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

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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 sequence located within intracellular loop-2 (R183AR) sequence identified an arginine-based (RXR) ER retention motif. Co-expression with PAR2 was shown to facilitate interaction with the chaperone protein 14-3-3, mutation of which allowed efficient membrane delivery of PAR4, effect produced through disruption of β-COP1 binding and facilitation of interaction with the chaperone protein 14–3–3ζ. 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 transduction. This work is likely to impact markedly upon the understanding of the receptor pharmacology of PAR4 in normal physiology and disease.

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.

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2 The abbreviations used are: PAR, proteinase-activated receptor; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; mEGFP/mEYFP/mCFP, 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 COPII-dependent retrograde transport. In the presence of PAR4, through PAR4/PAR2 heterodimer formation and interaction with 14-3-3ζ, 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. PAR4 is often co-expressed with PAR2 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 PAR2-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-amine (NH2) peptide (AYPKF-NH2), was synthesized by the University of Calgary Peptide Service (Calgary, Canada). ER-TrackerBlue-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 α1 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-14-3-3ζ 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(+) vector by PCR and digested with Kpn-Agel, whereas ECFP was amplified from the pECFP-N1 vector (Clontech) and digested with Agel-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 PAR2 to mutate potential arginine-based ER retention motifs (positions R183AR → A183AA (referred to as RAR mut) R186GRR → A186GAA and R183AR R186GRR → A183AA A186GAA) and the N-linked glycosylation site on the N-terminus of PAR4 (Asn56 → Ala56). A HA epitope tag (YFPDVPDYA) 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 μg ml⁻¹), and nonessential amino acids and passaged using 1× 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 μg ml⁻¹). NCTC-2544 cells were passaged using Versene (0.53 mM EDTA in PBS) to avoid trypsin exposure. All cells where 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 μg 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 μCi/well (1 Ci = 37 GBq) of myo-[2-3H]inositol (PerkinElmer Life Sciences) (0.5 μCi/well; 1 Ci = 37 GBq). Cells were pretreated with 20 mM lithium chloride for 30 min prior to agonist treatment (100 μM AYPKF-NH2 for 45 min). Measurement of the accumulation of inositol phosphates was carried out as previously described by Plevin et al. (39).
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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 μl of mowiol (Calbiochem). Cells were visualized using a Nikon TE300-E microscope (Nikon Instruments, New York) using a ×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 × 10⁵ cells per well in 24-well plates pre-coated with 0.1 ml/ml of poly-1-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 an 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 phosphatase substrate solution was prepared by dissolving p-nitrophenyl phosphate substrates 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-PAR4 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-PAR4 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 flask was rinsed in Tris-buffered saline (TBS) and centrifuged at 1,000 × g for 3 min. Supernatant was discarded and the cell pellets were washed 3 times in TBS followed by centrifugation at 1,000 × 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 × 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 × 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 × g. PAR4 expression was detected by Western blotting using antibodies specific for either PAR2 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 × 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 μg/ml of leupeptin, 10 μg/ml of aprotinin, and 1 μg/ml of PMSF). The cell lysate was homogenized using a precooled cell homogenizer (Isobiotec Precision Engineering, Germany, German Patent Office number 202 09 547.9) fitted with a size 10-μm clearance tungsten carbide ballbearing. The homogenate was centrifuged at 500 × 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 × g for 4 h at 4 °C to separate plasma membrane, endosomal, and ER fractions (40). Equal volume fractions (300 μl) were collected and precipitated in 37.5% TCA, incubated on ice for 15 min, and centrifuged at 14,000 × g for 15 min at 4 °C. The cell pellets obtained were resuspended in 2× 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

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a ×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 (FRETc) was calculated using a pixel-by-pixel methodology using the equation FRETc = FRET − (coefficient B × mECFP) − (coefficient A × 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 14-3-3ζ, 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 Na2VO4, 0.1 mM PMSF, 10 mg/ml of aprotinin, 10 mg/ml of leupeptin, and 1% (w/v) Triton X-100). After a 1-hour rotation at 4 °C, the cell lysates were clarified by centrifugation at 13,000 × g for 5 min at 4 °C. Supernatants were transferred to fresh Eppendorf tubes and 50 µl was removed for inputs. The remaining lysate was pre-cleared with 30 µl 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 × g and the pre-cleared lysate was transferred to fresh Eppendorf tubes containing 30 µl 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 × g, then washed with 500 µl of lysis buffer three times and proteins were eluted by incubation with 30 µl of anti-HA peptide (Sigma; 200 µg/ml) for 10 min at room temperature. Eluted proteins were removed and added to 10 µl of 5× 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 ± 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 monoclonal 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 PAR4 mEYFP or PAR4 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 → AXA). Of the possible motifs investigated, only mutation of the R183AR mutant resulted in an 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-localizing 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.
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-, 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.

**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 ×100 Plan Fluor objective. Images were merged to highlight distinct plasma membrane, nucleus, or ER compartments. Scale bars = 10 μm. 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.

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**PAR2 Facilitates Anterograde Transport of PAR4 and Interaction with 14-3-3**—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 (A405 nm) was detected (0.676 ± 0.05, *, p ≤ 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 A405 nm reading (1.016 ± 0.03, ***, p ≤ 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.
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 1–3). 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
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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 \( \zeta \) isoform of the ER export chaperone 14-3-3 was detected in co-immunoprecipitation experiments (Fig. 4D). When PAR2-HA was expressed in the parental NCTC-2544 cells, the ability of 14-3-3\( \zeta \) to interact with PAR2 was negligible. However, when PAR4-HA was expressed in NCTC-PAR2 cells, the ability of 14-3-3\( \zeta \) to interact with PAR4 was clearly shown. Differential interaction of PAR4 with 14-3-3\( \zeta \) was also demonstrated using GST pulldown assays employing GST-14-3-3\( \zeta \) fusion proteins (supplemental Fig. S3). PAR4 binding to GST-14-3-3\( \zeta \) was enhanced when expressed in NCTC-PAR2 cells. In addition, interaction between PAR4 and \( \beta \)-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/endo-somal compartments (Fig. 5C).

**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 \( \mu \)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.
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 proteins.

**FIGURE 4. PAR2 facilitates interaction between PAR4 and 14-3-3 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 proteins.
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→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

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**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 cell surface is shown by co-localization with a plasma membrane marker (white arrows). Cells were visualized using a ×100 Plan Fluor objective. Scale bars = 10 μm. 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.
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 μM agonist; 22.47 ± 0.45 and 100 μM agonist; 21.16 ± 2.62-fold of basal) was substantially greater than observed following expression of PAR4 in NCTC-2544 cells (50 μM agonist; 7.80 ± 0.46 and 100 μM 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 5HT3B 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 5HT3BR, 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 an ER retention motif are known to be shuttled back to the ER via COPI-containing vesicles (29). Our data in Fig. 4 show
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 PAR₄ 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 PAR₄ and in other cell 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 14-3-3 and at the same time loses its association with β-COP1. This competitive interaction is similar to that
observed for an ATP-sensitive potassium (K<sub>ATP</sub>) channel, in which COPI competes with 14-3-3/H9280 and -/H9256 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 PAR<sub>2</sub>/PAR<sub>4</sub> heterodimerization by FRET and co-immunoprecipitation, this suggests that interaction with PAR<sub>2</sub> increases the affinity of PAR<sub>4</sub> for 14-3-3 binding. Thus, we have identified a critical early checkpoint in the secretory pathway involved in the processing of PAR<sub>4</sub> and PAR<sub>2</sub>/PAR<sub>4</sub> heterodimer assembly, involving COPI and 14-3-3<zeta> 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 PAR<sub>2</sub> enhances surface expression with minimal intracellular compartmentalization, interaction at the membrane was negligible. Unlike the ability of class C GPCRs to form stable dimers, the current findings may indicate interaction between PAR<sub>2</sub> and PAR<sub>4</sub> 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 PAR<sub>4</sub> 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 PAR<sub>2</sub> in regulating PAR<sub>4</sub> trafficking in the context of N-linked glycosylation. This was initially detailed by pretreatment of wild type-PAR<sub>4</sub> (or the RAR mutant receptor) with tunicamycin or expression of PAR<sub>4</sub> mutated at the N-linked motif, located at position N<sup>56DS</sup> within the N-terminal domain. Both approaches abolished the presence of the mature PAR<sub>4</sub> form and prevented cell surface expression. Similar effects have previously been shown for the dopamine D<sub>3</sub> 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 (N<sup>56DS</sup>), PAR<sub>4</sub> was unable to undergo complex glycosylation, unless in the presence of PAR<sub>2</sub>. We also identified that N-glycosylation was a critical process in PAR<sub>4</sub>-mediated delivery of PAR<sub>4</sub> to the cell surface. Mutagenesis of the N-linked motif resulted in intracellular retention of PAR<sub>4</sub> despite the presence of PAR<sub>2</sub>.

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 PAR<sub>4</sub>/PAR<sub>4</sub> (57) and PAR<sub>1</sub>/PAR<sub>4</sub> heterodimerization (58), this is the first study to reveal a functional interaction between PAR<sub>2</sub>/PAR<sub>4</sub>. Heterodimer formation between PAR<sub>2</sub> and PAR<sub>4</sub> impacted significantly upon other protein interactions with COPI and 14-3-3<zeta>, and by affecting the subcellular localization of PAR<sub>4</sub> substantially enhanced PAR<sub>4</sub>-mediated signal transduction. PAR<sub>2</sub> and PAR<sub>4</sub> have been previously shown to be dual up-regulated by tumor necrosis factor α, a potent proinflammatory mediator (59, 60). An increase in PAR<sub>2</sub> has been linked to the progression of chronic inflammation (2, 61), with a role for PAR<sub>4</sub> in acute inflammation being implicated in other models of arthritis (62,
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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 heterodimeric interaction 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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