Transactivation of the PAR1-PAR2 Heterodimer by Thrombin Elicits β-Arrestin-mediated Endosomal Signaling*

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Background: Thrombin-cleaved PAR1 reveals a tethered ligand that can transactivate PAR2.

Results: The thrombin-activated PAR1-PAR2 heterodimer displays unique trafficking behavior, recruitment of β-arrestins to endosomes, and signaling responses compared with the receptor protomer.

Conclusion: PAR1 heterodimerization with PAR2 provides additional modes of thrombin-stimulated signaling responses.

Significance: Increased PAR2 expression associated with pathological conditions can modulate thrombin signaling via dimerization with PAR1 and β-arrestin signaling.

Thrombin cleaves the N terminus of PAR1, generating a new N-terminal domain that functions as a tethered ligand that binds intermolecularly to activate PAR2 in trans. The mechanisms that regulate PAR1-PAR2 heterodimer signaling and trafficking are not known. We now report that PAR1 and PAR2 form a heterodimer that exhibits unique trafficking and signaling behaviors compared with receptor protomers. Using bioluminescence resonance energy transfer, immunofluorescence microscopy, co-immunoprecipitation, and cells expressing receptors exogenously and endogenously, we show that PAR1 and PAR2 specifically interact and form stable dimers. Intriguingly, the PAR1-PAR2 heterodimer displays constitutive internalization that is driven by PAR1 C-terminal tail sorting motifs and is a process that enhances dimer formation. Upon thrombin activation, PAR1-PAR2 dimers co-internalize and recruit β-arrestins to endosomes. Remarkably, PAR1-PAR2 heterodimers appear to utilize a distinct interface for β-arrestin interaction compared with receptor protomers. Moreover, thrombin-activated PAR1-PAR2 heterodimers enhance β-arrestin-mediated ERK1/2 activation in the cytoplasm, whereas activated ERK1/2 induced by the thrombin-activated PAR1 protomer redistributes to the nucleus. Thus, the formation of PAR1-PAR2 heterodimers provides additional modes of thrombin-stimulated signaling responses that appear to be distinctly regulated compared with the receptor protomer.

G protein-coupled receptors (GPCRs) are the largest family of signaling receptors expressed in mammalian cells and mediate diverse cellular responses to numerous physiological stimuli. GPCRs are dynamic molecules that assume multiple distinct active conformations that can elicit unique signaling responses (1). This idea has been elegantly illustrated in studies using biased agonists. Biased agonists possess the capacity to bind to the same GPCR but stabilize distinct active conformations that preferentially interact with specific heterotrimeric G proteins or β-arrestins. In addition to biased agonists, it has become increasingly clear that GPCRs self-associate or associate with other GPCRs, resulting in dimeric complex formation that modulates receptor function. A plethora of biochemical and pharmacological evidence supports the idea that class A GPCRs exist as homodimers or heterodimers when expressed exogenously or endogenously in native tissues (2–4). In addition, several high-resolution crystal structures of class A GPCR homodimeric complexes have been solved, including the CXCR4 (5), μ-opioid (6), and κ-opioid (7) receptors. Although heterodimerization of class C GPCRs such as GABAB receptors and taste receptors is essential for expression and function, recent studies indicate that class A GPCRs also form functionally significant heterodimers (4), although in certain cases, the role of class A GPCR dimerization remains elusive. Moreover, GPCR heterodimerization is likely to influence specific receptor active conformations, resulting in unique signaling responses, providing an additional level of signaling complexity that is important to understand for drug development.

Protease-activated receptors (PARs) are a family of GPCRs that are activated by proteolysis. There are four PAR family members: PAR1, PAR2, PAR3, and PAR4. PARs are expressed in the vasculature and are best known to elicit cellular responses to coagulant proteases generated during vascular injury and in thrombotic diseases. The established paradigm of PAR activation involves proteolytic cleavage of the N-terminal domain, unmasking of a new N-terminal sequence that acts as a tethered ligand that binds intramolecularly to the receptor to initiate transmembrane signaling (8). PAR1, PAR3, and PAR4 are cleaved and activated by thrombin, the key effector protease of the coagulation cascade. PAR2 is activated by tryptase, trypsin, and upstream coagulation proteases factors VIIa and Xa, but not by thrombin. Besides the established paradigm of PAR activation, additional modes of PAR activation occur and involve cofactoring between different PARs. PAR1 and PAR3 are high-affinity receptors for thrombin (8–10). When the low-affinity thrombin receptor PAR4 is coexpressed with PAR1 or PAR3, the receptors act as cofactors by facilitating more rapid
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cleavage and activation of PAR4 by thrombin (11). PAR1 can also function as a cofactor for PAR2 activation through an unusual mechanism. Cleavage of the PAR1 N terminus by thrombin generates a tethered ligand domain that can bind in trans to PAR2 to initiate signaling (12). Moreover, a synthetic peptide representing the PAR1 tethered ligand sequence SFLLRN activates PAR2 with a similar potency to its own peptide agonist SLIGKV (13), further supporting the idea that PAR1 transactivates PAR2 through donation of its tethered ligand domain.

O’Brien et al. (12) were the first to show that the PAR1 tethered ligand domain is capable of transactivating PAR2. Numerous other studies have also demonstrated a function for PAR2 in thrombin-induced signaling responses (14, 15). Interestingly, Kaneider et al. (16) showed that PAR2 associates with PAR1 in endothelial cells during late stages of sepsis and switches thrombin signaling from barrier-disruptive to barrier-protective. In other work, PAR2 expression was shown to be necessary for PAR1-induced hyperplasia in vascular smooth muscle cells (17). The ability of PAR1 to transactivate PAR2 would necessitate that the two receptors be in close proximity, likely in the form of a heterodimer. Two previous studies have suggested that PAR1 and PAR2 associate (16, 17). However, the mechanisms that govern PAR1-PAR2 heterodimer formation, trafficking, and signaling have not been investigated.

Here, we demonstrate that PAR1 and PAR2 form stable dimers that localize to the cell surface and endocytic vesicles. Intriguingly, the PAR1 endocytic machinery drives PAR2 trafficking and appears to regulate PAR1-PAR2 heterodimer stability. We further demonstrate that thrombin activation of the PAR1-PAR2 heterodimer results in β-arrestin recruitment through an interface that is different from that utilized by receptor protomers. Remarkably, β-arrestins co-internalize with the thrombin-activated PAR1-PAR2 dimer and mediate ERK1/2 signaling in the cytosol while limiting nuclear ERK1/2 activation. These results indicate that the PAR1-PAR2 dimer utilizes a unique β-arrestin-binding interface and elicits signaling responses that are distinct from those induced by the PAR1 protomer.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The PAR2-specific peptide agonist SLIGKV was synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography at the Tufts University Core Facility (Boston, MA). Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Tumor necrosis factor-α was from PeproTech, Inc. (Rocky Hill, NJ). Rabbit anti-FLAG polyclonal antibody, mouse anti-FLAG monoclonal antibodies M1 and M2, peroxidase-conjugated mouse anti-FLAG monoclonal antibody M2, and mouse anti-β-actin antibody were purchased from Sigma-Aldrich. Mouse anti-PAR1 antibody WEDE was from Beckman Coulter (Fullerton, CA). Rabbit anti-PAR1 polyclonal antibody C5433 was described previously (18), and anti-PAR2 polyclonal antibody was from Dr. Wolfram Ruf (The Scripps Research Institute). Rabbit anti-β-arrestin polyclonal antibody was provided by Dr. Jeffrey Benovic (Thomas Jefferson University). Anti-μ2-adaptin AP50 antibody was obtained from BD Biosciences. Anti-β-arrestin antibody AICT was generously provided by Dr. Robert Lefkowitz (Duke University Medical Center). Anti-p44/42 ERK1/2 and anti-phospho-p44/42 ERK1/2 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-GAPDH and anti-p84 antibodies were from Gene’Tex (Irvine, CA). HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Bio-Rad. HRP-conjugated mouse anti-HA antibody was from Roche Applied Science. Goat anti-mouse and goat anti-rabbit antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 were from Invitrogen.

Cell Lines and cDNAs—COS-7 and HeLa cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. HeLa cells stably expressing various receptors were grown in complete DMEM supplemented with 250 μg/ml hygromycin. Human umbilical vein endothelial cell-derived EA.hy926 cells were grown and maintained as described (19).

The cDNA plasmids encoding N-terminally FLAG-tagged human wild-type PAR1, N-terminally FLAG-tagged PAR2, and C-terminal tail truncation mutants were described previously (20, 21). The N-terminally HA-tagged PAR2 construct was generated and cloned into the pcDNA3.1 vector. The PAR1 R41A mutant was generated by QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) and confirmed by dideoxy sequencing (Regenon Inc., San Diego, CA). β-Arrestin-1-GFP and β-arrestin-2-GFP were gifts from Dr. Marc Caron (Duke University Medical Center). Full-length PAR2 containing Renilla luciferase (Rluc) fused at the C terminus and full-length PAR1 with YFP at the C terminus were cloned into the pRk6 vector and generously provided by Dr. Jean-Philippe Pin (Montpellier University, Montpellier, France).

Cell Transfections—Cells were transiently transfected with various cDNA plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. COS-7 cells were transfected with plasmids using FuGENE 6 (Roche Applied Science) as recommended by the manufacturer for bioluminescence resonance energy transfer (BRET) assays. HeLa cells were transfected with 100 nm β-arrestin-1 siRNA (5′-CAUAGAAGCGUGACACAAAU-3′), 100 nm β-arrestin-2 siRNA (5′-GGACCGCAAAGUUCUGUGG-3′), 100 nm μ2-adaptin siRNA (5′-GUGGAUGCCUUUGCUGGUCA-3′), or 100 nm nonspecific siRNA (5′-CUACCCCAGGAGGCGACC-3′) using OligoFectamine (Invitrogen) and examined after 72 h.

Immunoprecipitations—Cells were plated in 6-well dishes at 5 × 105 cells/well and grown for 48 h. Cells were placed on ice, washed with PBS, and lysed with Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 1% Triton X-100) supplemented with protease inhibitors. Cell lysates were cleared by centrifugation, protein concentrations were determined using the BCA assay (Thermo Fisher Scientific), and equal amounts of lysates were immunoprecipitated with various antibodies. Immunoprecipitates were washed three times with lysis buffer, and proteins were eluted in 50 μl of 2X Laemmli buffer containing 0.2 mM DTT. Cell lysates or immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the appropriate antibodies. Membranes
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were developed by chemiluminescence, and band intensities were quantified by densitometry using ImageJ.

**Immunofluorescence Confocal Microscopy**—Cells were plated at 0.6 × 10⁵ cells on fibronectin-coated glass coverslips and grown overnight. Cells were transfected, grown for 24 h, either left untreated or treated with agonists, fixed in 4% paraformaldehyde, permeabilized with methanol, and then immunostained with various antibodies as described (22). Immunostaining of endogenous β-arrestins was performed essentially as described (23). Endothelial cells were counterstained with DAPI (Sigma-Aldrich). Coverslips were mounted in FluorSave reagent (Calbiochem) and imaged by confocal microscopy. Images were acquired using an Olympus IX81 DSU spinning disk confocal microscope fitted with a Plan Apo 60× oil objective and a Hamamatsu ORCA-ER digital camera. Fluorescent images of x-y 0.28-μm sections were collected sequentially using Intelligent Imaging Innovations SlideBook 4.2 software. Internalized receptors were quantified by generating masks excluding the plasma membrane and counting objects with fluorescence intensity above a threshold with SlideBook 4.2.

**ELISAs**—Expression of FLAG-PAR1 or HA-PAR2 at the cell surface was quantified by ELISA. Briefly, cells from 24-well plates were washed with PBS and fixed with 4% paraformaldehyde for 5 min at 4 °C. Cells were washed with PBS and incubated with primary antibody for 1 h at room temperature, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Cells were washed and incubated with 2,2′-azino-bis(3-ethylbenzthiazole-6-sulfonic acid) (Thermo Fisher Scientific) at room temperature. An aliquot was removed, and optical density (O.D.) was determined at 405 nm using a Molecular Devices SpectraMax Plus microplate reader.

**BRET Assays**—COS-7 cells were transfected for 48 h, detached with Cellstripper™ (Mediatech), washed three times with PBS, and resuspended in PBS containing 0.5 mM MgCl₂ and 0.1% glucose at a density of 5 × 10⁵ cells/ml. An aliquot (80 μl) of cells was added to a 96-well microplate in triplicate, and 10 μl of coelenterazine h substrate was added at a final concentration of 5 μM. After an 8-min delay, signals were determined with a TriStar LB 941 plate reader (Berthold Biotechnologies) using two filter settings (480 nm for Rluc and 530 nm for YFP). The BRET ratio was calculated as emission at 530 nm/emission at 480 nm, and net BRET was determined by subtracting the background BRET ratio (BRET ratio from cells expressing the Rluc construct only) using MicroWIN 2000 software (Berthold Technologies). The YFP signal was determined by excitation at 485 nm, and emission was detected at 535 nm. Total luminescence was measured by integrating the signal for 1 s/well without filter selection. Data were fitted with nonlinear regression using Prism software (GraphPad Software, San Diego, CA).

**ERK1/2 Activation Assays**—Cells plated in 24-well dishes were serum-starved overnight and treated with 10 nM thrombin for various times at 37 °C. Cells were lysed in 2× Laemmli buffer, sheared five times, resolved by SDS-PAGE, transferred to membranes, and immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. Membranes were developed using chemiluminescence and quantified with ImageJ. The phospho-ERK1/2 signal was normalized to the total ERK1/2 control and is expressed as the -fold increase over the 0-min control.

**Subcellular Fractionation**—Cells plated at 5.0 × 10⁶ cells/well in 6-well plates were grown overnight, and subcellular fractions were isolated as described previously (24). Cells were washed and resuspended in 500 μl of hypotonic buffer (20 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂) supplemented with protease inhibitors. Nonidet P-40 detergent was then added to a final concentration of 0.5%, and cells were incubated for 10 min with gentle rocking and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was collected and used as the cytoplasmic fraction. The pellet was washed once with hypotonic buffer without Nonidet P-40, resuspended, sonicated, and used as the nuclear fraction. Protein concentrations were determined by the BCA assay, and equivalent amounts of protein lysates were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with various antibodies.

**Data Analysis**—All data were analyzed using Prism 4.0 software. Statistical analysis was determined by performing Student’s t test or two-way analysis of variance with the Bonferroni post-test.

**RESULTS**

**PAR1-PAR2 Heterodimer Formation**—To investigate whether PAR1 and PAR2 have the capacity to physically interact, we examined heterodimer formation in living cells using BRET. BRET assays were performed utilizing full-length PAR2 and PAR1 containing Rluc or YFP fused in-frame to the C terminus, respectively. Both PAR2-Rluc and PAR1-YFP trafficked to the cell surface when expressed in COS-7 cells (Fig. 1A) and signaled normally (25). COS-7 cells were transfected with a constant amount of PAR2-Rluc and an increasing amount of PAR1-YFP, and the net BRET signal was quantified. We observed a hyperbolic increase in the BRET signal as the ratio of PAR1-YFP to PAR2-Rluc was increased (Fig. 1A), suggesting a specific interaction between these receptors. We also examined PAR1 and PAR2 association by co-immunoprecipitation. COS-7 cells were transfected with N-terminally FLAG-tagged PAR1 and/or N-terminally HA-tagged PAR2. Cells were lysed and immunoprecipitated with anti-PAR1 antibody WEDE, and the presence of HA-PAR2 in the immunocomplex was detected with HRP-conjugated anti-HA antibody. In cells coexpressing both receptors, HA-PAR2 was detected in co-immunoprecipitations using anti-PAR1 antibody WEDE, but not in co-immunoprecipitations with the IgG isotype control (Fig. 1B, lanes 4 and 5). These findings suggest that PAR1 and PAR2 form dimers at steady state (Fig. 1B). Immunofluorescence confocal microscopy studies indicated that PAR1 and PAR2 co-localized to the cell surface. HeLa cells were cotransfected with FLAG-PAR1 and HA-PAR2 and incubated with anti-FLAG and anti-HA antibodies for 1 h at 4 °C. Under these conditions, only the cell surface receptor cohorts were labeled with antibody. Cells were then fixed, processed, and imaged by confocal microscopy. FLAG-PAR1 and HA-PAR2 localized to the plasma membrane and displayed significant co-localization as indicated by the yellow color in the merged image (Fig. 1C). Together, these data suggest that PAR1-PAR2 heterodimers are formed and expressed on the plasma membrane.
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PAR1 Drives Heterodimer Constitutive Internalization—We next examined the trafficking behavior of the PAR1-PAR2 heterodimer. In HeLa cells expressing only the individual receptors, FLAG-tagged PAR1 localized predominantly to the cell surface at 0 min and exhibited constitutive internalization after 30 min of incubation at 37 °C in the absence of agonist (Fig. 2A). A further increase in PAR1 internalization was observed following a 10-min incubation with thrombin (Fig. 2A), as the majority of the receptor localized to endosomes after agonist exposure. Thus, PAR1 displays constitutive and agonist-induced internalization in HeLa cells, as we reported previously (18, 26). In contrast, incubation with neither thrombin nor medium alone for 30 min at 37 °C was sufficient to drive HA-PAR2 internalization (Fig. 2B), whereas a 30-min incubation with the PAR2-selective peptide agonist SLIGKV caused robust receptor internalization (Fig. 2B). However, the trafficking behavior of PAR2 switched upon coexpression with PAR1. Cell surface FLAG-PAR1 and HA-PAR2 were labeled with receptor-specific antibodies for 1 h at 4 °C and warmed to 37 °C, and constitutive internalization was allowed to proceed for 30 min. As expected, a fraction of PAR1 redistributed from the cell surface to endocytic vesicles at steady state (Fig. 2C). Surprisingly, however, a subpopulation of PAR2 also re-localized to the same endocytic vesicles containing PAR1 as indicated by the yellow color in the merged image (Fig. 2C). These findings suggest that constitutive internalization of the PAR1-PAR2 heterodimer is driven by the trafficking behavior of PAR1.

To further examine the possibility that PAR1 directs constitutive trafficking of the heterodimer, we utilized the PAR1 AKKAA mutant, which is defective in AP-2 ( adaptor protein complex-2) binding and constitutive internalization (18), and the ubiquitination-deficient PAR1 0K mutant, which exhibits enhanced constitutive internalization mediated by AP-2 (27). Cells were prelabeled with receptor-specific antibodies, washed, and incubated for either 0 or 30 min at 37 °C. The PAR1 AKKAA mutant and PAR2 were present at the cell surface at 0 min and remained localized on the cell surface even after prolonged incubation for 30 min at 37 °C (Fig. 2D), consistent with defective PAR1 AKKAA constitutive internalization. These data also indicate that antibody labeling does not induce PAR1 or PAR2 internalization. Conversely, the PAR1 0K mutant exhibited enhanced constitutive internalization and re-localized mainly to endocytic vesicles that immunostained for both PAR1 and PAR2 (Fig. 2E). Together, these findings indicate that constitutive internalization of the PAR1-PAR2 heterodimer is mediated by signals that regulate PAR1 trafficking.

PAR1-PAR2 Association Is Regulated by AP-2—To determine whether PAR1 mutants also affect PAR1-PAR2 association, we examined receptor interaction by co-immunoprecipitation. HeLa cells stably expressing similar amounts of FLAG-tagged wild-type PAR1, PAR1 0K, or PAR1 AKKAA were transiently transfected with HA-tagged PAR2. Cells were lysed and immunoprecipitated with anti-PAR1 antibody WEDE, and the association of HA-PAR2 was detected by immunoblotting. Although the amounts of immunoprecipitated wild-type PAR1 and mutants were similar (Fig. 3A, middle panels), the amount of co-association PAR2 was significantly enhanced by ∼7-fold in immunoprecipitates from PAR1 0K-expressing cells compared with cells expressing the wild-type receptor (Fig. 3A). In contrast, a minimal amount of PAR2 was detected in immunoprecipitates from cells expressing PAR1 AKKAA (Fig. 3A), a receptor mutant that cannot bind AP-2 (18). These findings suggest
that AP-2 interaction with the PAR1 C-terminal tail is important not only for PAR1-PAR2 constitutive internalization but may also have a function in stabilization of the heterodimer. To examine the role of AP-2 in PAR1-PAR2 heterodimer formation, we used siRNA targeting the μ2-adaptin subunit of AP-2, which ablates AP-2 expression and function in HeLa cells (18). HeLa cells coexpressing PAR1 and PAR2 transfected with μ2-adaptin siRNA showed loss of μ2-adaptin expression compared with nonspecific siRNA-transfected control cells (Fig. 3B, lower panels). The effect of AP-2 knockdown on PAR1-PAR2 heterodimer formation was then examined by co-immunoprecipitation. In cells lacking AP-2 expression, the capacity of wild-type PAR1 to associate with PAR2 was significantly reduced by ~50% (Fig. 3B). Similarly, the robust association of the PAR1 0K mutant with PAR2 was also substantially decreased by ~65% in AP-2-deficient cells compared with nonspecific siRNA-transfected control cells (Fig. 3B). Together, these findings suggest a function for AP-2 in PAR1-PAR2 dimer stabilization. Whether AP-2 functions directly to stabilize the PAR1-PAR2 dimer or indirectly by facilitating PAR1-PAR2 dimerization through another mechanism is not known.

**Thrombin Induces PAR1-PAR2 Heterodimer Conformational Changes and Internalization**—Thrombin binds to and cleaves the N terminus of PAR1, generating a new N-terminal domain...
that functions as a tethered ligand by binding intramolecularly to activate PAR1 or intermolecularly in trans to activate PAR2 (12). To test whether thrombin activation of PAR1 results in conformational changes of the PAR1-PAR2 heterodimeric complex, we performed BRET saturation assays. COS-7 cells were transfected with a fixed amount of PAR2-Rluc and an increasing amount of PAR1-YFP and treated with or without 10 nM thrombin for 10 min at 37 °C. The net BRET data (mean ± S.D., n = 3) were calculated and are representative of three independent experiments. HeLa cells expressing FLAG-PAR1 and HA-PAR2 were prelabeled with anti-PAR1 and anti-HA antibodies for 1 h at 4 °C. Cells were incubated with or without 10 nM thrombin at 37 °C for 10 min, fixed, processed, immunostained and imaged by confocal microscopy. The co-localization of PAR1 (red) and PAR2 (green) is revealed by the yellow color in the merged images. Scale bars = 10 μm. C, PAR2 internalization was quantitated and is expressed as the number of internal punctae (mean ± S.D., n = 30) from three independent experiments. The difference in the number of punctae detected in control cells versus thrombin-treated cells was significantly different as determined by Student’s t test. ***, p < 0.001.

**Figure 3. PAR1 association with PAR2 is mediated by AP-2.** A, HeLa cells expressing WT PAR1, PAR1 OK, or PAR1 AKKAA and HA-PAR2 were lysed, immunoprecipitated (IP) with anti-PAR1 antibody WEDE or IgG, resolved by SDS-PAGE, and immunoblotted (IB) as indicated. Cell lysates were immunoprecipitated with anti-HA or anti-actin antibody. The data (mean ± S.D.) are expressed as the fraction of PAR2 associated with wild-type PAR1 and are statistically significant as determined by Student’s t test. *, p < 0.05; **, p < 0.01 (n = 3).

**Figure 4. Thrombin modulates PAR1-PAR2 heterodimer conformation and internalization.** A, COS-7 cells expressing PAR2-Rluc and increasing amounts of PAR1-YFP were left untreated (control) or treated with 10 nM thrombin for 10 min at 37 °C. The net BRET data (mean ± S.D., n = 3) were calculated and are representative of three independent experiments. B, HeLa cells expressing FLAG-PAR1 and HA-PAR2 were prelabeled with anti-PAR1 and anti-HA antibodies for 1 h at 4 °C. Cells were incubated with or without 10 nM thrombin at 37 °C for 10 min, fixed, processed, immunostained and imaged by confocal microscopy. The co-localization of PAR1 (red) and PAR2 (green) is revealed by the yellow color in the merged images. Scale bars = 10 μm. C, PAR2 internalization was quantitated and is expressed as the number of internal punctae (mean ± S.D., n = 30) from three independent experiments. The difference in the number of punctae detected in control cells versus thrombin-treated cells was significantly different as determined by Student’s t test. ***, p < 0.001.

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**Figure 3.** PAR1 association with PAR2 is mediated by AP-2. A, HeLa cells expressing WT PAR1, PAR1 OK, or PAR1 AKKAA and HA-PAR2 were lysed, immunoprecipitated (IP) with anti-PAR1 antibody WEDE or IgG, resolved by SDS-PAGE, and immunoblotted (IB) as indicated. Cell lysates were immunoprecipitated with anti-HA or anti-actin antibody. The data (mean ± S.D.) are expressed as the fraction of PAR2 associated with wild-type PAR1 and are statistically significant as determined by Student’s t test. *, p < 0.05; **, p < 0.01 (n = 3).

**Figure 4.** Thrombin modulates PAR1-PAR2 heterodimer conformation and internalization. A, COS-7 cells expressing PAR2-Rluc and increasing amounts of PAR1-YFP were left untreated (control) or treated with 10 nM thrombin for 10 min at 37 °C. The net BRET data (mean ± S.D., n = 3) were calculated and are representative of three independent experiments. B, HeLa cells expressing FLAG-PAR1 and HA-PAR2 were prelabeled with anti-PAR1 and anti-HA antibodies for 1 h at 4 °C. Cells were incubated with or without 10 nM thrombin at 37 °C for 10 min, fixed, processed, immunostained and imaged by confocal microscopy. The co-localization of PAR1 (red) and PAR2 (green) is revealed by the yellow color in the merged images. Scale bars = 10 μm. C, PAR2 internalization was quantitated and is expressed as the number of internal punctae (mean ± S.D., n = 30) from three independent experiments. The difference in the number of punctae detected in control cells versus thrombin-treated cells was significantly different as determined by Student’s t test. ***, p < 0.001.

β-Arrestin Recruitment to Thrombin-activated PAR1-PAR2 Heterodimers—PAR1 and PAR2 display marked differences in the duration of β-arrestin recruitment. We previously showed that activation of PAR1 results in transient β-arrestin association at the plasma membrane (28), whereas activated PAR2 and β-arrestin form a stable complex that co-internalizes to endocytic vesicles (21, 29). Consistent with these studies, thrombin-activated PAR1 failed to remain associated with β-arrestin-2-GFP on endosomes in HeLa cells (Fig. 5A), whereas PAR2 activation resulted in substantial co-internalization and co-localization with β-arrestin-2-GFP on endocytic vesicles (Fig. 5B). Whether the thrombin-activated PAR1-PAR2 heterodimer recruits β-arrestins is not known and was next inves-
tigated. Cells coexpressing PAR1, PAR2, and β-arrestin-2-GFP were labeled with receptor-specific antibodies and either left untreated or treated with thrombin for 10 min at 37 °C. In the absence of agonist, β-arrestin-2-GFP remained diffusively distributed throughout the cytoplasm and did not co-localize with the PAR1-PAR2 heterodimer at the plasma membrane or endocytic vesicles (Fig. 5C). In striking contrast, thrombin-stimulated a marked redistribution of β-arrestin-2-GFP to endosomes that co-localized with PAR1 and PAR2 as indicated by the white color in the merged image (Fig. 5C). Similar results were obtained with β-arrestin-1-GFP (data not shown).

To confirm β-arrestin recruitment, we examined thrombin-induced PAR1-PAR2 co-association with endogenous β-arrestins by co-immunoprecipitation. HeLa cells expressing PAR1 alone or together with PAR2 were treated with thrombin for various times at 37 °C. Cells were lysed, PAR1 was immunoprecipitated, and the presence of β-arrestins was detected using anti-β-arrestin polyclonal antibody A1CT (30). In cells expressing PAR1, β-arrestin association with the receptor was not significantly increased following thrombin stimulation compared with untreated control cells (Fig. 6, lanes 1–4). In contrast, a significant ~1.5 -fold increase in endogenous β-arrestin association with the PAR1-PAR2 heterodimeric complex was observed following 10 min of thrombin stimulation (Fig. 6, lanes 5–8). These findings are consistent with the time course of thrombin-induced β-arrestin-2-GFP co-localization with the PAR1-PAR2 dimer on endosomes (Fig. 5C). Taken together, these data suggest that the thrombin-activated PAR1-PAR2 heterodimer forms a stable complex with β-arrestins that co-internalizes to endosomes.
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PAR1-PAR2 Heterodimer Recruits β-Arrestin through a Distinct Interface—We next examined the mechanism by which thrombin activation of the PAR1-PAR2 heterodimer recruits β-arrestin. To examine the importance of the thrombin-generated N-terminal tethered ligand domain of PAR1, we coexpressed a cleavage-defective PAR1 mutant in which Arg-41 was mutated to alanine, rendering a receptor mutant that can bind thrombin but is not cleaved by the protease (31). In the absence of agonist stimulation, PAR1 R41A and PAR2 localized to the cell surface and endocytic vesicles, whereas β-arrestin-2-GFP remained diffusively distributed (Fig. 7). Incubation with thrombin for 10 min at 37 °C failed to induce a marked change in PAR1 R41A, PAR2, or β-arrestin-2-GFP subcellular localization (Fig. 7), indicating that thrombin cleavage and generation of the N-terminal tethered ligand domain are necessary for recruitment of β-arrestin-2-GFP to the PAR1-PAR2 heterodimer.

Activation of PAR1 and PAR2 results in C-terminal tail phosphorylation and β-arrestin recruitment, although the receptors exhibit differences in β-arrestin-binding stability (21, 28, 32). To define the function of the PAR1 and PAR2 C-terminal tail domains in directing PAR1-PAR2 heterodimer recruitment of β-arrestins, we utilized C-terminal tail truncation mutants. The PAR2 C-terminal tail mutant K368Z (Z indicates stop codon) showed substantial internalization after 30 min of agonist stimulation, but the mutant receptor failed to recruit β-arrestins to endosomes in COS-7 cells compared with wild-type PAR2 (Fig. 8A), consistent with our previous study (21). Incubation with thrombin for 10 min at 37 °C induced PAR1-PAR2 K368Z mutant co-internalization (Fig. 8B), indicating that the receptors form dimers. Surprisingly, however, the PAR1-PAR2 K368Z mutant retained the capacity to recruit β-arrestins (Fig. 8B), suggesting that the C-terminal tail domain of PAR2 is not required for β-arrestin recruitment to the activated PAR1-PAR2 heterodimer. We next examined the PAR1 C-terminal tail truncation mutant Y397Z, which is defective in agonist-induced phosphorylation and internalization (33). Thrombin activation of the PAR1 Y397Z mutant failed to induce internalization of the PAR1 Y397Z-PAR2 heterodimer or β-arrestin recruitment (Fig. 8C), although PAR1 Y397Z and PAR2
PAR1-PAR2-stimulated ERK1/2 Activation Occurs via β-Arrestins—The ubiquitously expressed β-arrestins are multifunctional adaptors that control GPCR desensitization, internalization, and function as scaffolds that facilitate ERK1/2 activation (34). To define the function of β-arrestin recruitment to the thrombin-activated PAR1-PAR2 heterodimer, we examined ERK1/2 signaling. HeLa cells expressing PAR1 alone or together with PAR2 were treated with 10 nM thrombin for various times at 37 °C, and ERK1/2 activation was assessed using phosphospecific antibodies. HeLa cells expressing PAR1 alone or together with PAR2 showed a comparable amount of PAR1 on the cell surface (Fig. 9A, inset). In cells expressing PAR1-PAR2, thrombin induced a significantly greater ~10-fold increase in ERK1/2 phosphorylation compared with cells expressing PAR1 alone, which exhibited an ~5-fold increase in ERK1/2 phosphorylation (Fig. 9A). To confirm that enhanced ERK1/2 activation elicited by the thrombin-activated PAR1-PAR2 heterodimer is mediated by β-arrestins, we utilized siRNAs to deplete cells of β-arrestin expression. Thrombin activation of the PAR1-PAR2 heterodimer caused a significantly greater increase in ERK1/2 activation detected at 5 min in siRNA-transfected control cells compared with cells deficient in β-arrestin expression (Fig. 9B). We next examined the distribution of activated ERK1/2 in the cytoplasm versus the nucleus because the capacity of β-arrestins to function as scaffolds for ERK1/2 activation prolongs ERK1/2 activation in the cytoplasm and limits ERK1/2 nuclear translocation (35). The separation of cytosolic and nuclear fractions was verified by immunoblotting using antibodies directed against the p84 nuclear matrix protein and GAPDH, a cytosolic marker (Fig. 9C, lower panels). In HeLa cells expressing PAR1, thrombin induced a marked increase in ERK1/2 phosphorylation that was detected in the cytoplasm and nucleus at 5 min, remained elevated for 10 min, and then returned to basal levels after 30 min (Fig. 9C). As expected, thrombin stimulated persistent cytoplasmic ERK1/2 activation in cells expressing PAR1-PAR2 that remained elevated even after a 30-min thrombin incubation (Fig. 9C). In contrast, thrombin activation of the PAR1-PAR2 heterodimer caused a modest and transient translocation of ERK1/2 activation to the nucleus that was only detected at 5 min (Fig. 9C), consistent with prolonged ERK1/2 activation in the cytoplasm. These findings suggest that PAR2 potentiates and prolongs thrombin-induced ERK1/2 activation in the cytoplasm through dimerization with PAR1 and recruitment of β-arrestins to endosomes.

FIGURE 9. Thrombin-activated PAR1-PAR2 heterodimer exhibits enhanced cytosolic ERK1/2 signaling. A, HeLa cells expressing PAR1 alone or with PAR2 were incubated with 10 nM α-thrombin (αTh) for the indicated times at 37 °C and lysed. Cell lysates were immunoblotted with anti-phospho-ERK1/2 (pERK1/2) and anti-ERK1/2 antibodies. The data (mean ± S.D., n = 3) are expressed as the -fold increase in phospho-ERK1/2 over the 0-min control and were significantly different as determined by two-way analysis of variance. **, p < 0.01. Inset, HeLa cells expressing FLAG-PAR1 alone or with HA-PAR2 were fixed, and the amount of receptor on the cell surface was determined by ELISA. B, HeLa cells expressing PAR1-PAR2 were transfected with nonspecific (ns) siRNA or siRNA targeting both β-arrestin-1 and β-arrestin-2. Cells were then stimulated with 10 nM thrombin for the indicated times at 37 °C, lysed, and immunoblotted using anti-phospho-ERK1/2, anti-ERK1/2, and anti-β-arrestin antibodies. The data (mean ± S.D., n = 3) are expressed as the -fold increase in phospho-ERK1/2 over the 0-min control and were significantly different as determined by two-way analysis of variance. *, p < 0.05. C, HeLa cells expressing PAR1 or PAR1-PAR2 were incubated with 10 nM thrombin for the indicated times at 37 °C. Cytosolic and nuclear fractions were prepared and immunoblotted with various antibodies as indicated. These data are representative of three independent experiments.

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To examine whether endogenous PAR1 and PAR2 form heterodimers, we used cultured human endothelial EA.hy926 cells. Human endothelial cells express PAR1 and a low abundance of PAR2. Consistent with previous studies (36), we found that TNF-α increased the surface expression of PAR2 from 0.212 ± 0.036 to 0.326 ± 0.015 O.D. units as detected by ELISA without affecting PAR1 expression. In control cells, incubation with thrombin for 30 min resulted in substantial internalization of PAR1, but not PAR2 (Fig. 10A, upper panels). However, endothelial cells pretreated with TNF-α showed substantial co-internalization of PAR2 with PAR1 following thrombin stimulation (Fig. 10A, lower panels). In addition, thrombin induced β-arrestin recruitment to endocytic vesicles containing PAR1 in cells with increased PAR2 expression, but not in control cells expressing a low abundance of PAR2 (Fig. 10B), suggesting that PAR2 expression enhances thrombin-stimulated β-arrestin recruitment to PAR1. We next examined thrombin-stimulated ERK1/2 signaling in endothelial cells expressing a low abundance of PAR2 versus cells with increased PAR2 expression. In cells pretreated with TNF-α, thrombin caused a significant increase in ERK1/2 activation compared with untreated control cells (Fig. 10C). Together, these findings suggest that increased PAR2 expression increases dimer formation with PAR1, resulting in β-arrestin recruitment to endosomes and enhanced ERK1/2 signaling.

**DISCUSSION**

PAR1 has been shown to transactivate PAR2 by providing its N-terminal tethered ligand domain (12), which can act as a full agonist for PAR2 activation (13). In addition, PAR1 transactivation of PAR2 appears to occur in vivo and functions in late stages of sepsis and in hyperplastic responses to arterial injury in mouse models (16, 17). The ability of PAR1 to transactivate PAR2 would necessitate that the two receptors be in close proximity, likely in the form of a heterodimer. However, the mechanisms that control PAR1-PAR2 dimer formation, trafficking, and signaling have not been explored. In this study, we have shown that PAR1 and PAR2 form a stable heterodimer that displays distinct trafficking and signaling properties compared with the PAR1 protomer. We found that PAR1 drives the trafficking behavior of PAR2, but not vice versa. AP-2 is the critical mediator of PAR1 constitutive internalization (18). The PAR1

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**FIGURE 10.** Thrombin-induced endogenous PAR1-PAR2 heterodimer internalization, β-arrestin recruitment, and enhanced ERK1/2 activation. A, human cultured EA.hy926 endothelial cells were treated with 10 ng/ml TNF-α for 18 h, which increased PAR2 surface expression from 0.212 ± 0.036 to 0.326 ± 0.015 O.D. units (n = 3) at 405 nm as detected by ELISA. Control and TNF-α-pretreated cells were prelabeled with anti-PAR2 antibody for 1 h at 4 °C and stimulated with or without 10 nM thrombin for 30 min at 37 °C. Cells were fixed, processed, immunostained, and imaged by confocal microscopy. The co-localization of PAR1 (green) and PAR2 (red) is revealed by the yellow color in the merged images. Cell nuclei were counterstained with DAPI and are shown in blue. Scale bars = 10 μm. B, control and TNF-α-pretreated cells were incubated with or without 10 nM thrombin for 10 min at 37 °C. Cells were fixed, processed, immunostained for PAR1 (green) and β-arrestins (red), and imaged as described by confocal microscopy. The inset is a magnification of the boxed area. Scale bars = 10 μm. Images are representative of at least 30 cells examined in three independent experiments. βAr1, β-arrestin-1.C, control and TNF-α-pretreated cells were incubated with or without 10 nM α-thrombin (αTh) for various times, lysed, processed, and immunoblotted for phospho-ERK1/2 (pERK1/2) and total ERK1/2. The data (mean ± S.D., n = 3) are expressed as the fold increase in phospho-ERK1/2 over the 0-min control after normalization to total ERK1/2 and were significantly different as determined by two-way analysis of variance. *, p < 0.05.
AKKAA mutant cannot bind AP-2 and exhibits defective constitutive constitution. Coexpression of PAR1 AKKAA with PAR2 impaired heterodimer formation and constitutive internalization. Our studies further show that thrombin activation of the PAR1-PAR2 heterodimer results in a conformational change and requires the PAR1 tethered ligand domain to induce PAR1-PAR2 co-internalization and β-arrestin recruitment, an event that is not observed with the activated PAR1 protomer. Interestingly, β-arrestin recruitment to the thrombin-activated PAR1-PAR2 heterodimer occurs through a distinct interface compared with the PAR2 protomer. Consequently, thrombin activation of the PAR1-PAR2 dimer elicits a signaling response that is different from PAR1 protomer. Thus, PAR1-PAR2 dimer activation by thrombin results in biased signaling elicited by β-arrestins.

In the absence of ligand stimulation, GPCRs localize to the cell surface. However, some GPCRs constitutively internalize and recycle (37), whereas other GPCRs reside predominantly in intracellular compartments at steady state (38, 39). The distinct mechanisms that control the different trafficking behaviors of most GPCRs remain poorly understood. Previous studies have shown that a subpopulation of PAR2 is localized to the Golgi and trafficked to the cell surface following agonist stimulation (40, 41). Once at the cell surface, the cohort of plasma membrane-localized PAR2 is relatively stable and does not internalize constitutively (32). However, we found that coexpression of PAR1 switches the trafficking behavior of PAR2 from stable cell surface localization to constitutive co-internalization with PAR1. At steady state, the PAR1-PAR2 heterodimer localizes to both the plasma membrane and endocytic vesicles. These findings are different from a previous study showing that PAR1-PAR2 dimers were present mainly in the cytoplasm and re-localized to the plasma membrane of endothelial cells only after endotoxin treatment (16). These discrepancies could be due to differences in cell types or to receptor construct variations because our studies employed full-length PARs, and the previous work used receptors lacking the C-terminal tail domain.

We have further demonstrated that PAR1-PAR2 heterodimer constitutive internalization is mediated by the PAR1 C-terminal tail distal tyrosine motif and AP-2, the endocytic machinery that drives PAR1 constitutive internalization (16). Interestingly, AP-2 not only mediates PAR1-PAR2 heterodimer internalization it also regulates the stability of the heterodimer. We found that PAR1-PAR2 interaction is diminished in cells lacking AP-2 and in cells coexpressing the PAR1 AKKAA mutant, which cannot bind AP-2. It is possible that AP-2 directly links PAR1 and PAR2, facilitating dimer formation, although PAR2 lacks canonical AP-2-binding sites. However, PAR2 interacts with β-arrestins, which can bind to AP-2 and may facilitate indirect interaction with the receptor, but this has not been tested. The other possibility is that AP-2 affects dimer formation indirectly by facilitating PAR1-PAR2 heterodimer internalization to endosomes, which could enhance dimer stability, but how this might occur mechanistically is not known.

We have also shown in this study that PAR1-PAR2 association leads to a distinct capacity of thrombin to induce β-arrestin recruitment via a unique dimer interface. We and others have shown that PAR1 and PAR2 differentially interact with β-arrestins. Activation of PAR1 results in transient recruitment of β-arrestin (28, 42), whereas activated PAR2 forms a stable complex with β-arrestins that co-internalizes to endocytic vesicles and functions as a scaffold to promote ERK1/2 activation (21, 29). Here, we report that thrombin activation of the PAR1-PAR2 heterodimer results in β-arrestin recruitment and co-internalization to endosomes in cells expressing receptors exogenously and endogenously. Thus, PAR1-PAR2 dimer formation facilitates thrombin-dependent β-arrestin recruitment to endosomes, which does not occur with activation of the PAR1 protomer. Agonist stimulation of other GPCR heterodimers has also been shown to result in differential β-arrestin recruitment. The vasopressin 1A receptor binds weakly to β-arrestins, whereas the vasopressin 2 receptor forms a stable complex with β-arrestin; however, activation of the vasopressin 1A receptor-vasopressin 2 receptor dimeric complex results in co-internalization with β-arrestins and vasopressin 2 receptor-like trafficking properties (43). Similarly, β-arrestin does not co-internalize with the µ-opioid receptor in HEK293 cells. However, upon formation of a heterodimer with the substance P receptor, also known as the neurokinin-1 receptor, µ-opioid receptor agonist induces co-internalization of neurokinin-1-µ-opioid receptor heterodimers with β-arrestin into the same endosomal compartment (44). In most cases, it is not clear which molecular determinants mediate β-arrestin recruitment to receptors when present in heterodimeric complexes. We showed previously that activated PAR2 requires its C-terminal tail domain for stable association with β-arrestins, but not for receptor internalization (21). Surprisingly, we found that the PAR2 C-terminal tail is dispensable for thrombin-induced β-arrestin recruitment to the PAR1-PAR2 heterodimer on endosomes. In contrast, however, the PAR1 Y397Z-PAR2 C-terminal tail truncation mutant heterodimeric complex fails to internalize and to recruit β-arrestins. Together, these studies strongly suggest that PAR1-PAR2 heterodimer formation generates a new interface for interaction with β-arrestins, which appears distinct from that utilized by receptor protomers.

We have further demonstrated that PAR1-PAR2 heterodimer formation leads to a unique capacity of thrombin to induce β-arrestin-dependent signaling responses. Activation of the PAR1 protomer by thrombin results in preferential coupling to Gq and Gi proteins and induction of ERK1/2 signaling in many cell types (45, 46), whereas activated PAR2 interacts with β-arrestins to induce ERK1/2 signaling from endosomes (21, 29). Our results indicate that the thrombin-activated PAR1-PAR2 heterodimer utilizes β-arrestins to induce ERK1/2 activation. These findings are consistent with activated PAR1-PAR2-mediated β-arrestin recruitment to endosomes. Interestingly, Kaneide et al. (16) showed that thrombin signaling switched from RhoA to Rac1 in endotoxin-treated endothelial cells in which a PAR1-PAR2 complex was detected, indicating that the PAR1-PAR2 heterodimer may signal differently compared with the PAR1 protomer. However, these studies did not determine the function of β-arrestins. We recently showed that activation of PAR1 with the anticoagulant protease activated protein C results in biased β-arrestin-2-dishevelled-2 activation of Rac1 signaling in endothelial cells (45). Thus, it remains to be determined whether β-arrestins also mediate thrombin-
induced Rac1 activation mediated by the PAR1-PAR2 dimer in endotoxin-treated cells.

In summary, this work illustrates an important function for PAR1-PAR2 heterodimer-induced β-arrestin-mediated signaling to the repertoire of thrombin-induced cellular responses. In a mouse model of sepsis, formation of the PAR1-PAR2 dimer in endothelial cells appears to switch thrombin signaling from barrier-disruptive to barrier-protective (16), whereas in vascular smooth muscle cells, the PAR1-PAR2 heterodimer mediates hyperplasia following vascular injury (17). Clearly, PAR1-PAR2 heterodimer signaling is important for progression of certain pathological diseases. However, the role of β-arrestins in PAR1-PAR2-induced pathological responses is not known and is important to investigate. In addition, β-arrestins have been implicated in activated PAR2-induced cytoskeleton reorganization and chemotaxis through activation of cofilin (47). Whether PAR1-PAR2 heterodimer-mediated recruitment of β-arrestins also regulates cell migration and/or endothelial adherens junction and barrier maintenance is not known but is critical to understand.

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