serum-starved in DMEM containing 1 mg/ml BSA and 10 mM HEPES for 1 h at 37 °C. Both Dami and HeLa cells were then stimulated with agonist at 37 °C as indicated, and samples were collected at specific time points by direct lysis in 2× Laemmli sample buffer containing 200 mM DTT and analyzed by immunoblotting.

Data Analysis—Statistical significance was determined by one-way ANOVA, two-way ANOVA, or Student's *t* test using Prism 4.0 software (GraphPad).

Author Contributions—T. H. S. designed and conducted most of the experiments. Assistance was provided by L. J. C. (Fig. 1*C*, Fig. 5*B*, Fig. 5*C*, and Fig. 7*A*), J. G. L. (Fig. 5*D*, Fig. 6*B*, and Fig. 7*B*), and M. R. D/ (Fig. 8*B* and Fig. 8*C*). T. H. S. analyzed the data and wrote most of the paper with assistance from J. T. J. T. and M. T. N. reviewed the paper, and M. T. N. provided the PAR4 antibody as well as subject area expertise.

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