

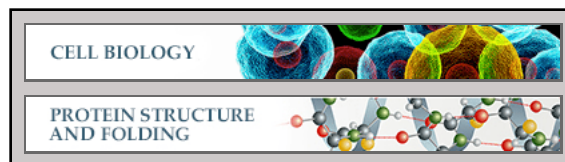
Cell Biology:

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Novel Role for Proteinase-activated Receptor 2 (PAR₂) in Membrane Trafficking of Proteinase-activated Receptor 4 (PAR₄)*[§]

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Background: Bioinformatic analysis revealed that PAR₄ possesses an ER retention motif.

Results: PAR₂ both abrogates and facilitates chaperone protein interaction with PAR₄ to allow PAR₄ to evade ER retention and be delivered to the plasma membrane.

Conclusion: PAR₂ regulates PAR₄ localization and cell signaling through heterodimerization.

Significance: Impact upon understanding PAR₂ and PAR₄ in inflammation where clear roles are defined.

Proteinase-activated receptors 4 (PAR₄) is a class A G protein-coupled receptor (GPCR) recognized through the ability of serine proteases such as thrombin and trypsin to mediate receptor activation. Due to the irreversible nature of activation, a fresh supply of receptor is required to be mobilized to the cell surface for responsiveness to agonist to be sustained. Unlike other PAR subtypes, the mechanisms regulating receptor trafficking of PAR₄ remain unknown. Here, we report novel features of the intracellular trafficking of PAR₄ to the plasma membrane. PAR₄ was poorly expressed at the plasma membrane and largely retained in the endoplasmic reticulum (ER) in a complex with the COPI protein subunit β -COPI. Analysis of the PAR₄ protein sequence identified an arginine-based (RXR) ER retention sequence located within intracellular loop-2 (R¹⁸³AR → A¹⁸³AA), mutation of which allowed efficient membrane delivery of PAR₄. Interestingly, co-expression with PAR₂ facilitated plasma membrane delivery of PAR₄, an effect produced through disruption of β -COPI binding and facilitation of interaction with the chaperone protein 14-3-3 ζ . Intermolecular FRET studies confirmed heterodimerization between PAR₂ and PAR₄. PAR₂ also enhanced glycosylation of PAR₄ and activation of PAR₄ signaling. Our results identify a novel regulatory role for PAR₂ in the anterograde traffic of PAR₄. PAR₂ was shown to both facilitate and abrogate protein interactions with PAR₄, impacting upon receptor localization and cell signal transduc-

tion. This work is likely to impact markedly upon the understanding of the receptor pharmacology of PAR₄ in normal physiology and disease.

Proteinase-activated receptors (PARs)² are a class A GPCR family comprised of four family members, PAR₁ through to PAR₄, which play key roles in aspects of both physiology and pathophysiology including platelet aggregation, wound healing, and various aspects of inflammation (1–3). Detailed characterization of the protein structure of the PAR family has identified proteolytic cleavage sites at the receptor N-terminal (4–7). For each receptor a unique tethered ligand is exposed within the N-terminal that interacts with the second extracellular loop to mediate receptor activation. The structural determinants that regulate PAR activation have long been of interest and there has been considerable focus placed upon the mechanisms underpinning membrane trafficking and signal termination particularly for PAR₁ (8–14) and PAR₂ (15–19). The most recent family member to be cloned, PAR₄ (20, 21), is distinct from both PAR₁ and PAR₂. Although a thrombin-activated receptor, it lacks the hirudin-like domain required for thrombin selectivity and is activated by several other ligands including trypsin. In addition it has a shorter C terminus than PAR₁ and PAR₂ and lacks essential phosphorylation sites within intracellular domains, which are present in other family members and have been shown to be necessary for receptor desensitization (22). PAR₄ has been poorly studied relative to either PAR₁ or PAR₂ but has been shown to signal via Ca²⁺ mobilization and to

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² The abbreviations used are: PAR, proteinase-activated receptor; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; mEGFP/mEYFP/mECFP, monomeric enhanced green/yellow/cyan fluorescent protein; RXR, arginine-based ER retention motifs; COPI, coat protein I complex; RFRET, ratiometric FRET.

regulate the MAP kinases (23). However, very little is known of the mechanisms regulating receptor trafficking.

Due to the irreversible nature of activation of PARs, for responsiveness to agonist to be retained, fresh supplies of receptor are required to be mobilized to the cell surface. Delivery to the membrane requires efficient transport between the ER/Golgi/plasma membrane, which can be facilitated through discrete motifs that reside within the synthesized protein (24, 25). Many chaperone proteins, such as coat protein complexes (COPI and COPII), can assist transport of recently synthesized proteins through motif-based sorting (26–28). Properly assembled proteins are packaged for export into COPII vesicles where they progress to the ER-Golgi intermediate complex, a process known as anterograde transport. Misfolded proteins or those exposing sequences encoding ER retention motifs (for example, RXR, KDEL, or KKAA motifs) are shuttled back to the ER via COPI vesicles, in a process known as retrograde transport (29). During the assembly of multimeric proteins, such as GPCR homo/heterodimers, proteins possessing ER retention signals have been shown to evade ER retention through the steric masking of motifs during protein folding (30–33). 14-3-3 proteins have previously been shown to assist motif masking to ensure export of proteins to the Golgi (25, 34). Once proper protein folding has been achieved, post-translational modifications, such as complex glycosylation, will occur (35).

Here we identify for the first time the presence of an arginine-based ER retention motif within intracellular loop-2 of PAR₄, which results in ER retention through COPI-dependent retrograde transport. In the presence of PAR₂, through PAR₂/PAR₄ heterodimer formation and interaction with 14-3-3 ζ , PAR₄ was able to evade ER retention and undergo N-linked complex glycosylation. This resulted in efficient delivery to the plasma membrane. The impact of enhanced cell surface expression was reflected in enhanced PAR₄-mediated cell signal transduction. PAR₂ is often co-expressed with PAR₄, and they are dual up-regulated by various pro-inflammatory mediators and have been shown to be co-activated by common agonists (20, 36, 37). In the presence of PAR₂, a significant increase in PAR₄-mediated total inositol phosphate accumulation was observed. This work demonstrates for the first time a novel regulatory role for PAR₂ in the anterograde traffic and signaling of PAR₄. This is mediated by selective interaction with COPI or 14-3-3 proteins, offering a new paradigm for class A GPCR trafficking and control.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The PAR₄ activating peptide, Ala-Tyr-Pro-Gly-Lys-Phe-amidated (NH₂) peptide (AYPGKF-NH₂), was synthesized by the University of Calgary Peptide Service (Calgary, Canada). ER-Tracker™ Blue-White DPX Dyes (Molecular Probes) for ER labeling and the anti-transferrin receptor mouse monoclonal antibody were purchased from Invitrogen Ltd. Rabbit polyclonal anti-Na⁺,K⁺-ATPase α 1 antibody was purchased from Cell Signaling Technology Inc. Living Colors® full-length A.v. GFP rabbit polyclonal antibody was purchased from Clontech-TaKaRa Bio Europe (France). PKH26 Red Fluorescent Cell Linker kit for general cell membrane labeling, anti-PAR₄ goat polyclonal, anti-14-3-3 ζ rabbit

polyclonal antibodies, monoclonal anti-HA-agarose conjugate, HA peptide, and tunicamycin were from Sigma. The anti-PAR₄ rabbit polyclonal antibody was obtained from Abcam (Cambridge, UK). Anti- β coatomer protein (β -COP1) rabbit polyclonal antibody was purchased from Pierce and Thermo Fisher Scientific (Loughborough, UK). Mouse monoclonal anti-HA antibody was purchased from Cambridge Bioscience (Cambs, UK). Pierce Cell Surface Protein Isolation Kit was purchased from Thermo Scientific. The alkaline phosphatase substrate kit was obtained from Bio-Rad.

Epitope-tagged PAR Constructs—Human PAR₂ was amplified by polymerase chain reaction (PCR) from a pRSV-PAR₂ vector.

The PCR product was then digested with HindIII-BamHI and cloned into the respective sites of a pEYFP-N1 vector (Clontech). Human PAR₄ was amplified from a pcDNA3.1(+)-hPAR₄ vector by PCR and digested with Kpn-AgeI, whereas ECFP was amplified from the pECFP-N1 vector (Clontech) and digested with AgeI-XbaI. PAR₄ and ECFP were ligated and cloned into the KpnI-XbaI sites of the pcDNA3.1(+) vector. Monomeric ECFP and EYFP constructs were generated by amino acid substitution of Ala²⁰⁶ to Lys²⁰⁶ (38), through site-directed mutagenesis using the Gene Tailor™ Site-directed Mutagenesis System (Invitrogen). Amino acid substitutions were similarly made within the primary sequence of PAR₄ to mutate potential arginine-based ER retention motifs (positions R¹⁸³AR \rightarrow A¹⁸³AA, (referred to as RAR mut) R¹⁸⁸GRR \rightarrow A¹⁸⁸GAA and R¹⁸³AR R¹⁸⁸GRR \rightarrow A¹⁸³AA A¹⁸⁸GAA) and the N-linked glycosylation site on the N-terminal of PAR₄ (Asn⁵⁶ \rightarrow Ala⁵⁶). A HA epitope tag (YPYDVPDYA) was incorporated into the C-terminal of PAR₄ by PCR to generate PAR₄-HA. All constructs were confirmed by sequencing.

Cell Culture—HEK293 cells were maintained in minimal essential medium with Earle's salts, L-glutamine supplemented with 10% fetal calf serum (FCS), penicillin (100 units ml⁻¹), streptomycin (100 μ g ml⁻¹), and nonessential amino acids and passaged using 1 \times SSC (sodium citrate, pH 7.4). NCTC-2544 cells and NCTC-PAR₂ cells were grown in Medium 199 with Earle's salts (Sigma) containing 10% FCS, sodium bicarbonate (50 mM), L-glutamine (2 mM), penicillin (100 units ml⁻¹), and streptomycin (100 μ g ml⁻¹). NCTC-2544 cells were passaged using Versene (0.53 mM EDTA in PBS) to avoid trypsin exposure. All cells were then incubated at 37 °C in a humidified atmosphere with 5% CO₂ with medium replaced every 2 days.

Transient Transfection—Cells were grown in 12- and 6-well plates or T75 flasks prior to transient transfection at 70–80% confluence with 1, 2, or 10 μ g of endo-free plasmid DNA, respectively, using Lipofectamine 2000 (Invitrogen) following the recommended manufacturer's guidelines. Maximal gene expression was observed 48 h post-transfection.

Inositol Phosphate Accumulation Assay—Following transient transfection for 24 h, cells were serum starved for a further 24 h in serum-free medium supplemented with 0.5 μ Ci/well (1 Ci = 37 GBq) of myo-[2-³H]inositol (PerkinElmer Life Sciences) (0.5 μ Ci/well; 1 Ci = 37 GBq). Cells were pretreated with 20 mM lithium chloride for 30 min prior to agonist treatment (100 μ M AYPGKF-NH₂ for 45 min). Measurement of the accumulation of inositol phosphates was carried out as previously described by Plevin *et al.* (39).

Fluorescence Microscopy—Cells were washed in PBS prior to methanol fixation for 15 min at room temperature. After further washes with PBS, cells were stained using 4',6-diamidino-2-phenylindole (DAPI) nuclear dye or ER Tracker™ dye then mounted onto glass microscope slides with 15 μ l of mowiol (Calbiochem). Cells were visualized using a Nikon TE300-E microscope (Nikon Instruments, New York) using a $\times 100$ (numerical aperture; NA 1.3) oil immersion Fluor lens. Emitted fluorescence was detected using a photometric Cool Snap-HQ monochrome camera (Roper Scientific, Trenton, NJ) set up in 12-bit mode (0–4095 gray tones). Metamorph software (version 7.0, Molecular Devices Corp., Downing, PA) was used to control image acquisition and modify image settings. Images were background corrected, based on statistical correction of average background regions from defined regions of interest.

Cell Surface ELISA—Changes in cell surface expression of PAR₄ were measured by Enzyme-linked ImmunoSorbent Assay (ELISA). Cells were transfected with PAR₄ for 24 h prior to being seeded at a density of 1×10^5 cells per well in 24-well plates pre-coated with 0.1 ml/ml of poly-L-lysine. Cells were grown overnight to recover. Surface receptors were pre-labeled with anti-PAR₄ (1/1000 dilution) at 4 °C for 1 h. Cells were fixed in 3.7% paraformaldehyde for 5 min and then washed three times in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl). Cells were blocked with 1% BSA in TBS for 45 min at room temperature followed by a 1-h incubation with a alkaline phosphatase-conjugated goat anti-rabbit antibody (1/1000 dilution) in 1% BSA in TBS. Cells were washed four times in TBS to remove unbound secondary antibody. Alkaline phosphate substrate solution was prepared by dissolving *p*-nitrophenyl phosphate tablets in diethanolamine buffer (Bio-Rad). Substrate solution was added to cells and the plates were incubated at 37 °C for 10–30 min. Absorbance was measured at 405 nm using a microplate reader (Dynex MRX revelation).

Cell Surface Biotinylation—Surface expression of PAR₄ in NCTC-2544 and NCTC-PAR₂ cells was measured by a biotinylation assay using Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). Briefly, four T75 cm² flasks of NCTC-2544 or NCTC-PAR₂ cells were transfected with PAR₄ mECFP. Cells were labeled with Sulfo-NHS-SS-Biotin for 30 min at 4 °C on a rocking platform. The biotinylation reaction was stopped through the addition of a quench solution followed by further incubation at 4 °C for 15 min. Cells were scraped and the flasks were rinsed in Tris-buffered saline (TBS) and centrifuged at $1,000 \times g$ for 3 min. Supernatant was discarded and the cell pellets were washed 3 times in TBS followed by centrifugation at $1,000 \times g$ for 3 min. Cells were lysed using the provided lysis buffer containing complete protease inhibitor mixture (Roche Diagnostics) and sonicated on ice at low power to disrupt using five 1-s bursts, then incubated at 4 °C for 30 min on an orbital rotator. The cell lysates were then centrifuged at $10,000 \times g$ for 2 min at 4 °C. Clarified supernatants were transferred to a new tube and incubated with NeutrAvidin-agarose for 60 min at room temperature with end-over-end mixing using a rotator. Supernatant/agarose slurry was centrifuged for 1 min at $1,000 \times g$ and the supernatant was discarded. The agarose pellet was washed 3 times in the wash buffer provided with the addition of complete protease inhibitor mixture. SDS-PAGE sample

buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 50 mM DTT) was added to the sample, which was then heated in a heat block for 5 min at 95 °C. The tubes were then centrifuged for 2 min at $1,000 \times g$. PAR₄ expression was detected by Western blotting using antibodies specific for either PAR₄ or GFP. Equal expression of total levels of PAR₄ mECFP in transfected cells was confirmed by resolving the corresponding whole cell lysates prepared from the same cells used for the biotinylation experiments.

Western Blotting—Proteins were separated by 8–10% SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked for nonspecific binding in 2% BSA (w/v) diluted in NATT buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% (v/v) Tween 20) for 2 h. The blots were then incubated overnight with 50 ng/ml of primary antibody diluted in 0.2% BSA (w/v) in NATT buffer then washed with NATT buffer at 15-min intervals for a further 90 min. The blots were then incubated with HRP-conjugated secondary antibody (20 ng/ml) in 0.2% BSA (w/v) diluted in NATT buffer for 2 h. After a further 90-min wash, the membranes were treated with ECL reagent and exposed to Kodak x-ray film.

Subcellular Fractionation of ER and Plasma Membrane Compartments—Cells were grown to 70–80% confluence in $5 \times T150$ cm tissue culture flasks prior to transient transfection with PAR₄ mECFP. The cells were harvested and the cell pellet resuspended in 3 ml of HES buffer (25 mM HEPES, 1 mM EDTA, and 250 mM sucrose, pH 7.4) supplemented with protease inhibitors (25 μ g/ml of leupeptin, 10 μ g/ml of aprotinin, and 1 μ g/ml of PMSF). The cell lysate was homogenized using a pre-cooled cell homogenizer (Isobiotec Precision Engineering, Germany, German Patent Office number 202 09 547.9) fitted with a size 10- μ m clearance tungsten carbide ballbearing. The homogenate was centrifuged at $500 \times g$ for 2 min at 4 °C and the supernatant was transferred to a fresh tube and resuspended in Opti-prep (Invitrogen) density gradient medium to create a 45% (v/v) density sample solution. A density gradient (30–10%) was prepared using Opti-prep medium mixed in HES buffer followed by ultracentrifugation at $72,000 \times g$ for 4 h at 4 °C to separate plasma membrane, endosomal, and ER fractions (40). Equal volume fractions (300 μ l) were collected and precipitated in 37.5% TCA, incubated on ice for 15 min, and centrifuged at $14,000 \times g$ for 15 min at 4 °C. The cell pellets obtained were resuspended in 2 \times Laemmli sample buffer supplemented with 1 M urea and resolved by Western blotting. Subcellular fractionation of ER and plasma membrane compartments were determined using Na⁺,K⁺-ATPase, transferrin receptor, and calnexin antibodies as markers for plasma membrane, endosomal, and ER fractions, respectively.

Intermolecular FRET—Wide-field intermolecular FRET microscopy was performed at room temperature in living cells (41–43) on a Nikon TE2000-E inverted microscope (Nikon Instruments, Melville, NY). Cells were grown on 0 thickness on coverslips and transiently transfected with the appropriate monomeric donor mECFP or acceptor mEYFP-tagged constructs. Coverslips were placed into a microscope chamber containing physiological HEPES-buffered saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10 mM D-glucose, pH 7.4). FRET imaging was performed using

a $\times 40$ (numerical aperture; NA 1.3) oil immersion Fluor lens. Emitted fluorescence was detected using a photometric Cool Snap-HQ monochrome camera (Roper Scientific, Trenton, NJ) set up in 12-bit mode (0–4095 gray tones). MetaMorph software (version 7.6.4 Molecular Devices Corp.) was used to control both the microscopy hardware and multiwavelength fluorescence image acquisition required for intermolecular FRET detection. Donor 430 nm or acceptor 500 nm excitation light was generated using a computer controlled Optoscan monochromator (Cairn Research, Faversham, Kent, UK) coupled to a 103/W2 mercury (Hg) arc lamp source (Cairn Research). Optimization of illumination excitation center wavelength and band pass settings was performed to prevent cross-excitation, minimize donor and acceptor bleed-through into the FRET channel, and ensure no recorded pixels within the channel images were saturated above a gray level intensity value of 4095. The risk of motion occurring during the sequential FRET imaging process was minimized by using a high-speed filter wheel (Prior Scientific Instruments, Cambridge, UK). Metamorph imaging software was used to quantify the FRET images using the specified bleed-through FRET method. Corrected FRET (FRET_c) was calculated using a pixel-by-pixel methodology using the equation $\text{FRET}_c = \text{FRET} - (\text{coefficient } B \times \text{mECFP}) - (\text{coefficient } A \times \text{mEYFP})$, where mECFP, mEYFP, and FRET values correspond to background corrected images obtained through the donor mECFP, mEYFP, and FRET channels. *B* and *A* correspond to the values obtained for the mECFP (donor) and mEYFP (acceptor) bleed-through coefficients, respectively, calculated using cells transfected with either the mECFP or mEYFP protein alone. Ratiometric FRET (RFRET) values were calculated from the measurements taken from raw FRET fluorescence and dividing this value by the total spectral bleed-through of the acceptor and donor into the FRET channel, *i.e.* raw FRET divided by (acceptor multiplied by (a)) + (donor multiplied by (b)). In the absence of energy transfer (*i.e.* no FRET occurrence), the RFRET value measured is ~ 1 , values greater than 1 represent the occurrence of FRET, thus indicative of protein interaction.

Co-immunoprecipitation—To measure PAR₄ interaction with 14-3-3 ζ , cells transiently expressing PAR₄-HA were washed with PBS prior to solubilization in lysis buffer (20 mM HEPES buffer, pH 7.7, containing 50 mM NaCl, 0.1 mM EDTA, 0.1 mM Na₃VO₄, 0.1 mM PMSF, 10 mg/ml of aprotinin, 10 mg/ml of leupeptin, and 1% (w/v) Triton X-100). After a 1-h rotation at 4 °C, the cell lysates were clarified by centrifugation at $13,000 \times g$ for 5 min at 4 °C. Supernatants were transferred to fresh Eppendorf tubes and 50 μ l was removed for inputs. The remaining lysate was pre-cleared with 30 μ l of protein G/protein A-agarose (Calbiochem) and placed in an rotator for 1 h at 4 °C. Samples were centrifuged at 4 °C for 5 min at $5,000 \times g$ and the pre-cleared lysate was transferred to fresh Eppendorf tubes containing 30 μ l of monoclonal anti-HA-agarose conjugate (Sigma) and rotated overnight at 4 °C. Samples were centrifuged at 4 °C for 5 min at $5,000 \times g$, then washed with 500 μ l of lysis buffer three times and proteins were eluted by incubation with 30 μ l of anti-HA peptide (Sigma; 200 μ g/ml) for 10 min at room temperature. Eluted proteins were removed and added to

10 μ l of 5 \times SDS sample buffer and boiled at 95 °C for 10 min prior to SDS-PAGE.

Statistical Analysis—Where experimental data are shown as a blot, this represents one of at least 3 experiments and data represent the mean \pm S.E. Statistical analysis was by one-way analysis of variance with Dunnett's post-test (*, $p < 0.05$; **, $p < 0.01$).

RESULTS

Intracellular Retention of PAR₄ in the Endoplasmic Reticulum—To monitor the expression level and localization of PAR₄, the receptor was tagged at the C terminus with a monomeric variant form of enhanced cyan fluorescent protein (mECFP) and transiently expressed in keratinocyte-derived NCTC-2544 cells. These cells provided an ideal model for these investigations, due to the lack of endogenous PAR expression (44). The localization of PAR₄ was initially monitored using fluorescence microscopy of NCTC-2544 cells transiently expressing PAR₄ mECFP (Fig. 1A). In comparison to cells expressing either PAR₁ mEYFP or PAR₂ mEYFP, PAR₄ mECFP was largely retained inside the cell with only weak membrane localization observed. Further microscopy in cells treated with an ER tracker dye (Fig. 1B) highlighted that PAR₄ mECFP was predominantly retained in the ER.

Presence of a Functional Arginine-based ER Retention Motif within PAR₄—Analysis of the protein sequence for PAR₄ identified two potential arginine-based (RXR) ER retention motifs located within the intracellular loop-2 of the receptor (supplemental Fig. S1). Alignment of the primary sequences for all PAR family members found that these motifs were unique to PAR₄. The contribution of these motifs in controlling the cellular localization of PAR₄ was assessed by removing the arginine residues by alanine substitution (RXR \rightarrow AXA). Of the possible motifs investigated, only mutation of the R¹⁸³AR to A¹⁸³AA resulted in a loss of ER retention and allowed PAR₄ to translocate to the plasma membrane (Fig. 2A). Receptor expression levels were determined by Western blotting (Fig. 2B). Following expression of PAR₄ mECFP the appearance of a protein band, resolving around 65 kDa, was observed. This corresponded well with the predicted molecular mass of PAR₄ mECFP (38 kDa for PAR₄ combined with 27 kDa for the mECFP). As Fig. 2B shows, as the expression of the R¹⁸³AR mutant increased, the appearance of multiple protein forms was observed, a doublet resolving around 65 kDa and a slightly larger species resolving between 70 and 80 kDa. Subcellular fractionation of cells expressing either PAR₄ mECFP or the R¹⁸³AR mutant was carried out to separate plasma membrane, endosomal, and ER compartments followed by Western blot (Fig. 2C). The 65-kDa protein species observed in cells expressing PAR₄ mECFP or mutant receptor reached maximal levels in ER and endosomal fractions (lanes 4–7), co-locating with calnexin and transferrin markers, respectively. These experiments identified that the higher molecular mass species observed in cells expressing the R¹⁸³AR mutant reflected receptors located in the plasma membrane and endosome compartments (lanes 1–4) as shown using Na⁺, K⁺-ATPase and transferrin receptor markers, respectively.

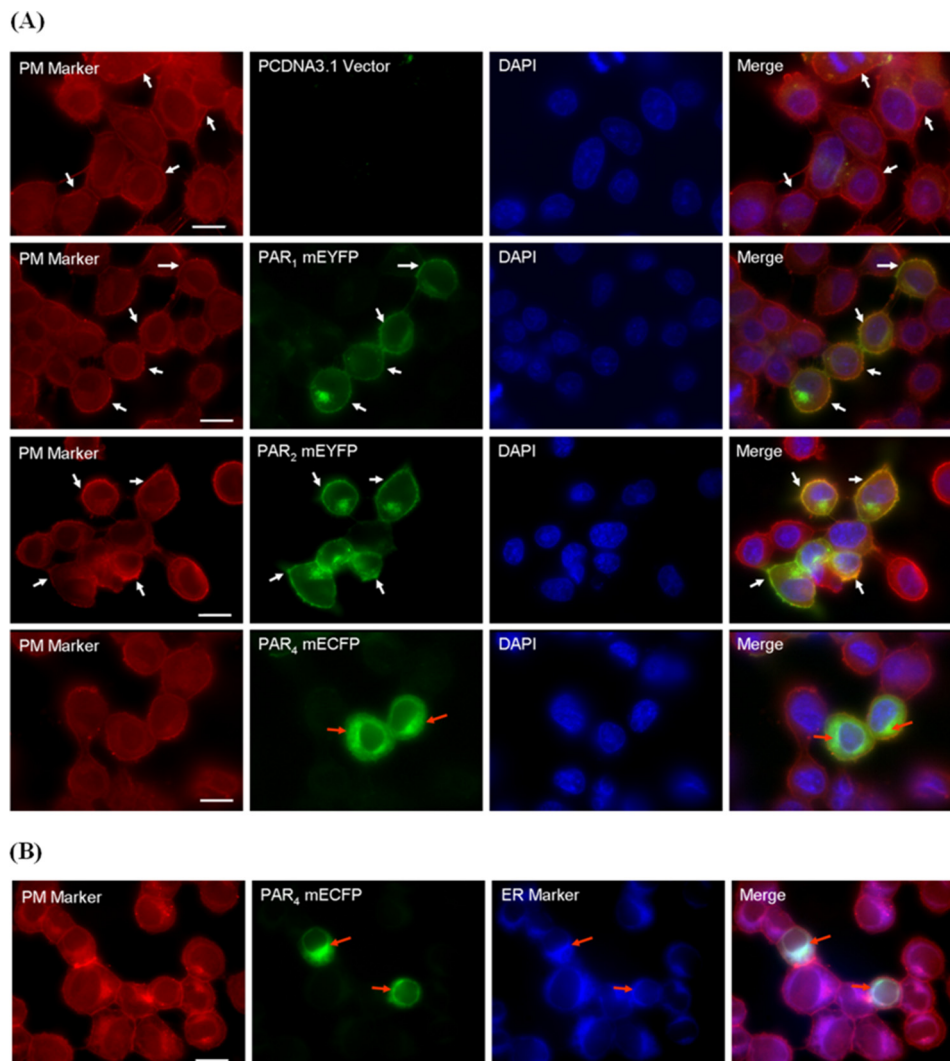


FIGURE 1. Retention of PAR₄ in the ER. NCTC-2544 cells expressing pcdNA3.1 empty vector, PAR₁ mEYFP, PAR₂ mEYFP, or PAR₄ mECFP (green) were treated with PKH26 red fluorescent cell linker dye to stain the plasma membrane (PM, red). Cells were fixed and treated with either (A) 4',6-diamidino-2-phenylindole (DAPI) to identify nuclei (blue) or (B) ER marker (ER-Tracker Blue-White DPX Dyes, Molecular Probes) to identify the ER (blue). Cells were visualized using a $\times 100$ Plan Fluor objective. Images were merged to highlight distinct plasma membrane, nucleus, or ER compartments. Scale bars = 10 μ m. White arrows point to the plasma membrane, whereas red arrows are indicative of the intracellular/ER compartmentalization of PAR₄. Image set representative of three separate experiments.

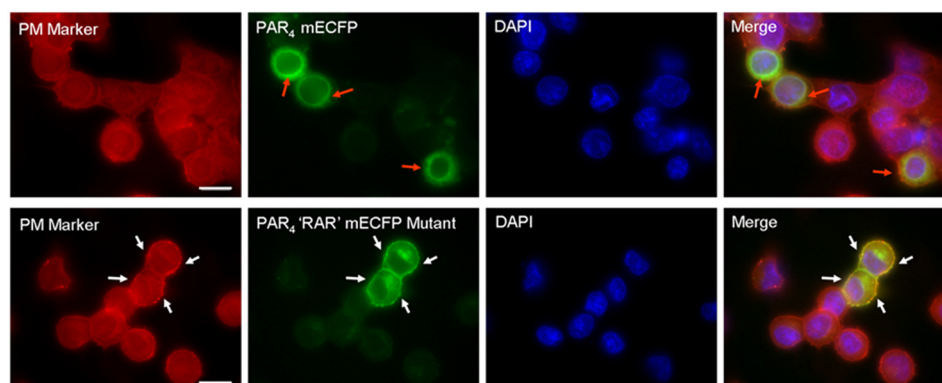
Coat protein I complex (COPI) can target proteins for retention through recognition and interaction with RXR motifs (29). COPI is comprised of multiple subunits including α -, β -, β' -, γ -, δ -, ϵ -, and ζ -COP. The ability of COPI to interact with PAR₄ was demonstrated through co-immunoprecipitation with the β -COP subunit of the COPI complex as shown in Fig. 2D.

PAR₂ Facilitates Anterograde Transport of PAR₄ and Interaction with 14-3-3 ζ —The presence of the R¹⁸³AR ER motif in the protein sequence contributed greatly to the retention of PAR₄ in the ER. Despite the presence of this ER retention motif, as the earlier original characterization studies show, PAR₄ has the ability to reach the plasma membrane and respond to protease activation (20). During the assembly of multimeric proteins it has been shown that ER motifs, particularly arginine-based ER retention motifs (25), may be masked to allow proteins to evade the quality control processes in the ER (31, 45). Interestingly when co-expressed with PAR₂ mEYFP, PAR₄ mECFP was found to be localized at the plasma membrane with minimal intracellular compartmentalization, as shown by confocal

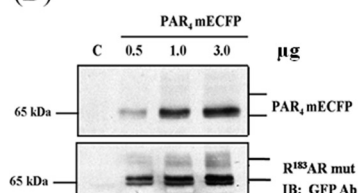
microscopy in Fig. 3A. This was explored further by cell surface ELISA using an N-terminal PAR₄-specific antibody to quantify differences in surface receptor levels in NCTC-2544 cells and NCTC-2544 cells stably expressing PAR₂ (NCTC-PAR₂), as shown in Fig. 3B. When PAR₄ mECFP was expressed in NCTC-2544 cells a small increase in absorbance ($A_{405\text{ nm}}$) was detected (0.676 ± 0.05 , $p \leq 0.05$, $n = 4$) compared with control untransfected cells (0.495 ± 0.05 , $n = 4$). When expressed in NCTC-PAR₂ cells, PAR₄ surface expression was significantly increased, as demonstrated by the enhanced $A_{405\text{ nm}}$ reading (1.016 ± 0.03 , $N4$, $***$, $p \leq 0.001$ compared with PAR₄ in NCTC-2544 cells). Enhanced translocation of PAR₄ mECFP to the plasma membrane was not replicated when co-expressed with PAR₁ mEYFP (supplemental Fig. S2). PAR₄ was still largely localized intracellularly, with PAR₁/PAR₄ co-localization observed predominantly within vesicles.

As shown in Fig. 4A, similar membrane translocation was observed for PAR₄ mECFP when transfected into NCTC-PAR₂.

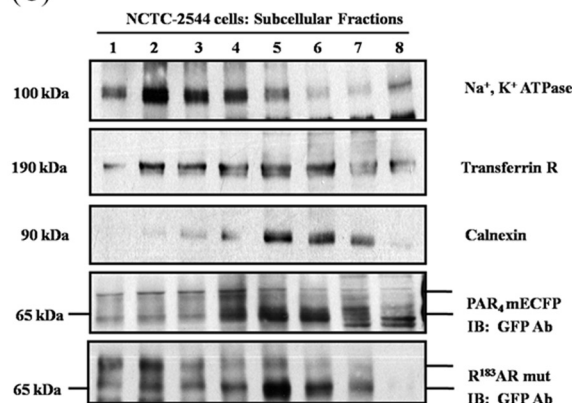
(A)



(B)



(C)



(D)

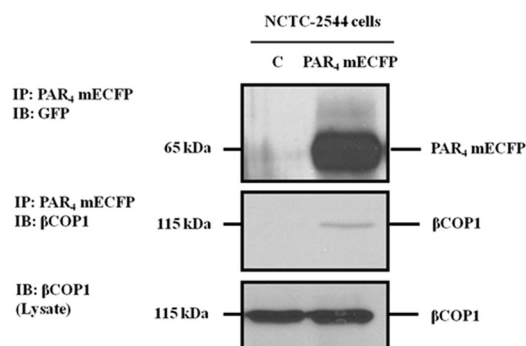


FIGURE 2. Mutation of the arginine-based ER retention motif (R¹⁸³AR) enhances the cell surface expression of PAR₄. A, NCTC-2544 cells expressing PAR₄ mECFP or PAR₄ RAR mECFP mutant (green) were treated to stain for the plasma membrane (PM, red) and nucleus (blue) as previously described. Cells were visualized using a ×100 Plan Fluor objective. Scale bars = 10 μm. Intracellular retention of PAR₄ mECFP is highlighted (red arrows), whereas notable membrane localization of the PAR₄ RAR mECFP is evident (white arrows). B, protein expression was assessed using Western blotting of whole cell lysates expressing increasing amounts of PAR₄ mECFP or PAR₄ RAR mECFP constructs as indicated. C, changes in the surface expression of PAR₄ were confirmed by subcellular fractionation using differential ultracentrifugation on an iodixanol gradient in cells expressing PAR₄ mECFP or PAR₄ RAR mECFP. Fractions were precipitated and resolved by SDS-PAGE followed by Western blotting. PAR₄ mECFP (predicted band size ~65 kDa) was detected using a polyclonal GFP antibody capable of recognizing the ECFP at the C-terminal of PAR₄. Na⁺, K⁺-ATPase (~100 kDa), transferrin receptor (~190 kDa), and calnexin (90 ~kDa) antibodies were used for the detection of membrane, endosomal, and ER compartments, respectively. D, interaction between PAR₄ and COPI complex proteins was confirmed by co-immunoprecipitation. Lysates from NCTC-2544 cells expressing PAR₄ mECFP were subjected to immunoprecipitation (IP) and then probed for β-COP1 interaction. Images and blots are representative of three separate experiments.

In addition, when PAR₄ mECFP was resolved by Western blotting two clear protein forms were detected when expressed in NCTC-PAR₂ cells (Fig. 4B). These results were similar to the observations made in previous experiments resolving the ER retention motif mutant PAR₄ protein. Subsequent subcellular fractionation of NCTC-PAR₂ cells expressing PAR₄ mECFP highlighted the distinct differences in the compartmentalization of PAR₄. As Fig. 4B shows, the more rapidly migrating

65-kDa species was confined to ER and endosomal compartments (lanes 4–6), whereas the distribution of the less rapidly migrating form strongly correlated with ER, endosomal, and plasma membrane fractions (lanes 1–6). Enhanced surface expression of PAR₄ was subsequently quantified using cell surface biotinylation, as shown in Fig. 4C. Following biotinylation of surface proteins, expression of PAR₄ was probed using both anti-GFP and anti-PAR₄ specific antibodies in NCTC-2544 and

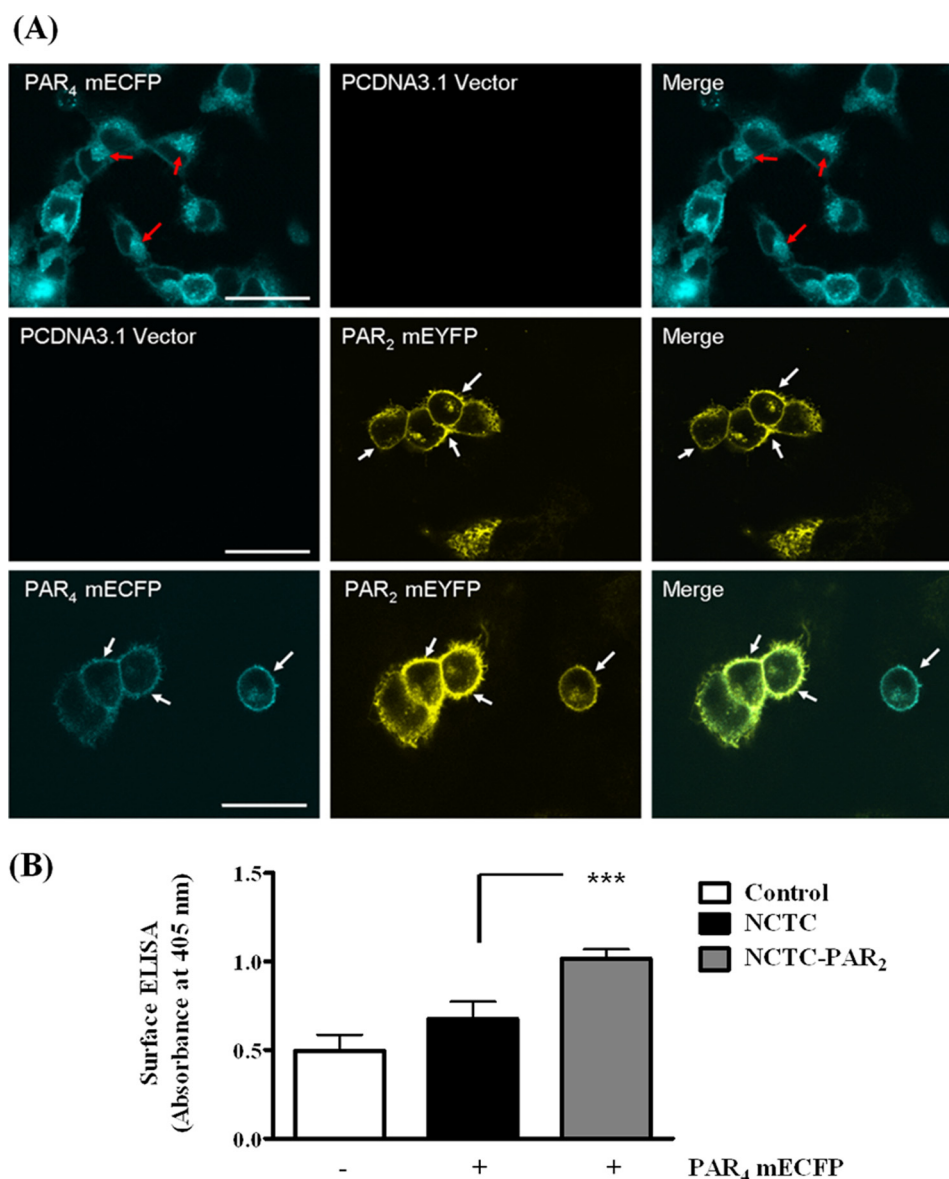


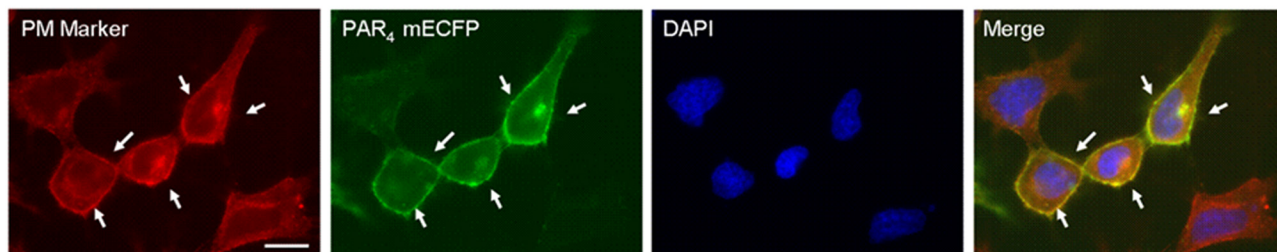
FIGURE 3. PAR₂ enhances surface expression of PAR₄ when co-expressed in NCTC-2544 cells. NCTC-2544 cells expressing PAR₄ mECFP (cyan), PAR₂ mEYFP (yellow), or co-expressing both constructs were fixed for confocal microscopy. Scale bars = 25 μ m. *A*, membrane (white arrows) receptor expression is highlighted, whereas intracellular receptor pools are indicated using red arrows. Images shown are representative of at least five independent experiments. *B*, enhancement of PAR₄ surface expression in the presence of PAR₂ was subsequently quantified using a surface ELISA approach with an N-terminal anti-PAR₄ antibody to detect changes in surface levels of PAR₄. Data are presented as the optical density at 405 nm obtained from NCTC-2544 cells only (Control), NCTC-2544 or NCTC-PAR₄ cells expressing PAR₄ mECFP performed in at least three independent experiments. ***, $p = 0.001$ one-way analysis of variance with Dunnett's post-test.

NCTC-PAR₂ cells transfected with PAR₄ mECFP. Although detection of surface PAR₄ was negligible in transfected NCTC-2544 cells (0.725 ± 0.30 -fold increase over mock transfected cells, $n = 4$), a significant increase in surface PAR₄ was detected in NCTC-PAR₂-transfected cells (5.199 ± 0.85 -fold increase over mock cells, $n = 4$). In addition to enhanced cell surface expression of PAR₄ in the presence of PAR₂, a notable increase in the ability of PAR₄ to interact with the ζ isoform of the ER export chaperone 14-3-3 was detected in co-immunoprecipitation experiments (Fig. 4D). When PAR₄-HA was expressed in the parental NCTC-2544 cells, the ability of 14-3-3 ζ to interact with PAR₄ was negligible. However, when PAR₄-HA was expressed in NCTC-PAR₂ cells, the ability of 14-3-3 ζ to interact with PAR₄ was clearly shown. Differential interaction of PAR₄

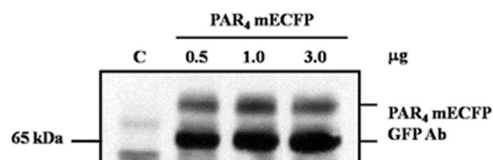
with 14-3-3 ζ was also demonstrated using GST pulldown assays employing GST-14-3-3 ζ fusion proteins (supplemental Fig. S3). PAR₄ binding to GST-14-3-3 ζ was enhanced when expressed in NCTC-PAR₂ cells. In addition, interaction between PAR₄ and β -COP1 was no longer observed during co-expression of PAR₂ and PAR₄ (Fig. 4E and supplemental Fig. S3).

The localization of PAR₄ was further explored in HEK293 cells, which have an endogenous level of PAR₂ (Fig. 5). When expressed, PAR₄ mECFP was observed both at the plasma membrane and in intracellular compartments (Fig. 5A), which when resolved by Western blot (Fig. 5B) identified similar protein species as in NCTC-2544 cells, representative receptor populations were expressed at the cell surface and in ER/endosomal compartments (Fig. 5C).

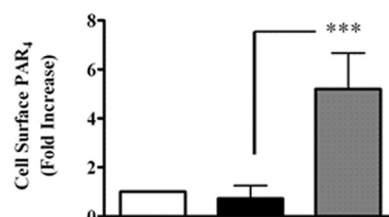
(A)



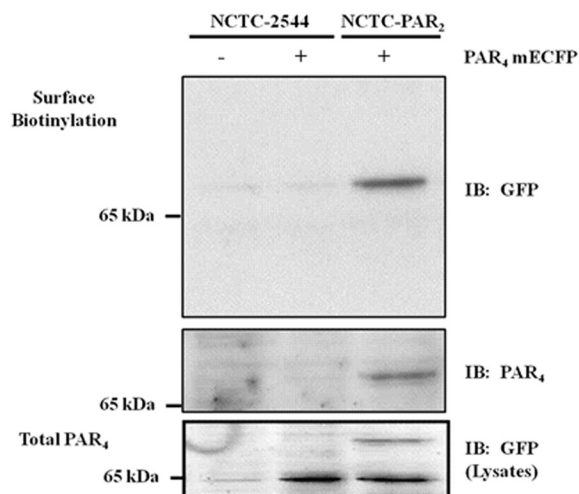
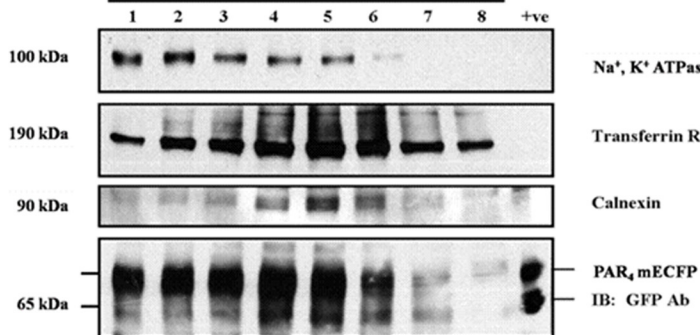
(B)



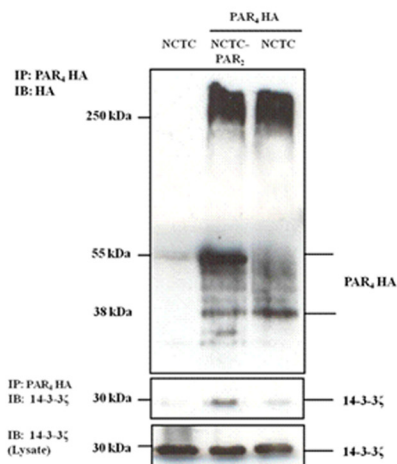
(C)



NCTC PAR₂ cells (with PAR₄ mECFP): Subcellular Fractions



(D)



(E)

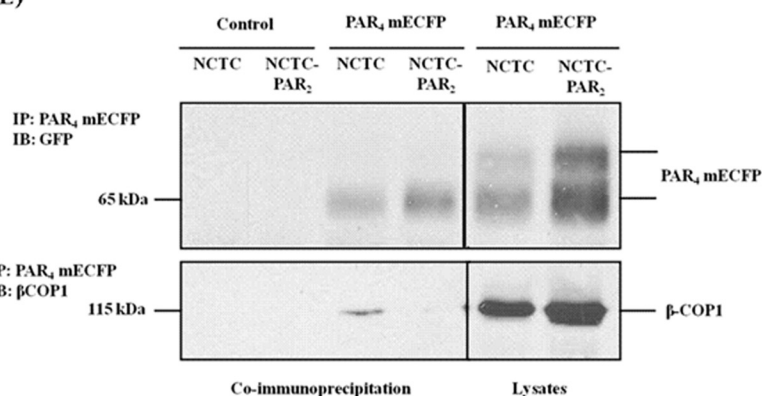


FIGURE 4. PAR₂ facilitates interaction between PAR₄ and 14-3-3ζ but disrupts interaction with β-COP1. PAR₄ mECFP was transiently transfected into NCTC overexpressing PAR₂ (NCTC-PAR₂) cells. *A*, cells were treated, as described previously, to identify the plasma membrane (red) and nuclei (blue). Cells were visualized using a ×100 Plan Fluor objective. Images were merged to highlight distinct plasma membrane/nuclear compartments. Scale bars = 10 μm. Enhanced surface expression of PAR₄ in NCTC-PAR₂ cells is indicated by white arrows. *B*, protein expression was assessed using Western blotting in cells expressing increasing amounts of PAR₄ mECFP with the protein bands were detected separated by subcellular fractionation in NCTC-PAR₂ cells and resolved by Western blotting, as previously shown. *C*, enhanced surface expression was then quantitatively assessed by cell surface biotinylation of NCTC-2544 and NCTC-PAR₂ cells expressing PAR₄ mECFP. Interaction between (D) PAR₄-HA and 14-3-3ζ or (E) PAR₄-mECFP and the βCOP subunit of COPI was assessed by co-immunoprecipitation (IP) in NCTC-2544 and NCTC-PAR₂ cells. Images and blots are representative of at least four independent experiments.

The novel features of PAR₂/PAR₄ co-expression were investigated further to identify if enhancement of the PAR₄ cell surface expression was a result of interaction between PAR₂ and

PAR₄. For this purpose, wide field intermolecular FRET imaging was carried out (41, 42) in HEK293 cells expressing either PAR₄ mECFP or PAR₂ mEYFP alone or co-expressing these two

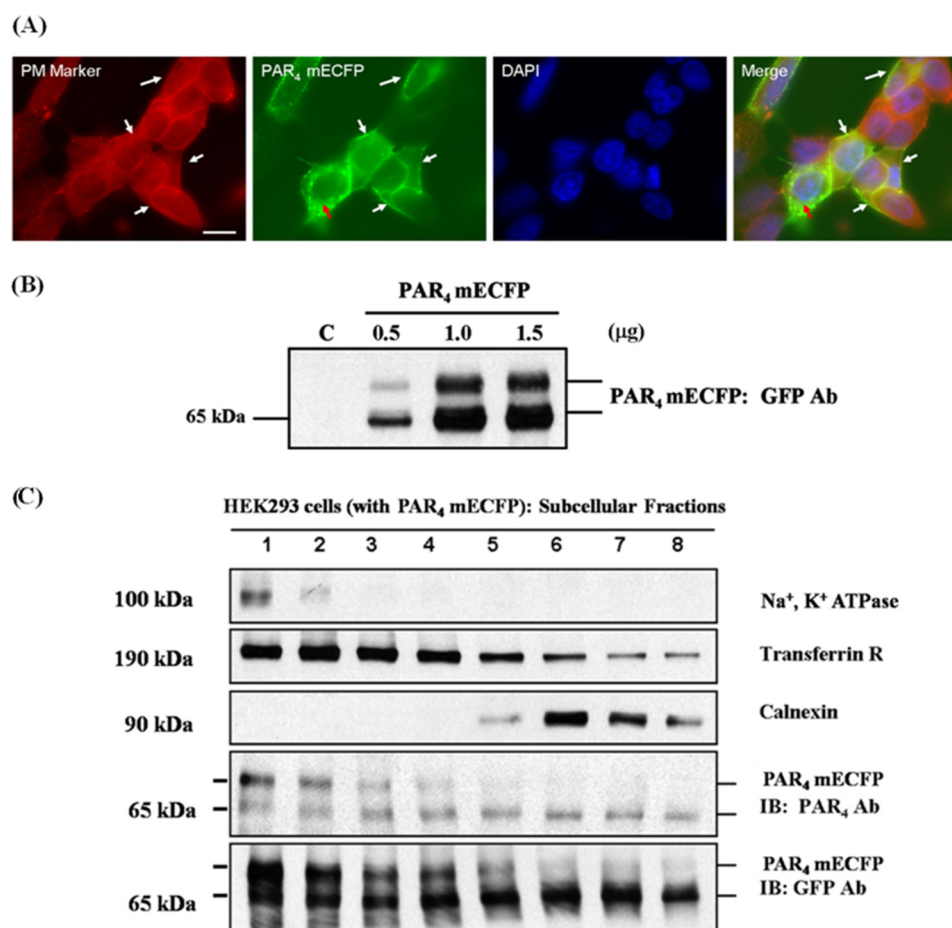


FIGURE 5. Membrane localization of PAR₄ mECFP in HEK293 cells. PAR₄ mECFP was transiently transfected into HEK293 cells that endogenously express PAR₂. *A*, localization of PAR₄ at the cells surface is shown by co-localization with a plasma membrane marker (white arrows). Cells were visualized using a $\times 100$ Plan Fluor objective. Scale bars = 10 μ m. *B*, the protein band pattern of PAR₄ expression was further assessed by Western blot of transfected whole cell lysates followed by subcellular fractionation (*C*) as previously described. Images and blots are representative of three separate experiments.

constructs. As shown in Fig. 6A, an intracellular FRET signal was observed, presumably in the ER and/or Golgi complex, with a weak signal observed at the plasma membrane. When quantified, co-expression of PAR₂ mEYFP and PAR₄ mECFP resulted in a significant increase in RFRET (1.883 ± 0.003) when compared with experimental conditions where collisional FRET could occur, *i.e.* co-expression of mEYFP and mECFP in cells yielded a RFRET value of 1.173 ± 0.055 . Interaction between PAR₂ and PAR₄ was also demonstrated by co-immunoprecipitation in HEK293 cells as shown in Fig. 6B. These data indicate that PAR₂/PAR₄ heterodimerization occurs and is likely responsible for enhanced cell surface expression of PAR₄.

We then examined the role of PAR₂ in regulating further post-translational modification of PAR₄ in the context of receptor maturation and cell surface expression. *N*-Linked complex glycosylation is an important post-translational modification for efficient cell surface delivery of GPCRs (46). Analysis of the protein sequence of PAR₄ identified an Asn-Xxx-serine/threonine *N*-glycosylation motif located at position 56 (N⁵⁶DS) in the N terminus of the receptor (supplemental Fig. S1), suggesting that PAR₄ may undergo *N*-linked glycosylation. To assess this, first, pharmacological inhibition of glycosylation was carried out using the GlcNAc phosphotransferase inhibitor tunicamycin,

which prevents all *N*-linked glycosylation. Fig. 7A shows that pretreatment of HEK293 cells with tunicamycin eliminated the higher molecular mass form of PAR₄, whereas the lower 65-kDa protein was still retained, with a minor intermediate band indicated. This effect was replicated in the R¹⁸³AR to A¹⁸³AA mutant, known to be preferentially expressed at the membrane. Experiments were then conducted using an N-terminal mutant of PAR₄ (Asn⁵⁶ \rightarrow Ala⁵⁶) to determine the effect of PAR₂ upon protein species expression (Fig. 7B) and receptor localization (Fig. 7C). In control NCTC cells the PAR₄ N⁵⁶A mutant construct (Fig. 7B, *top panel*) was expressed as a single 65-kDa protein form that corresponded to wild type PAR₄ mECFP. When expressed in NCTC-PAR₂ cells (Fig. 7B, *middle panel*) a loss in the higher molecular mass protein form was observed in comparison to wild type PAR₄ mECFP, however, the 65-kDa species was expressed alongside the slightly higher molecular mass form previously observed in the tunicamycin inhibition experiments. Similar results were obtained following expression in HEK293 cells (Fig. 7B, *lower panel*). Corresponding fluorescence microscopy images are shown in Fig. 7C. Lack of PAR₄ cell surface expression was observed following expression of the PAR₄ N⁵⁶A mutant in NCTC-PAR₂ cells.

Finally, the effect of enhanced cell surface expression of PAR₄ in the presence of PAR₂ was explored further in relationship to

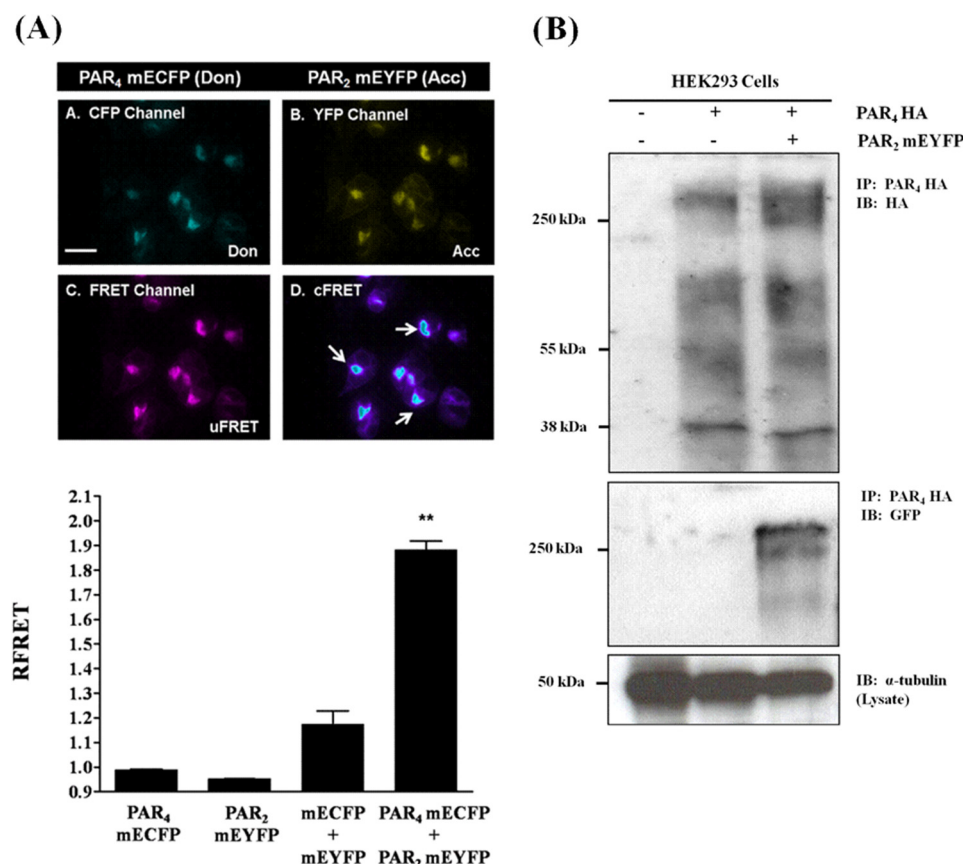


FIGURE 6. FRET imaging and co-immunoprecipitation reveals heterodimer formation between PAR₂ and PAR₄ in HEK293 cells. PAR₄ mECFP and PAR₂ mEYFP were co-expressed in HEK293 cells. Wide field FRET imaging was performed in live cells. *A*, images were acquired for CFP, YFP, uncorrected FRET (uFRET), with the uFRET channel corrected for spectral bleed-through/contamination (cFRET). Scale bars = 25 μ m. Corresponding ratio-metric FRET values were then quantified and graphed. Data are expressed as mean \pm S.E. from three separate FRET experiments ($n = 72$ single cell measurements), **, $p = 0.001$ one-way analysis of variance with Dunnett's post-test. *B*, interaction between PAR₄-HA and PAR₂ mEYFP was further assessed by co-immunoprecipitation (IP). Blots are representative of at least three independent experiments.

PAR₄-mediated cell signal transduction. As shown in Fig. 8, NCTC-2544 and NCTC-PAR₂ cells transiently expressing PAR₄ mECFP produced an increase in basal inositol phosphate generation (NCTC, 4.59 ± 0.45 ; NCTC-PAR₂, 8.28 ± 0.65 -fold of basal) compared with mock transfected cells. When each of these cell systems was treated with the PAR₄-specific activating peptide, AYPGKF-NH₂, a further increase in the inositol phosphate response was observed. When PAR₄ was expressed in NCTC-PAR₂ cells total inositol phosphate (InsP₁₋₄) accumulation (50 μ M agonist; 22.47 ± 0.45 and 100 μ M agonist; 21.16 ± 2.62 -fold of basal) was substantially greater than observed following expression of PAR₄ in NCTC-2544 cells (50 μ M agonist; 7.80 ± 0.46 and 100 μ M agonist; 9.47 ± 2.45 -fold of basal).

DISCUSSION

Receptor traffic from the ER to the plasma membrane involves highly coordinated events that in many cases may require numerous accessory proteins and motif-based sorting processes (35). The work presented here explores the mechanisms through which PAR₄ localization may be regulated. We demonstrate for the first time the fundamental structural properties and protein interactions that govern PAR₄ trafficking to the plasma membrane. Furthermore, we reveal the critical role of PAR₂ in aiding plasma membrane expression of PAR₄.

Initially we used bioinformatic analysis, which indicated that PAR₄ possesses a potential arginine-based (RXR) ER retention motif in intracellular loop 2, similar in sequence to those of the KA2 kainate (47) and 5HT_{3B} serotonin (45) receptors. As such, we considered that this motif might be responsible for retention of PAR₄ in the ER as an immature protein as shown in Fig. 1. Unlike the KA2R and 5HT_{3B}R, however, where mutation of the motif did not affect ER retention, mutation of the R¹⁸³AR sequence of PAR₄ resulted in enhanced cell surface expression as assessed by both indirect immunofluorescence and subcellular fractionation. These findings are similar to previous studies showing efficient surface delivery following mutagenesis of a RSRR retention motif located within the C-terminal of the GABA_{B1} polypeptide (24, 48). Protein complexes such as COPI can target other proteins for retention through recognition and interaction with RXR ER retention sequences (29), as demonstrated for retention of KA2 receptors (33) and, indeed, our studies showed PAR₄ interaction with the β -COP subunit of the COPI complex (Fig. 2D). Equally, ER retention of KA2R has been shown to correlate with an interaction with subunits that comprise the COPI chaperone system (33). Misfolded proteins or those containing sorting motifs such as an ER retention motif are known to be shuttled back to the ER via COPI-containing vesicles (29). Our data in Fig. 4 show

PAR₂ Regulation of PAR₄ Trafficking

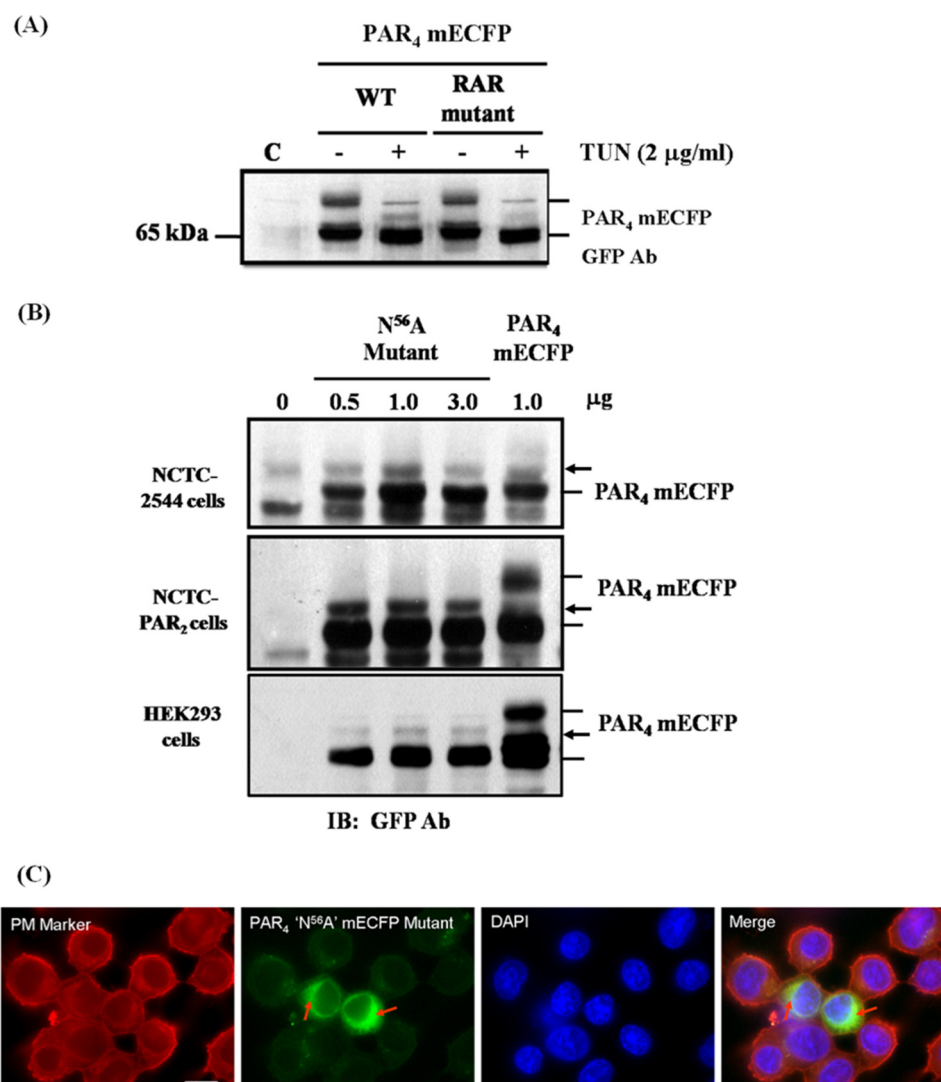


FIGURE 7. The presence of PAR₂ allows N-linked glycosylation of PAR₄, which promotes membrane localization. HEK293 cells were transfected with (A) PAR₄ mECFP or PAR₄ RAR mutant constructs. Prevention of *de novo* N-glycosylation was carried out by the addition of tunicamycin (2 μg/ml) to transfected cells as indicated for 16 h. B, NCTC, NCTC-PAR₂, and HEK293 cells were transfected with increasing concentrations of the PAR₄ N⁵⁶A mECFP mutant or PAR₄ mECFP. Whole cell lysates were prepared and resolved by Western blotting. C, fluorescent microscopy confirmed a loss of surface localization of PAR₄ following mutation of N-glycosylation site (red arrows). Cells were visualized using a ×100 Plan Fluor objective. Scale bars = 10 μm. Blots and images are representative of three independent experiments.

that indeed PAR₄ is retrieved via a COPI-mediated system, as found for KA2R.

Having defined the subcellular retention of PAR₄, we sought to determine what mechanisms might facilitate transport to the membrane because PAR₄ is able to signal in a number of cell types (49–51) and must reach the cell surface to be cleaved by the proteinase agonists. One potential candidate was PAR₂ because this receptor is often co-expressed with PAR₄ and has been shown to be co-activated by common agonists (20, 36, 37). However, a key issue in exploring this possibility was the fact that most cell lines currently studied express endogenous PAR₂ at some level. In this present study, characterization of PAR₄ subcellular distribution utilized NCTC-2544 cells because these express negligible PAR receptor levels and this allowed a direct assessment of the cellular distribution and function of PAR₄ in the absence and presence of PAR₂. Indeed, prior stable expression of PAR₂ in this cell line revealed enhanced PAR₄ plasma membrane expression compared with control NCTC

cells. These results were recapitulated in HEK293 cells that are known to express endogenous PAR₂ and in other cells types we examined including human umbilical vein endothelial cells and PC3 cells (not shown), which are also known to express PAR₂ endogenously. Significantly, we show that enhanced plasma membrane delivery of PAR₄ by PAR₂ has functional sequelae, including increasing PAR₄-mediated inositol phosphate accumulation stimulated by a selective PAR₄ agonist. To our knowledge this is the first study to reveal such an interaction.

How may the presence of PAR₂ facilitate PAR₄ trafficking to the membrane? In the secretory pathway proper folding and assembly of GPCRs is essential for their efficient export to the cell membrane and function (52). Isoforms of 14-3-3 proteins have been shown to recognize and mask RXR motifs to direct ER/Golgi export of multimeric proteins (34, 53). Interestingly, we found that in the presence of PAR₂, PAR₄ becomes associated with 14-3-3ζ and at the same time loses its association with β-COP1. This competitive interaction is similar to that

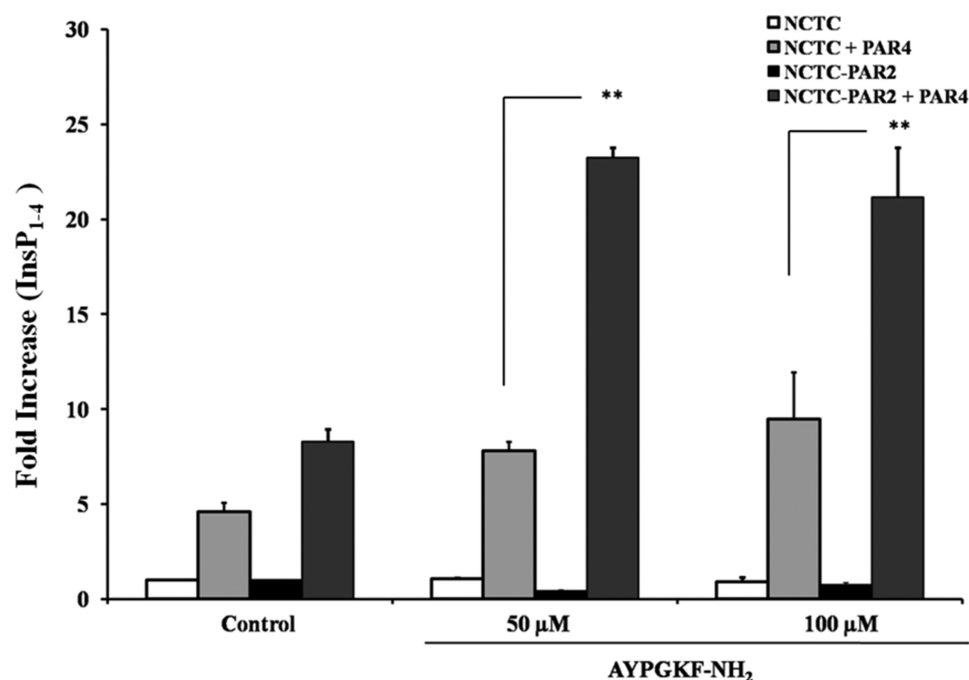


FIGURE 8. **Enhanced PAR₄-mediated [³H]inositol phosphate accumulation in the presence of PAR₂.** NCTC-2544 and NCTC-PAR₂ cells transiently expressing PAR₄ mECFP were serum starved with serum-free growth medium supplemented with 0.25 μ Ci of *myo*-[2-³H]inositol for 24 h. Cells were pretreated with 10 mM lithium chloride for 15 min prior to stimulation with AYPGKF-NH₂ as indicated for 45 min. Total inositol phosphate (InsP₁₋₄) accumulation was measured via anion exchange. The data are representative values performed in triplicate (mean \pm S.E.) over three independent experiments (**, $p < 0.01$).

observed for an ATP-sensitive potassium (K_{ATP}) channel, in which COPI competes with 14-3-3 ϵ and - ζ isoforms for interaction with the RKR motif on the cytosolic domain of each α subunit of the channel to facilitate ER retention (34). Because we also demonstrate PAR₂/PAR₄ heterodimerization by FRET and co-immunoprecipitation, this suggests that interaction with PAR₂ increases the affinity of PAR₄ for 14-3-3 binding. Thus, we have identified a critical early checkpoint in the secretory pathway involved in the processing of PAR₄ and PAR₂/PAR₄ heterodimer assembly, involving COPI and 14-3-3 ζ chaperone systems, which explains the effects described above. Interestingly, when similar FRET experiments were conducted in the NCTC-2544 cell model, where co-expression with PAR₂ enhances surface expression with minimal intracellular compartmentalization, interaction at the membrane was negligible.³ Unlike the ability of class C GPCRs to form stable dimers, the current findings may indicate interaction between PAR₂ and PAR₄ to be of a reversible and transient nature. This is akin to a number of other recent examples of GPCR dimerization that are transient and presumably defined by Mass-Action (54, 55). This would both allow PAR₄ to evade intracellular retention and, once at the cell surface, to be free to function in a monomeric or homodimeric state. This concept is currently under investigation.

We provided further confirmation of a role of PAR₂ in regulating PAR₄ trafficking in the context of *N*-linked glycosylation. This was initially detailed by pretreatment of wild type-PAR₄ (or the RAR mutant receptor) with tunicamycin or expression of PAR₄ mutated at the *N*-linked motif, located at position

N⁵⁶DS within the N-terminal domain. Both approaches abolished the presence of the mature PAR₄ form and prevented cell surface expression. Similar effects have previously been shown for the dopamine D₅ receptor (46) where mutagenesis of specific Asn-linked motifs resulted in ER retention of the receptor. Despite the presence of an *N*-linked glycosylation motif (N⁵⁶DS), PAR₄ was unable to undergo complex glycosylation, unless in the presence of PAR₂. We also identified that *N*-glycosylation was a critical process in PAR₂-mediated delivery of PAR₄ to the cell surface. Mutagenesis of the *N*-linked motif resulted in intracellular retention of PAR₄, despite the presence of PAR₂.

To our knowledge, despite work detailing the involvement of COPI/14-3-3 in the intracellular transport of class C GPCRs and ion channels, our data are the first to describe such interactions for a well established class A GPCR family such as the PAR family. However, recent studies on the intracellular trafficking of a class A orphan GPCR, GPR15, detailed a critical role for the binding of 14-3-3 (to an RXR motif located at the extreme C-tail of the receptor) in its surface localization (56).

Although studies have demonstrated PAR₁/PAR₄ (57) and PAR₁/PAR₃ heterodimerization (58), this is the first study to reveal a functional interaction between PAR₂/PAR₄. Heterodimer formation between PAR₂ and PAR₄ impacted significantly upon other protein interactions with COPI and 14-3-3 ζ , and by affecting the subcellular localization of PAR₄, substantially enhanced PAR₄-mediated signal transduction. PAR₂ and PAR₄ have been previously shown to be dual up-regulated by tumor necrosis factor α , a potent proinflammatory mediator (59, 60). An increase in PAR₂ has been linked to the progression of chronic inflammation (2, 61), with a role for PAR₄ in acute inflammation being implicated in other models of arthritis (62,

³ M. R. Cunningham, J. D. Pediani, G. Milligan, and R. Plevin, unpublished observations.

63). Co-expression of these receptors in these pathophysiological environments, where tumor necrosis factor α is abundant, may be pivotal to the progression of a PAR₂/PAR₄-mediated proinflammatory response. In this study we identified a novel heterodimer partnership formed between PAR₂/PAR₄, which allows PAR₄ to bypass COPI-dependent retrograde transport and exit the ER to undergo post-translational modification to be delivered to the plasma membrane as a mature glycoprotein. These findings may be important in the understanding of the roles of each receptor in the context of inflammation.

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