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Journal

Pharmacological Reviews, 65(4)

ISSN

0031-6997

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Publication Date

2013-10-01

DOI

10.1124/pr.111.004747

Peer reviewed

ASSOCIATE EDITOR: ARTHUR CHRISTOPOULOS

# Cofactoring and Dimerization of Proteinase-Activated Receptors

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**Abstract**—Proteinase-activated receptors (PARs) are G protein-coupled receptors that transmit cellular responses to extracellular proteases and have important functions in vascular physiology, development, inflammation, and cancer progression. The established paradigm for PAR activation involves proteolytic cleavage of the extracellular N terminus, which reveals a new N terminus that functions as a tethered ligand by binding intramolecularly to the receptor to trigger transmembrane signaling. Most cells express more than one PAR, which can influence the mode of PAR activation and signaling. Clear examples include murine PAR3 cofactoring of PAR4 and transactivation of PAR2 by PAR1. Thrombin binds to and cleaves murine PAR3, which facilitates PAR4 cleavage and activation. This process is essential for thrombin signaling and platelet activation, since murine PAR3 cannot signal

alone. Although PAR1 and PAR4 are both competent to signal, PAR1 is able to act as a cofactor for PAR4, facilitating more rapid cleavage and activation by thrombin. PAR1 can also facilitate PAR2 activation through a different mechanism. Cleavage of the PAR1 N terminus by thrombin generates a tethered ligand domain that can bind intermolecularly to PAR2 to activate signaling. Thus, PARs can regulate each other's activity by localizing thrombin when in complex with PAR3 and PAR4 or by cleaved PAR1, providing its tethered ligand domain for PAR2 activation. The ability of PARs to cofactor or transactivate other PARs would necessitate that the two receptors be in close proximity, likely in the form of a heterodimer. Here, we discuss the cofactoring and dimerization of PARs and the functional consequences on signaling.

This work is supported by the National Institutes of Health [Grant R01-GM090689] (to J.T.); by the National Institutes of Health [Grant DP2-HL117748] (to A.P.L.); and by a National Institutes of Health Initiative for Maximizing Student Diversity [Grant R25-GM083275] (to T.H.S.).

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dx.doi.org/10.1124/pr.111.004747.

## I. Introduction

Proteinase-activated receptors (PARs) are a family of G protein-coupled receptors (GPCRs) composed of four members: PAR1, PAR2, PAR3, and PAR4. PARs are expressed in many cell types in the vasculature, including platelets, endothelial cells, smooth muscle cells, fibroblasts, immune cells, as well as gastrointestinal epithelial cells and neurons, astrocytes, and microglia of the central nervous system (Coughlin, 2005; Ramachandran et al., 2012). The discovery of PARs resulted from a search for a receptor that mediated responses to thrombin, the key effector protease of the coagulation cascade. PAR1 was the first family member identified and is the major effector of thrombin signaling in most cell types, and was originally dubbed the “thrombin receptor” (Vu et al., 1991a). PAR3 and PAR4 were subsequently discovered and also signal in response to thrombin (Ishihara et al., 1997; Kahn et al., 1998; Xu et al., 1998), whereas PAR2 is activated by trypsin-like serine proteases and upstream coagulant proteases, but not by thrombin (Nystedt et al., 1994). Thrombin activation of PARs is critical for hemostasis and thrombosis as well as for inflammatory and proliferative responses associated with vascular injury (Coughlin, 2005). PARs have also been implicated in vascular development (Griffin et al., 2001), neural tube closure (Camerer et al., 2010), inflammation (Ramachandran et al., 2012), cancer progression (Arora et al., 2007), and modulation of the innate immune response during viral infection (Antoniak et al., 2013).

The activation of PARs occurs by proteolytic cleavage of the extracellular N terminus, which generates a new N-terminal domain that functions as a tethered ligand by binding intramolecularly to the receptor to trigger transmembrane signaling (Fig. 1) (Coughlin, 1999). The mechanism of PAR1 activation by thrombin is the best characterized. The PAR1 N-terminal LPDR41-S42 sequence of PAR1 is essential for thrombin recognition and cleavage of the R41-S42 peptide bond (Vu et al., 1991b). A second interaction occurs between thrombin's anion-binding exosite I and an acidic region “WEDEE” of the PAR1 N terminus termed the “hirudin-like” domain based on its sequence similarity to the leech anticoagulant peptide hirudin (Fig. 1) (Rydel et al., 1990). The hirudin-like binding site increases thrombin's affinity and potency toward cleavage and activation of PAR1. Thus, the function of thrombin in the activation of PAR1 is to unmask the cryptic N-terminal-tethered ligand domain. Consistent with this mode of activation, synthetic peptides that represent

the tethered ligand sequence can activate PAR1 independent of thrombin and proteolytic cleavage (Scarborough et al., 1992; Chen et al., 1994). PAR3 also contains a hirudin-like binding site and is a high-affinity thrombin receptor. However, PAR4 lacks this sequence and displays low affinity for thrombin. PAR2 is the only PAR not activated by thrombin, but is cleaved by Factors Xa/VIIa, trypsin and tryptase at the N terminus, resulting in intramolecular liganding and activation (Nystedt et al., 1994; Molino et al., 1997). In addition to thrombin, activated Protein C (APC) and matrix metalloproteinases-1 and -13 have been shown to cleave and activate PAR1, but cleavage of the N terminus occurs at distinct sites (Mosnier et al., 2012; Austin et al., 2013). PAR2, and to a lesser extent PAR3 and PAR4, have also been shown to be cleaved and activated by other proteases (Ramachandran et al., 2012).

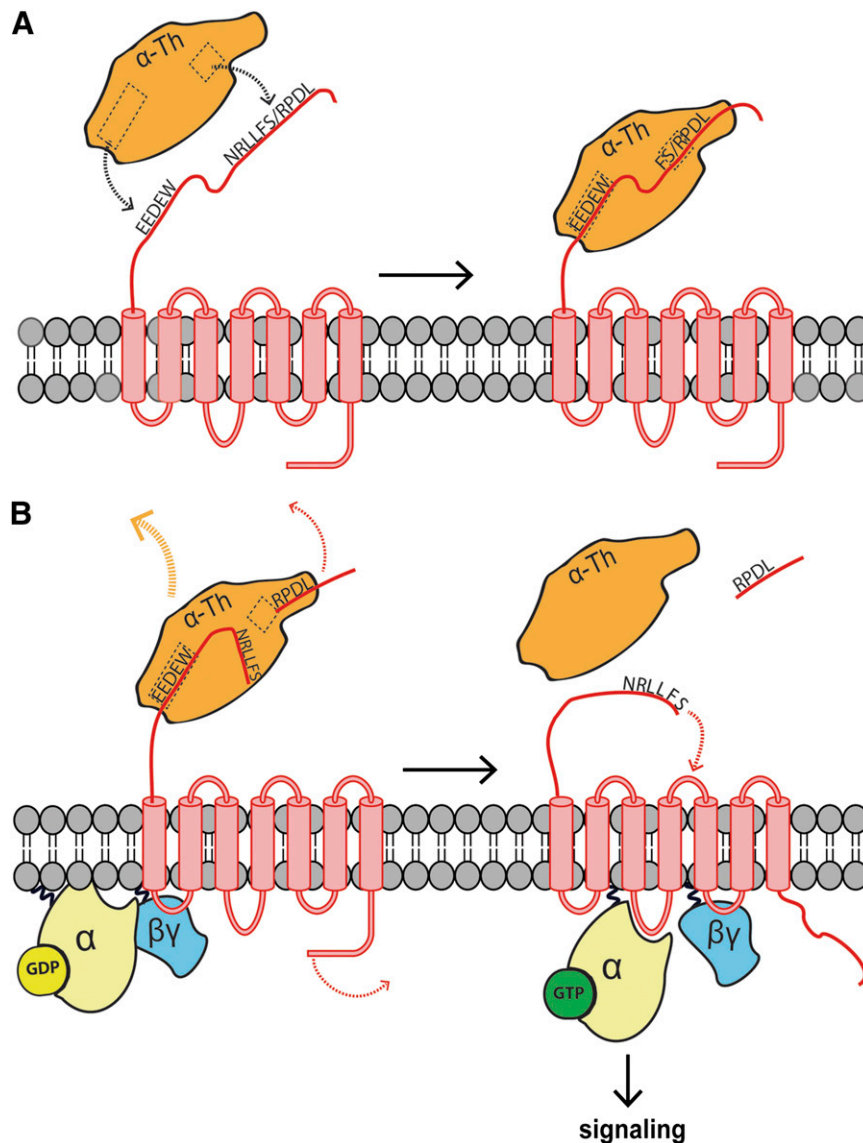
Once cleaved and activated, PARs undergo conformational changes within the transmembrane (TM) helices that expose cytoplasmic surfaces important for interaction with  $\alpha$  subunits of heterotrimeric G proteins localized at the inner leaflet of the plasma membrane (Oldham and Hamm, 2008). Similar to other GPCRs, PARs function as guanine-nucleotide exchange factors and promote the exchange of GDP for GTP on the  $\alpha$  subunit leading to  $\beta\gamma$  subunit dissociation and effector signaling (Fig. 1). Activated PAR1 and PAR2 couple to multiple heterotrimeric G protein subtypes, including  $G\alpha_i$ ,  $G\alpha_q$ , and  $G\alpha_{12/13}$  (Soh et al., 2010). PAR4 also signals through  $G\alpha_q$  and  $G\alpha_{12/13}$  activation, whereas mouse PAR3 does not appear to signal autonomously (Nakanishi-Matsui et al., 2000). In addition to heterotrimeric G proteins, activated PARs can also signal via interaction with  $\beta$ -arrestins and other adaptor proteins to promote cellular responses (Soh et al., 2010).

Most cell types express more than one PAR, and their activity can be modulated by interaction with each other. In human platelets, PAR1 is expressed together with PAR4, whereas PAR3 and PAR4 are coexpressed in murine platelets. Although thrombin binds to and cleaves murine PAR3, it does not appear to signal autonomously but rather functions as a cofactor to facilitate more efficient cleavage and activation of PAR4 (Nakanishi-Matsui et al., 2000). A similar situation may exist between PAR1 and PAR4 in human platelets (Leger et al., 2006). PAR1 and PAR3 are coexpressed in endothelial cells (O'Brien et al., 2001) and murine podocytes (Madhusudhan et al., 2012) and function together to regulate certain signaling responses (McLaughlin et al., 2007; Madhusudhan

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**ABBREVIATIONS:** APC, activated protein C;  $\beta$ 1-AR,  $\beta$ 1-adrenergic receptor; BiFC, biomolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; CFP, cyan fluorescent protein; C-tail, cytoplasmic tail; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinases-1 and -2; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEK293, human embryonic kidney 293; MOR,  $\mu$ -opioid receptor; NK1, neurokinin-1; PAR, proteinase-activated receptor; Rluc, *Renilla* luciferase; TM, transmembrane; TR-FRET, time-resolved fluorescence energy resonance transfer; YFP, yellow fluorescent protein.

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**Fig. 1.** Thrombin activates PAR1 through proteolytic cleavage. (A)  $\alpha$ -Thrombin ( $\alpha$ -Th) binds to the extracellular N terminus of PAR1 through two distinct binding sites. The PAR1 N-terminal LDPR41-S42 is recognized by thrombin's active site (dashed square) and an acidic region of PAR1 "WEDEE," termed the hirudin-like domain. (B)  $\alpha$ -Th cleaves the PAR1 N-terminal R41-S42 peptide bond and then dissociates. The newly formed PAR1 N terminus beginning with the peptide sequence SFLLRN then functions as a tethered ligand and binds intramolecularly to the receptor, causing conformational changes and G protein activation. Activated PAR1 functions as a guanine-nucleotide exchange factor and facilitates exchange of GDP for GTP on the heterotrimer  $\alpha$  subunit, resulting in dissociation of  $\beta\gamma$  and effector signaling.

et al., 2012). PAR2 expression is typically low in naïve endothelial cells and is increased following endothelial stimulation with inflammatory mediators (Nystedt et al., 1996). Intriguingly, the thrombin-cleaved PAR1-tethered ligand domain can bind intermolecularly and transactivate PAR2 (O'Brien et al., 2001), a situation that is favored when PAR2 expression is increased (Kaneider et al., 2007; Lin and Trejo, 2013). Thus, PARs can modulate each other's signaling activity by localizing thrombin to facilitate efficient receptor cleavage or by providing a tethered ligand domain to an adjacent PAR. The capacity of PARs to function in this manner suggests that the receptors are in close proximity, likely in the form of a dimer. Several

published studies now indicate that PARs are capable of forming homodimers and heterodimers with each other (Table 1).

PARs are members of the class A family of rhodopsin-like GPCRs (Coughlin, 1994). Although isolated class A GPCR monomers reconstituted in nanodiscs *in vitro* can couple to G protein activation (Whorton et al., 2007) and interact with  $\beta$ -arrestins (Bayburt et al., 2011), it remains unclear whether monomers are the major functional entities in native tissues. GPCRs are also known to form homodimers and heterodimers, and can exist as part of larger oligomeric complexes (Fig. 2). The metabotropic glutamate-like class C family of GPCRs are obligate dimers and only function when

TABLE 1  
Proteinase-activated receptor cofactoring and dimerization

This table highlights the publications that provide evidence for PAR cofactoring and dimerization.

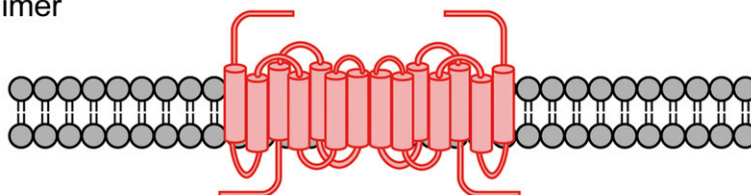
Receptors	Function	Technique	Reference
PAR1-PAR1	Intermolecular liganding Ca <sup>2+</sup> signaling	Functional reconstitution, <i>X. laevis</i> oocytes	Chen et al., 1994
PAR1-PAR1	Not studied	BRET, HEK293 cells	McLaughlin et al., 2007
PAR1-PAR2	Intermolecular liganding, Ca <sup>2+</sup> signaling	Blocking antibodies, functional reconstitution, endothelial cells and COS7 cells	O'Brien et al., 2000
PAR1-PAR2	Intermolecular liganding, Cytoprotective signaling	Blocking and activating agents FRET, co-IP, in vitro and in vivo studies, endothelial cells	Kaneider et al., 2007
PAR1-PAR2	Intermolecular liganding, smooth muscle hyperplasia	Blocking and activating agents, co-IP, in vitro and in vivo studies, smooth muscle cells and COS7 cells	Seigny et al., 2011
PAR1-PAR2	Intermolecular liganding, $\beta$ -arrestin signaling	Functional reconstitution, BRET, co-IP, microscopy, COS7 cells, HeLa cells, endothelial cells	Lin and Trejo, 2013
PAR1-PAR3	Allosteric modulation of G protein signaling	BRET, fluorescence microscopy, HEK293 and endothelial cells	McLaughlin et al., 2007
mPAR1-mPAR3	Cytoprotective signaling	Blocking agents, co-IP in vitro and in vivo studies, podocytes and mesangial cells	Madhusudhan et al., 2012
PAR1-PAR4	Cofactoring platelet activation	Blocking agents, co-IP, FRET, in vitro and in vivo studies, platelets and COS7 cells	Leger et al., 2006
PAR2-PAR2	Intermolecular liganding, inflammatory signaling	Functional reconstitution, co-IP, pepducins, in vitro and in vivo studies, COS7 cells and inflammatory cells	Seigny et al., 2011
PAR2-PAR3	Cytoprotective signaling	Blocking agents, co-IP in vitro studies, human podocytes and mesangial cells	Madhusudhan et al., 2012
PAR2-PAR4	Anterograde trafficking	FRET, co-IP, microscopy HEK293 cells	Cunningham et al., 2012
mPAR3-mPAR4	Cofactoring platelet activation	Functional reconstitution, COS7 cells	Nakanishi-Matsui et al., 2000
PAR4-PAR4	Ca <sup>2+</sup> signaling	BRET, BiFC HEK293 cells	De La Fuente et al., 2012
mPAR4-mPAR3	Not studied	BRET, HEK 293 cells	Arachiche et al., 2013

co-IP, coimmunoprecipitation.

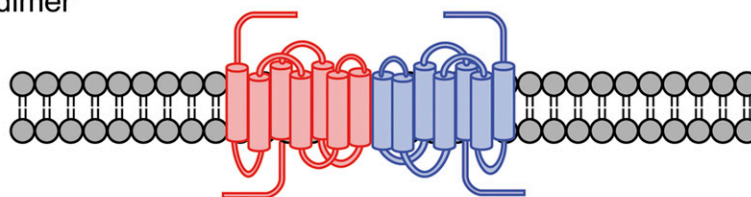
bound to their dimeric partner (Pin et al., 2003). Although numerous studies have documented class A GPCR dimerization in various model systems, including in native cells (Albizu et al., 2010), the prerequisite of dimer or oligomer formation with

relation to class A GPCR function has not been demonstrated unequivocally. Here, we discuss the evidence supporting cofactoring and dimerization of PARs and the functional consequences on signaling responses.

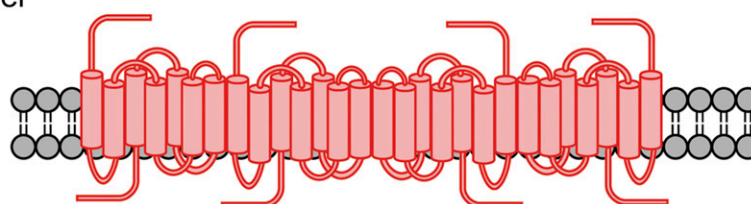
### Homodimer



### Heterodimer



### Oligomer



**Fig. 2.** GPCR dimer formation. Class A family of rhodopsin-like GPCRs are composed of several hundred members, and a vast literature indicates that many of these receptors can self-associate to form homodimers or interact with other GPCRs to form heterodimers or exist as higher-order oligomeric complexes. Several recent structures of GPCR dimers have been solved and indicate that distinct regions of various TMs mediate the dimer interface, depending on the receptor. Our illustration of a GPCR dimer, heterodimer, and oligomer association are not meant to reflect actual TM interactions, since these have not been definitively determined for any PAR dimers.

## II. PAR Cofactoring

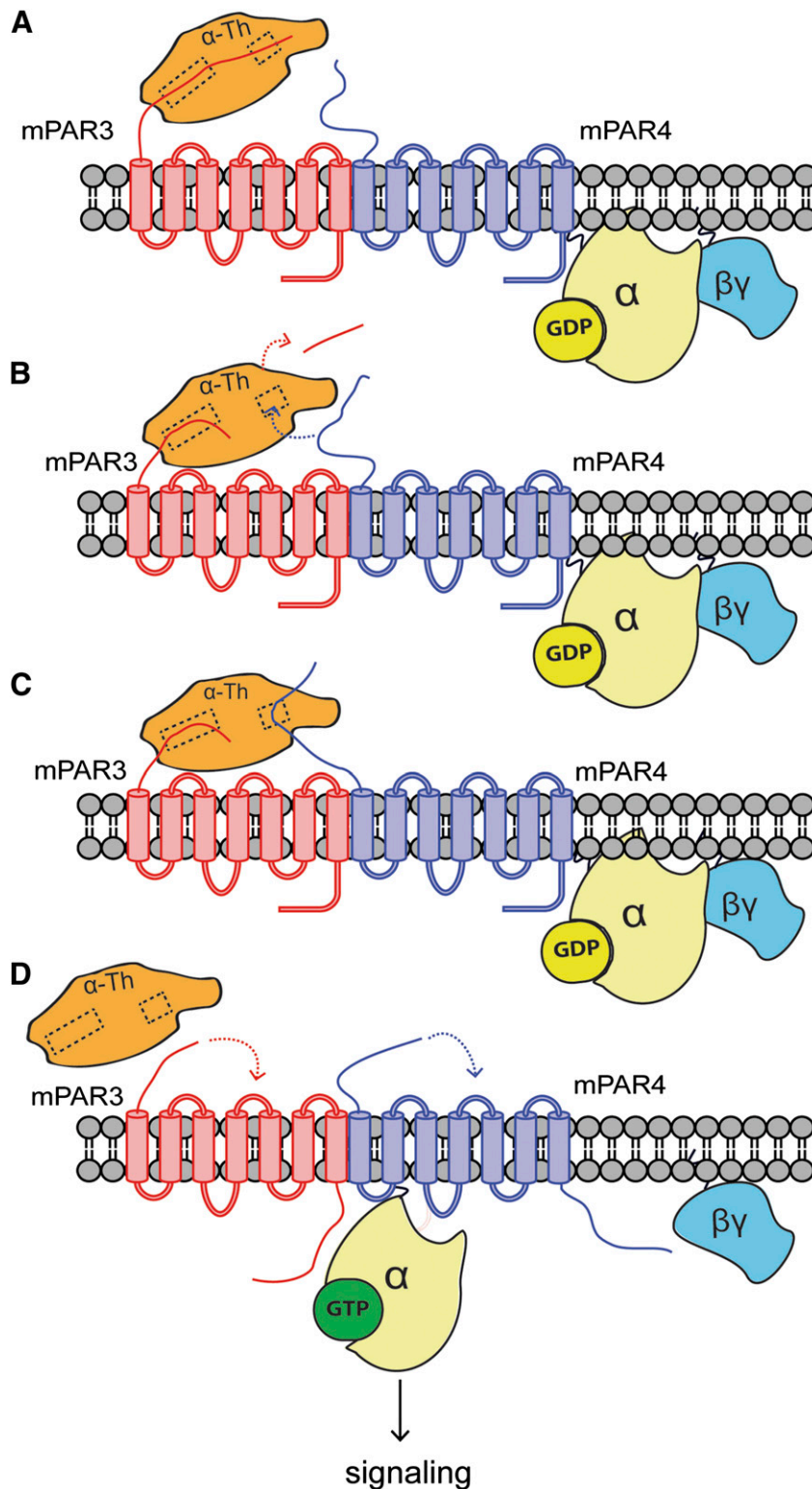
The concept that a PAR can function as a cofactor to facilitate activation of an adjacent PAR was initially described in studies of mouse platelets (Nakanishi-Matsui et al., 2000). These studies also provided a probable explanation for the existence of mPAR3, a receptor that fails to signal after cleavage by thrombin. Murine PAR3 cofactoring of mPAR4 is critical for rapid and efficient platelet activation induced by thrombin (Nakanishi-Matsui et al., 2000). There is also evidence that hPAR1 functions as a cofactor for hPAR4 activation (Leger et al., 2006). However, in the latter case, both receptors are competent to signal and each may contribute to a distinct biologic process during platelet activation.

*A. Mouse PAR3 Cofactoring of mPAR4.* Thrombin is a potent activator of platelets and provokes platelet shape change, secretion, and integrin activation that culminates in platelet aggregation. Platelet secretion and aggregation induced by thrombin is mediated by  $G\alpha_q$  signaling, whereas  $G\alpha_{13}$ , but not  $G\alpha_{12}$ , is necessary for platelet shape change and aggregation in response to low thrombin concentrations (Offermanns et al., 1997; Moers et al., 2003). Mouse platelets express two thrombin receptors—mPAR3 and mPAR4. Platelets isolated from mPAR3 knockout mice exhibited impaired or delayed responses to thrombin stimulation, suggesting a function for mPAR3 in thrombin signaling (Kahn et al., 1998). Antibodies directed against mPAR3 also abrogated platelet activation by thrombin (Ishihara et al., 1998). However, thrombin cleavage of mPAR3 failed to promote a  $Ca^{2+}$  response when expressed ectopically (Nakanishi-Matsui et al., 2000). The signaling deficiency of mPAR3 is inconsistent with the receptor's role in mediating robust thrombin-induced platelet activation. One explanation for this phenomenon is that mPAR3 acts as a cofactor for mPAR4 and facilitates its cleavage by thrombin. This is supported by studies performed in transfected COS7 cells. The coexpression of mPAR3 together with mPAR4 at comparable cell surface levels resulted in a significant increase in the efficiency of thrombin-stimulated signaling (Nakanishi-Matsui et al., 2000). The capacity of mPAR3 to enhance thrombin signaling requires mPAR4 cleavage and cell surface localization of the mPAR3 N-terminal domain containing the critical site for thrombin binding. In platelets deficient in mPAR4, thrombin failed to elicit platelet activation or aggregation, confirming that mPAR3 is not sufficient to promote transmembrane signaling itself, but instead functions as a cofactor that facilitates thrombin cleavage and activation of mPAR4 (Fig. 3) (Sambrano et al., 2001). The cofactor effect observed with mPAR3 and mPAR4 is further supported by X-ray crystallography studies using N-terminal fragments of murine PAR3 and PAR4 bound to thrombin (Bah et al., 2007). This work nicely illustrates that cleaved mPAR3 remains bound to exosite I of thrombin,

leaving thrombin's active site free and accessible to other substrates, such as mPAR4 (Fig. 3) (Bah et al., 2007). Thus, thrombin activation of mPAR4 can occur with exosite I bound to cleaved mPAR3, which may promote substrate diffusion into the active site by modulating thrombin's conformation (Bah et al., 2007). These elegant studies by Coughlin and colleagues provided the first example of mammalian GPCR cofactoring (Nakanishi-Matsui et al., 2000).

*B. Human PAR1 Cofactoring of hPAR4.* Similar to mouse platelets, human platelets express two thrombin receptors—hPAR1 and hPAR4. However, in contrast to murine PAR3, both of these receptors efficiently couple to  $G\alpha_q$  and  $G\alpha_{13}$  signaling pathways in platelets and other cell types. Thus, the biologic significance of this "dual thrombin receptor system" in the context of human platelets was not immediately obvious (Kahn et al., 1999). Some initial hypotheses regarding the existence of a dual thrombin receptor system were that it simply provided redundancy for a very important physiologic process such as hemostasis, or that the system allowed platelets to more finely tune signaling responses to a wide range of thrombin concentrations, with hPAR1 mediating responses to low thrombin concentrations and hPAR4 signaling contributing at high thrombin concentrations (Kahn et al., 1999). Although the precise contribution of hPAR1 cofactoring of hPAR4 to thrombin-stimulated human platelet activation remains controversial, subsequent investigations suggest that hPAR4 has a distinct function. Studies in transfected Rat-1 fibroblasts showed that thrombin activation of hPAR4 resulted in  $G\alpha_q$  signaling responses that persisted much longer than those induced by thrombin activation of hPAR1 (Shapiro et al., 2000). Another study used receptor-specific ligands and inhibitors to tease apart each PAR's individual contribution to platelet activation and found differences in the temporal kinetics of receptor activation and signaling (Covic et al., 2000). Thrombin activation of hPAR1 was shown to result in an initial rapid increase in intracellular  $Ca^{2+}$  and was followed by a subsequent hPAR4-mediated sustained  $Ca^{2+}$  response. Intriguingly, the activation of hPAR4 by thrombin appeared more effective at sustaining the secondary signaling responses due to granule secretion and ADP-mediated purinergic receptor stimulation (Covic et al., 2000; Holinstat et al., 2006), a process important for the late phase of platelet aggregation.

Despite the role of hPAR4 in facilitating prolonged platelet activation by thrombin (Covic et al., 2000), it was not clear whether thrombin activation of hPAR4 was dependent on hPAR1 in human platelets, similar to mPAR3 cofactoring of mPAR4 in mouse platelets. High-affinity thrombin receptors, such as PAR1 and PAR3, contain an N-terminal localized hirudin-like domain that enables their rapid association with thrombin's exosite I (Figs. 1 and 3) (Jacques et al.,



**Fig. 3.** Model of PAR3 cofactoring of mPAR4. (A) The mPAR3-thrombin complex is shown. Similar to PAR1, the mPAR3 hirudin-like sequence binds with high affinity to thrombin's  $\alpha$ -Th exosite I (dashed rectangle), facilitating the interaction between mPAR3's cleavage site and thrombin's active site (dashed square). (B) After cleavage, mPAR3 remains associated with thrombin due to the high-affinity interaction at exosite I (dashed rectangle). This conformation leaves thrombin's active site unobstructed (dashed square) and accessible to the mPAR4 N-terminal region. (C) The mPAR3-thrombin-mPAR4 complex is shown. The localization of catalytically active thrombin by mPAR3 allows the mPAR4 N terminus to interact with thrombin's active site (dashed square) and results in efficient thrombin cleavage of mPAR4. (D) After cleavage of mPAR4, thrombin dissociates from the complex. The newly exposed N-terminal-tethered ligand domain of mPAR4 is then able to bind intramolecularly to the second extracellular loop and triggers G protein signaling.

2000). The lower-affinity PAR4 lacks such a domain, and must rely on proline residues and a cluster of anionic residues to interact with the active site of thrombin (Jacques and Kuliopulos, 2003). A nuclear magnetic resonance structure of thrombin-cleaved hPAR1 exodomain and theoretical docking models indicated that hPAR1 behaves similarly to mPAR3 in that it remains associated with thrombin's exosite I and leaves the active site of thrombin free to potentially cleave other proteins, such as hPAR4 (Fig. 3) (Seeley et al., 2003). A more recent study used a PAR1-specific antagonist, a direct thrombin inhibitor (bivalirudin), and blocking agents, and found that hPAR1 can function as a cofactor for thrombin activation of PAR4 on human platelets and other cell types (Leger et al., 2006). This study showed that PAR4 was capable of being activated by low thrombin concentrations under conditions in which thrombin-cleaved PAR1 signaling was blocked with the peptide mimetic RWJ-56110, a small-molecule antagonist. The subsequent addition of bivalirudin, which prevents thrombin exosite I interaction with the PAR1 hirudin-like domain, resulted in complete loss of PAR4 activation by low thrombin concentrations. Thus, as with mPAR3-mPAR4 cofactoring, coexpression of hPAR1 enhanced thrombin's cleavage and activation of PAR4. However, unlike mPAR3-PAR4, direct activation of either human PAR1 or PAR4 is sufficient to induce platelet activation, although PAR1 is the more potent activator (Kahn et al., 1999).

### III. PAR1 Homodimer

The first evidence to suggest that PAR1 self-associates and forms homodimers was demonstrated in studies examining the mechanism of thrombin activation of PAR1 (Chen et al., 1994). These studies showed that the exposed tethered ligand domain of cleaved PAR1 can bind intermolecularly to activate an adjacent PAR1, albeit with less efficiency than intramolecular liganding. In more recent work, bioluminescence resonance energy transfer (BRET) was used to demonstrate that PAR1 is capable of forming homodimers constitutively (McLaughlin et al., 2007).

**A. PAR1 Intermolecular Liganding.** PAR1 mutants containing either a defective tethered ligand domain or specific mutations within the intracytosolic regions that ablate G protein coupling were used to assess function by reconstitution in *Xenopus laevis* oocytes and COS7 cells (Chen et al., 1994). These studies used a PAR1 F43A mutant where the critical phenylalanine (F) was converted to alanine (A) of the tethered ligand domain, rendering it inactive. PAR1 signaling defective mutants were generated by converting tyrosines (Y) to alanines at Y371, Y372, and Y373 residues located toward the end of TM VII, or by transposing the critical aspartate (Asp199) and arginine (Arg200) residues of the DRY motif present in TM III. Expression of either the PAR1

inactive tethered ligand mutant or signaling-defective mutants alone failed to elicit a signaling response to thrombin (Chen et al., 1994). Coexpression of the PAR1 F43A mutant together with YYY or DR-RD mutants, however, resulted in reconstitution of thrombin signaling. These findings suggest that the thrombin-generated tethered ligand domain of a signaling-deficient receptor can bind intermolecularly to an adjacent receptor to provoke intracellular signaling, although the intermolecular liganding mechanism is considerably less efficient than the intramolecular tethered ligand activation of PAR1 (Chen et al., 1994). However, in certain cell types, PAR1 is compartmentalized in caveolae (Russo et al., 2009; Soh and Trejo, 2011), and compartmentalization could facilitate intermolecular liganding by enhancing receptor association, but this remains to be tested.

**B. PAR1 Homodimer Formation.** PAR1-PAR1 homodimerization has also been demonstrated in transfected human embryonic kidney 293 (HEK293) cells using BRET (McLaughlin et al., 2007). BRET is widely used to monitor the dynamics of GPCR dimerization in living cells and relies on nonradiative transfer of energy between donor and acceptor fluorophores (Perroy et al., 2004; Hamdan et al., 2006). BRET does not require external illumination to initiate fluorescence transfer and avoids photobleaching issues associated with other resonance transfer approaches. In BRET, *Renilla* luciferase (Rluc) is typically fused to the GPCR C-tail domain and upon oxidation of its substrate, coelenterazine, releases photons that can excite an appropriate acceptor fluorophore, such as a receptor fused to yellow fluorescent protein (YFP) or green fluorescent protein (GFP) that is in close proximity, which then emits photons with longer wavelengths. The two common BRET configurations are BRET<sup>1</sup> and BRET<sup>2</sup>, which use different fluorescent acceptors and substrates (Lohse et al., 2012). The advantage of BRET<sup>2</sup>, which uses GFP2 and DeepBlueC, over BRET<sup>1</sup>, which uses YFP and coelenterazine, is that BRET<sup>2</sup> has superior donor and acceptor emission peak separation and results in lower background signal. However, because DeepBlueC has low quantum yield, more cells and more sensitive instruments are required. One disadvantage of BRET is substrate instability, so that real-time monitoring of protein-protein interactions is limited to short timescales (e.g., minutes), but recent generation of more stable substrates may circumvent these issues (Pfleger et al., 2006). BRET is typically performed on a large number of cells using a luminometer, and is thus limited in its ability to distinguish the origin of the signal from distinct cellular compartments. However, a recent study suggests that BRET can be used for imaging single cells (Coulon et al., 2008), although the spatial and temporal resolutions for BRET imaging require further improvements before it will be broadly applicable to the study of receptor dynamics. BRET also requires the overexpression of GPCRs tagged with fluorescent proteins, making it difficult to determine



if the signal is derived from a dimer or higher-order oligomer.

Specific receptor-receptor interactions display a hyperbolic increase in BRET that is directly proportional to the acceptor-donor ratio, whereas nonspecific interactions typically yield a linear BRET increase (Hamdan et al., 2006). As the ratio of PAR1-GFP to PAR1-Rluc expression was increased in HEK293 cells, a hyperbolic increase in BRET was observed compared with a linear increase in BRET produced by increasing the ratio of GABA<sub>B</sub>-GFP and PAR1-Rluc (McLaughlin et al., 2007). These findings suggest that PAR1 forms constitutive homodimers. Confocal microscopy imaging was then used to examine the subcellular localization of PAR fusion proteins and revealed the presence of PAR1-GFP and PAR1-Rluc at both the cell surface and in intracellular compartments, indicating that BRET arises from different cellular locations. Although the addition of thrombin had no immediate effect on BRET detected between PAR1 homodimers, prolonged stimulation caused a significant decrease in BRET. Conformational changes between proteins can be reflected as either increases or decreases in BRET (Salahpour et al., 2012). Direct activation of PAR1 with peptide agonist failed to affect the BRET signal after immediate or prolonged agonist stimulation. The underlying mechanisms for the differential effects of thrombin versus peptide agonist on the changes in the PAR1-PAR1 elicited BRET signal are not known. It is unlikely that these changes are attributed to differences in PAR1 redistribution from the cell surface since both thrombin and agonist peptide are equally effective at stimulating PAR1 internalization (Chen et al., 2011). It is possible that thrombin induces distinct conformational changes in the PAR1 dimer that are more efficiently detected by BRET. However, the absence of rapid changes in BRET following agonist activation is inexplicable unless such conformational changes result from slower intermolecular liganding rather than rapid efficient intramolecular activation of PAR1. Previous studies indicated that, in many instances, agonist addition has a minimal effect on GPCR protomer interaction based on the use of resonance energy transfer approaches (Canals et al., 2003, 2004). However, certain GPCR dimer pairs, such as the melatonin MT<sub>2</sub> receptors (Ayoub et al., 2002) and the CXCR4-CCR3 receptor dimers (Percherancier et al., 2005), clearly displayed ligand-induced conformational changes that were detectable by BRET.

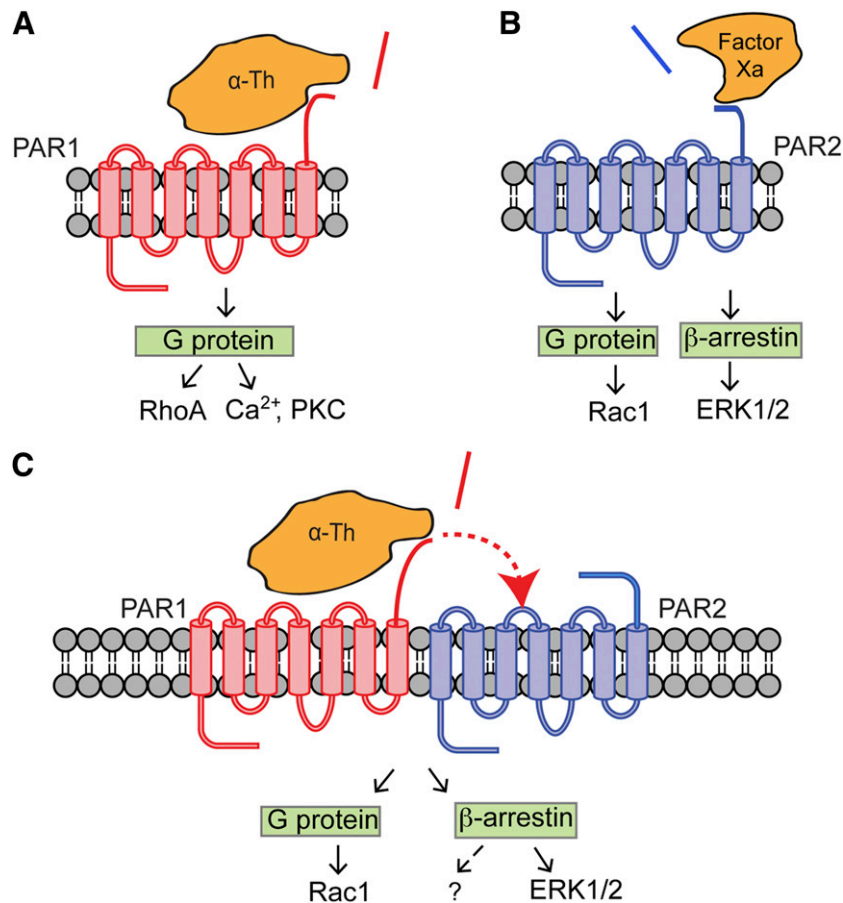
#### IV. PAR1 Transactivation of PAR2

In contrast to PAR cofactoring (Nakanishi-Matsui et al., 2000), a substantial amount of literature provides evidence that PAR1 can modulate the activity of PAR2 through a distinct mechanism. In this case, thrombin cleavage of the PAR1 N terminus unmask a tethered

ligand domain that can bind *in trans* to activate PAR2 via an intermolecular liganding mechanism, which elicits distinct signaling response compared with either receptor protomer.

A. *PAR1 Intermolecular Liganding of PAR2.* The idea that the PAR1 tethered ligand can bind intermolecularly to activate PAR2 *in trans* was based on studies examining synthetic peptide ligand cross-reactivity between PARs (Blackhart et al., 1996). The PAR1 and PAR2 tethered ligand sequences, SF<sup>2</sup>LLRN and SL<sup>2</sup>IGKV, respectively, are similar in amino acid sequence, but differ considerably at position 2, which is the critical residue important for the agonist peptide potency at PAR1 (Scarborough et al., 1992). The peptide agonist of PAR2 contains a leucine rather than phenylalanine at position 2 and fails to activate PAR1 signaling in a *X. laevis* oocyte expression system. In contrast, the PAR1 agonist peptide is able to activate PAR2 with a similar potency as the native agonist peptide of PAR2 examined in the same system. These findings indicate that PAR2 is able to tolerate a wide range of substitutions at critical residues of its peptide agonist compared with PAR1, and raise the possibility that the tethered ligand domain of thrombin-cleaved PAR1 might transactivate a neighboring PAR2.

The original study that examined the capacity of PAR1 to transactivate PAR2 through intermolecular liganding was performed in COS7 cells and endothelial cells (O'Brien et al., 2000). These studies used a PAR1 L258P mutant harboring a leucine to proline substitution within the second extracellular loop that remained sensitive to thrombin cleavage, but could not signal. COS7 cells expressing either the PAR1 L258P mutant or PAR2 alone failed to signal in response to thrombin. However, coexpression of the mutant receptors restored thrombin-stimulated signaling responses. PAR1-blocking antibodies and cross-desensitization experiments in endothelial cells were also used and confirmed transactivation of PAR2 by thrombin-cleaved PAR1 (O'Brien et al., 2000). These studies were the first to show that intermolecular liganding can occur between endogenous PAR1 and PAR2 in native cells (Fig. 4C). Subsequent work showed that PAR1 transactivates PAR2 on endothelial cells during progression of sepsis (Kaneider et al., 2007), a systemic inflammatory response characterized by exacerbated coagulation (Hotchkiss and Karl, 2003). Under these pathologic conditions, PAR1 signaling switched from vascular-disruptive to vascular-protective (Kaneider et al., 2007), as a consequence of increased PAR2 expression. Consistent with these studies, in cytokine-treated endothelial cells and HeLa cells, expression of PAR2 enhanced the capacity of thrombin-activated PAR1 to stimulate extracellular signal-regulated kinases-1 and -2 (ERK1/2) signaling (Fig. 4) (Lin and Trejo, 2013). Other studies have also demonstrated a function for PAR2 in thrombin-induced signaling responses in



**Fig. 4.** PAR1 transactivation of PAR2. (A) PAR1 is likely to exist as a monomer in naïve endothelial cells, and upon thrombin activation, efficiently couples to G proteins to promote RhoA activation, Ca<sup>2+</sup> mobilization, and protein kinase C (PKC) activation, important mediators of endothelial barrier disruption. (B) In contrast, PAR2 activation by factor Xa is known to stimulate barrier protection through G protein coupling to Rac1 activation. However, activation of PAR2 is also known to result in recruitment of β-arrestins, which function as a scaffold to facilitate ERK1/2 activation. (C) In inflammatory conditions, PAR2 expression is increased in endothelial cells and forms dimers with PAR1. Thrombin cleavage of PAR1 results in the generation of a tethered ligand that can bind to an adjacent PAR2 *in trans* to promote a distinct signaling response that results in Rac1 activation and β-arrestin-mediated ERK1/2 activation and vascular protective responses.

various cellular contexts (Shi et al., 2004; Lidington et al., 2005). Thus, PAR1 appears to modulate the activity of PAR2 by providing its tethered ligand domain, strongly suggesting that the two receptors are likely to form a physical heterodimer.

**B. PAR1-PAR2 Heterodimer Formation.** PAR1 and PAR2 interaction was first studied using single-cell confocal fluorescence resonance energy transfer (FRET) imaging and coimmunoprecipitation in endothelial cells (Kaneider et al., 2007). FRET relies on the nonradiative transfer of energy between cyan fluorescent protein (CFP) and YFP, which have overlapping spectral properties, and is an approach used commonly to assess GPCR dimerization (Lohse et al., 2012). The GPCR pairs are typically fused in frame to either CFP or YFP, and excitation of CFP results in YFP emission when the two fluorophores are in close proximity and aligned correctly. The FRET efficiency is typically determined by measuring changes in CFP emission before and after photobleaching of YFP, which results in a stronger emission for the CFP donor. To examine PAR1-PAR2 association by FRET, C-tail truncation mutants of PAR2

fused to CFP and PAR1 fused to YFP were first expressed separately in endothelial cells and revealed no specific FRET increase (Kaneider et al., 2007). Upon coexpression of PAR1 ΔC-tail-YFP and PAR2 ΔC-tail-CFP, a punctate signal was detected in the cytoplasm and then relocalized to the cell periphery after stimulation with endotoxin, a bacterial inflammatory mediator that increases endogenous PAR2 expression (Nystedt et al., 1996). Although this is an interesting observation, confirmatory experiments were necessary since PAR C-tail mutants are defective in signaling and trafficking (Shapiro et al., 1996; Ricks and Trejo, 2009). Thus, endogenous PAR1-PAR2 interaction was evaluated by coimmunoprecipitation using a membrane-impermeable cross-linking agent (Kaneider et al., 2007). The use of coimmunoprecipitation to study GPCR dimer formation is one of the few techniques that do not require expression of exogenous epitope-tagged proteins but requires antibodies that specifically recognize the receptors. Preincubation of endothelial cells with cross-linking reagent failed to affect the mobility of PAR1, which migrated as a broad band and likely represents

a fully mature monomeric PAR1 that is extensively glycosylated (Vouret-Craviari et al., 1995; Soto and Trejo, 2010). However, the addition of cross-linking reagent after treatment with endotoxin resulted in the formation of a high-molecular-weight species that was detected with anti-PAR1 antibodies and is likely to represent either PAR1 homodimers or heterodimers with PAR2. A similar high-molecular-weight PAR2 species was detected after endotoxin preincubation and cross-linking, suggesting the formation of either PAR2 homodimers or heterodimeric complexes with PAR1. Although coimmunoprecipitation is commonly used to assess protein-protein interaction, it does have limitations. The solubilization of membrane proteins is necessary for coimmunoprecipitation, and therefore provides minimal information on the subcellular localization of protein-protein interactions, and does not exclude the possibility that receptors are present in the same membrane microdomain but are not physically associated. Because of the equilibrium conditions used for coimmunoprecipitation, the most abundant interactions will be identified, leaving out transiently interacting proteins.

In more recent work, a full-length PAR2 fused to Rluc and PAR1 containing YFP fused in frame to the C-tail domain were used to assess heterodimerization using BRET in COS7 cells. A hyperbolic increase in BRET was observed as the ratio of PAR1-YFP to PAR2-Rluc was increased in unstimulated cells (Lin and Trejo, 2013), suggesting that the receptors interact constitutively. Coimmunoprecipitation of epitope-tagged PAR1 and PAR2 confirmed receptor association in HeLa cells. Thrombin also induced a statistically significant change in the maximal BRET signal elicited by PAR2-Rluc and PAR1-YFP, without altering the BRET<sub>50</sub>. These findings indicate that thrombin cleavage of PAR1 induces a conformational change between receptor protomers but does not affect the affinity of receptor association (Lin and Trejo, 2013). In addition, coexpression of PAR2 with PAR1 resulted in constitutive cointernalization of the heterodimer, a process that was driven by the trafficking behavior of PAR1, since PAR2 does not constitutively internalize in a manner similar to that observed with PAR1 in these cell types (Paing et al., 2006; Ricks and Trejo, 2009). The addition of thrombin caused an even greater increase in PAR1-PAR2 cointernalization. A similar phenomenon was observed with endogenous PAR1 and PAR2 expressed in endothelial cells in which PAR2 expression was increased by cytokine pretreatment (Lin and Trejo, 2013). Thus, increased expression of PAR2 facilitates heterodimer formation with PAR1 in both native and transfected cell systems and suggests that, in pathologic inflammatory diseases, the PAR1-PAR2 heterodimer may predominate.

*C. PAR1-PAR2 Heterodimer Signaling.* Several studies indicate that class A GPCR heterodimers elicit

distinct signaling responses compared with receptor protomers (Rozenfeld and Devi, 2011). Thus, a function for class A GPCR heterodimers may be to provide signaling diversity in certain cellular contexts. This is best described for PAR2 transactivation by PAR1 in cultured endothelial cells.

Thrombin was shown to stimulate expression of the complement-inhibitory protein decay-accelerating factor, an important mediator of cytoprotection, via PAR1 transactivation of PAR2 in endothelial cells (Lidington et al., 2005). A second study showed that thrombin-cleaved PAR1 transactivated PAR2 during progression of sepsis and resulted in vascular protection rather than disruption (Kaneider et al., 2007). In normal conditions, thrombin-activated PAR1 preferentially couples to G $\alpha_q$  G $\alpha_{12/13}$ , resulting in Ca<sup>2+</sup> mobilization, RhoA activation, and endothelial barrier disruption (Fig. 4A) (Komarova et al., 2007), whereas activated PAR2 signals to G $\alpha_i$  and Rac1 activation to promote endothelial barrier protection (Fig. 4B) (Feistritzer et al., 2005; Kaneider et al., 2007). The generation of thrombin during early phases of sepsis is generally vascular disruptive, suggesting that signaling occurs mainly through PAR1 activation of G $\alpha_q$  G $\alpha_{12/13}$  signaling. However, during late stages of sepsis, thrombin activation of PAR1 switched from RhoA to Rac1 signaling, a process that required PAR2 expression and function (Kaneider et al., 2007). These findings suggest that signaling by the PAR1-PAR2 heterodimer is different compared with the PAR1 protomer.

Despite numerous studies documenting differences in PAR1-PAR2 heterodimer signaling compared with receptor protomers, the mechanistic basis for the phenomenon remained unclear. In recent work,  $\beta$ -arrestins were shown to be differentially recruited to the thrombin-activated PAR1 protomer versus the PAR1-PAR2 heterodimer (Fig. 4) (Lin and Trejo, 2013). PAR1 and PAR2 display marked differences in  $\beta$ -arrestin recruitment. Activation of PAR1 results in transient  $\beta$ -arrestin association that rapidly dissociates before receptor internalization (Chen et al., 2004; Lin and Trejo, 2013). In contrast, activated PAR2 and  $\beta$ -arrestin form a stable complex that cointernalize into endocytic vesicles (DeFea et al., 2000; Stalheim et al., 2005). Remarkably, thrombin activation of the PAR1-PAR2 heterodimer expressed exogenously or endogenously resulted in substantial recruitment of  $\beta$ -arrestins that cointernalized with the heterodimer. Unlike the PAR2 protomer, recruitment of  $\beta$ -arrestin to the thrombin-activated PAR1-PAR2 heterodimer did not require the C-tail domain of PAR2 (Lin and Trejo, 2013), suggesting that  $\beta$ -arrestins interact with the PAR1-PAR2 heterodimer through a distinct interface. In addition,  $\beta$ -arrestins were shown to enhance thrombin-activated PAR1-PAR2 heterodimer stimulation of cytosolic ERK1/2 signaling; however, the role of  $\beta$ -arrestins in endothelial cytoprotective signaling was not examined.

Similar to the PAR1-PAR2 heterodimer, agonist activation of other GPCR heterodimers has been shown to cause differential recruitment of  $\beta$ -arrestins. A relevant example is the  $\mu$ -opioid receptor (MOR) and neurokinin-1 (NK1) receptor heterodimer recruitment of  $\beta$ -arrestin (Pfeiffer et al., 2003). MOR does not cointernalize with  $\beta$ -arrestins in HEK293 cells (Pfeiffer et al., 2003). However, upon dimerization with the NK1 receptor, activation of MOR resulted in cointernalization of the MOR-NK1 heterodimer together with  $\beta$ -arrestins to endosomes and caused delayed resensitization. Thus, the differential recruitment of  $\beta$ -arrestins to GPCR dimers versus monomers may be important for distinguishing their function.

### V. PAR1-PAR3 Heterodimer Formation and Signaling

The capacity of PAR1 to heterodimerize with PAR3 has been examined in HEK293 cells (McLaughlin et al., 2007) and in murine podocytes (Madhusudhan et al., 2012). These studies showed that PAR1 and PAR3 form a heterodimeric complex and function to modulate the efficiency of G protein coupling and cytoprotective signaling.

The expression of PAR1-Rluc together with increasing amounts of PAR3-GFP in HEK293 cells yielded a hyperbolic increase in BRET, indicating that PAR1 and PAR3 interaction is specific. A similar BRET response was observed in cells expressing PAR3-Rluc and PAR3-GFP, suggesting that, in addition to forming heterodimers with PAR1, PAR3 can self-associate and form homodimers (McLaughlin et al., 2007). To evaluate the relative affinity of PAR1 for itself versus PAR3, the BRET<sub>50</sub> values were determined. This analysis revealed no major differences in the capacity of PAR1 to form homodimers versus heterodimers with PAR3. In contrast to the PAR1 homodimer, both thrombin and peptide agonist activation of the PAR1-PAR3 heterodimer caused a decrease in BRET after prolonged stimulation, which was attributed to differences in desensitization. However, these studies cannot exclude the possibility that decreases in BRET are due to conformational changes not associated with desensitization.

PAR3 also modulated the activity of PAR1 by potentiating its response to thrombin (McLaughlin et al., 2007). Although ablation of PAR3 expression by small interfering RNA had no effect on thrombin-stimulated increases in intracellular Ca<sup>2+</sup>, endothelial barrier permeability was significantly attenuated. To determine whether PAR3 affected the capacity of PAR1 to couple to distinct G protein subtypes, BRET assays were performed. The PAR1 homodimer was shown to associate with both G $\alpha_q$  and G $\alpha_{13}$ , and a change in net BRET was observed following thrombin incubation, indicating that agonist induced a conformational change in the receptor-G protein complex. The PAR1-PAR3

heterodimer appeared to interact with G $\alpha_q$  and G $\alpha_{13}$  similarly. However, an obvious difference in BRET signal was observed with G $\alpha_{13}$  interaction with PAR1-PAR1 homodimers versus PAR1-PAR3 heterodimers, suggesting that, in the presence of PAR3, PAR1 interaction with G $\alpha_{13}$  is different. These data combined with the functional responses suggest that PAR3 modulates the activity of PAR1 to preferentially couple to G $\alpha_{13}$  signaling.

The anticoagulant protease APC is known to cleave and activate PAR1 and PAR3 to promote cytoprotective signaling in endothelial cells and neurons (Mosnier et al., 2007), but how APC signals to cytoprotective responses in podocytes was not clear. APC was shown to cleave PAR3 and to inhibit apoptosis in mouse podocytes (Madhusudhan et al., 2012). Because murine PAR3 is not able to signal autonomously, a search for an associated signaling receptor was pursued and focused on PAR1, since mouse podocytes express a modest amount of PAR2 and no PAR4. Blocking antibodies directed against PAR1 or PAR3 attenuated the capacity of APC to inhibit apoptosis, suggesting that both receptors are required for APC cytoprotective signaling (Madhusudhan et al., 2012). Intriguingly, the addition of APC also induced endogenous PAR1-PAR3 heterodimer formation based on coimmunoprecipitation experiments, suggesting that PAR3 activation regulates dimer formation. This idea was confirmed using transfected mesangial cells and a cleavage-deficient murine PAR3 S38P mutant, which failed to interact with endogenous PAR1 compared with wild-type PAR3. Together, these findings indicate that APC cleavage of PAR3 drives interaction with PAR1, but mechanistically how this occurs is not known.

The early studies of GPCR dimer formation suggested that receptor-receptor interaction occurred during biogenesis (Bouvier, 2001), which is clearly the case for the class C GPCRs such as the GABA<sub>B1</sub>-GABA<sub>B2</sub> dimer (Pin et al., 2003). However, the nature of class A GPCR interaction remains controversial. The idea that class A GPCRs can form dimers is supported by a vast literature, including several recent high-resolution structures of CXCR4 (Wu et al., 2010), MOR (Manglik et al., 2012),  $\kappa$ -opioid receptor (Wu et al., 2012), and the  $\beta$ 1-adrenergic receptor ( $\beta$ 1-AR) (Huang et al., 2013); however, it remains unclear where, when, and how class A GPCR dimerization occurs. In fact, recent work suggests that certain class A GPCRs associate and dissociate rapidly at the cell surface. Studies using fluorescence recovery after photobleaching together with  $\beta$ 1-AR containing an N-terminal YFP and C-terminal-tagged CFP fusion and anti-YFP antibody to retard mobility showed that  $\beta$ 1-ARs transiently interact, whereas  $\beta$ 2-ARs formed stable dimers (Dorsch et al., 2009). A similar phenomenon using antibody immobilization and fluorescence recovery after photobleaching indicated D2-dopamine receptors form transient dimers

(Fonseca and Lambert, 2009). These findings raise important questions regarding the nature of transient GPCR association and dissociation. Studies of PARs have shown that, depending on the receptor pair, constitutive and ligand-induced dimer formation can occur, suggesting that PARs can form stable and transient interactions.

## VI. PAR1-PAR4 Heterodimer Formation

The capacity of hPAR1 to function as a cofactor for thrombin-induced hPAR4 activation in platelets and other cell types (Leger et al., 2006) suggests that the receptors associate with each other in a complex. To assess hPAR1 and hPAR4 interaction, coimmunoprecipitation assays of endogenous receptors from thrombin-treated human platelets were performed. Using anti-hPAR4-specific antibodies, hPAR1 was detected in hPAR4 immunoprecipitates, but not in immunoprecipitates from nonspecific IgG controls (Leger et al., 2006). These studies suggest that endogenous hPAR1 and hPAR4 are present in a complex. The formation of an hPAR1-hPAR4 complex was confirmed in COS7 cells ectopically expressing epitope-tagged receptors. PAR1-PAR4 association was also examined by FRET using a PAR1 C-tail truncation mutant fused to CFP and a PAR4 C-tail truncation mutant fused to YFP (Leger et al., 2006). In FRET studies, specific interactions are demonstrated by titration experiments with increasing amounts of YFP acceptor protein in the presence of a constant amount of CFP donor protein, which should yield a hyperbolic curve that saturates at a high acceptor-to-donor ratio (Lohse et al., 2012). FRET experiments between protein pairs that are unlikely to interact should yield a linear FRET signal with an increasing acceptor-to-donor ratio and serve as a control. Because the FRET titration experiments performed with hPAR1  $\Delta$ C-tail CFP and hPAR4  $\Delta$ C-tail YFP lacked data points in the linear range of the saturation curve, and since PAR C-tail deletion mutants displayed impaired signaling and trafficking (Shapiro et al., 1996; Leger et al., 2006; Ricks and Trejo, 2009); thus, further studies are necessary to confirm hPAR1 and hPAR4 dimer formation in living cells.

As with many approaches, FRET has limitations, including overlapping emission spectra of the donor and acceptor fluorophores resulting in a low signal to noise, photobleaching issues, and the large size of the fluorophore that could interfere with normal protein function. In recent studies, time-resolved FRET (TR-FRET) between a lanthanide and fluorophore conjugated to ligands for four different GPCRs was used successfully to monitor GPCR oligomerization in native tissues (Albizu et al., 2010). TR-FRET has a much higher signal to noise ratio and is an attractive approach to study PAR dimerization. The majority of approaches used so far to examine PAR dimerization have relied

on resonance energy transfer between receptors fused to fluorescent proteins, which cannot be applied to receptors expressed in native tissues. Although several specific antagonists for PAR1 have been generated (Ramachandran et al., 2012), there are currently no selective antagonists for PAR2, PAR3, or PAR4 which will hamper the use of TR-FRET to examine PAR heterodimerization. Although antibodies can be used with TR-FRET, the bivalent nature of antibody binding could stabilize non-native complexes. An emerging technology to study GPCR dimerization by TR-FRET involves the use of SNAP-tags. A SNAP-tag is an engineered variant of the human repair protein *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase protein that covalently reacts with benzylguanine substrates. As proof of concept, GABA<sub>B</sub> receptors containing N-terminal SNAP-tags were labeled with either europium cryptate or a red fluorophore d2, and TR-FRET was used to examine GPCR dimerization (Maurel et al., 2008). Thus, several new alternatives offer greater sensitivity and can be used to study PAR dimerization.

## VII. PAR2 Homo- and Heterodimerization

*A. PAR2 Intermolecular Liganding and Signaling.* PAR2 can self-associate to form homodimers and signal via an intermolecular liganding mechanism, as recently demonstrated (Sevigny et al., 2011). Similar to previous studies on PAR1, a functional reconstitution strategy was used with a noncleavable PAR2 R36A mutant, where the critical arginine was converted to alanine and a cleavable PAR2 R172, Q173 signaling-deficient mutant, in which the glutamine (Q) and arginine located in the DRY motif present at the end of TM III were transposed. As expected, HEK293 cells expressing the PAR2 R36A or RQ mutant alone failed to migrate or signal in response to proteolytic activation by trypsin. In contrast, coexpression of the PAR2 mutants resulted in recovery of cellular responses, suggesting that the tethered ligand domain of a signaling-defective PAR2 can function *in trans* to activate an adjacent PAR2. To confirm PAR2-PAR2 interaction, coimmunoprecipitation assays were performed using cells coexpressing differentially epitope-tagged receptors and revealed that T7-tagged PAR2 associated with myc-tagged PAR2 in COS7 cells (Sevigny et al., 2011). The authors further discovered that a PAR2-specific pepducin P2pal-18S that blocked agonist activation of PAR2 also inhibited PAR2 dimerization as assessed by functional reconstitution of mutant receptors expressed in HEK293 cells. The P2pal-18S pepducin also blocked endogenous PAR2-mediated human neutrophil migration and paw edema in mice (Sevigny et al., 2011). However, it remains unclear whether the effects of P2pal-18S are due specifically to inhibition of endogenous PAR2 monomers or homodimers in human neutrophils.

**B. PAR2-PAR3 Heterodimerization and Signaling.** In contrast to mouse podocytes, human podocytes express PAR2 and PAR3 and were evaluated as potential candidates for mediating APC-induced cytoprotective signaling (Madhusudhan et al., 2012). Both an anti-PAR3 antibody and small interfering RNA-mediated ablation of PAR3 abolished APC-promoted antiapoptotic effects, indicating that PAR3 is essential for this process. Podocytes lacking PAR2 also exhibited complete loss of APC-mediated antiapoptotic effects, whereas an anti-PAR2 SAM 11 antibody was without effect, indicating that PAR2 signaling, but not necessarily cleavage, is important. However, many PAR2 antibodies lack specificity (Adams et al., 2012); thus, further experiments were necessary to confirm these findings. To validate PAR2-PAR3 interaction, coimmunoprecipitation was used. Similar to PAR1-PAR3 association, APC activation of PAR3 induced heterodimerization with PAR2, an effect that was completely blocked with an anti-PAR3 antibody. These findings demonstrate a function for both PAR2 and PAR3 activity in APC-induced cytoprotection and suggest that activation of PAR3 has the capacity to modulate PAR2 activity, perhaps through formation of a physical dimer. Whether PAR2-PAR3 association is regulated through TM interactions and/or via donation of its tethered ligand domain remains to be determined.

**C. PAR2-PAR4 Heterodimerization and Anterograde Trafficking.** During biogenesis, GPCRs are properly folded through complex interactions with chaperones in the endoplasmic reticulum (ER) and then exported to the Golgi where additional modifications such as N-linked glycosylation occur (Achour et al., 2008). Previous reports have suggested that GPCR dimerization occurs early in the ER and is also important for export to the cell surface. Using HEK293 cells,  $\beta$ 2-AR mutants lacking an ER export motif or harboring an ER retention signal dimerized with wild-type  $\beta$ 2-AR and impaired its trafficking to the cell surface (Salahpour et al., 2004). Disruption of a putative  $\beta$ 2-AR dimerization motif GXXXGXXXL in TM VI also prevented wild-type  $\beta$ 2-AR trafficking to the plasma membrane, suggesting that the mutants exert a dominant affect. PAR4 was also found to contain a functional arginine-based ER retention signal within the second intracellular loop (Cunningham et al., 2012). It is intriguing that PAR2 coexpression enhanced PAR4 trafficking to the cell surface in both a keratinocyte cell line NCTC-2544 and HEK293 cells, indicating that PAR2 drives PAR4 trafficking through the biosynthetic pathway. As a consequence, PAR2 enhanced N-linked glycosylation of PAR4 and signaling (Cunningham et al., 2012). To evaluate PAR4-PAR2 interaction, coimmunoprecipitation and intermolecular FRET were used together with full-length PAR4 fused to CFP and PAR2 fused to YFP in HEK293 cells. The data suggest that PAR4-PAR2 association is robust in HEK293 cells,

which display a large reservoir of intracellular receptors, whereas in NCTC-2544 cells, receptors are present mainly on the cell surface and exhibited minimal FRET signal. These findings suggest that PAR4-PAR2 interaction is transient and may dissociate once the receptor dimer is trafficked to the plasma membrane, but this remains to be tested directly.

### VIII. PAR4 Homo- and Heterodimerization

The formation of PAR4 homodimers was recently examined by BRET and biomolecular fluorescence complementation (BiFC) (De la Fuente et al., 2012). In BiFC, PAR4 fused to either the N-terminal half of the Venus fluorophore or the C-terminal half of the Venus was expressed in HEK293 cells. The individual PAR4 split Venus variants did not exhibit fluorescence unless they were coexpressed in the same cells. Because split fluorescent proteins have a propensity to reassociate, critical controls are essential to demonstrate the specificity of GPCR interaction using BiFC strategies (Ejendal et al., 2013). Complementary experiments were performed with BRET and confirmed PAR4 homodimerization (De la Fuente et al., 2012). The PAR4-PAR4 homodimer interaction was then mapped using chimeras generated between PAR4 and rhodopsin, a GPCR that does interact with PAR4, and PAR4 point mutants using BRET. These studies showed that hydrophobic residues within the TM IV helix bundle mediate PAR4 dimer formation, and disruption resulted in diminished  $\text{Ca}^{2+}$  signaling. BRET was also used to demonstrate that murine PAR3 and PAR4 form constitutive homodimers and heterodimers when expressed in HEK293 cells (Arachiche et al., 2013). Given the presence of an ER retention signal in PAR4 (Cunningham et al., 2012), it will be important to determine if PAR4 trafficking through the biosynthetic pathway is regulated by homodimerization and/or heterodimerization with either PAR1 or PAR3, which to our knowledge has not been investigated.

The mechanisms that regulate class A GPCR dimerization remain poorly understood. Many studies have substantiated that class A GPCRs form homo- and heterodimers; however, only a few studies have confirmed dimerization in native tissues. Despite concern about the nature of class A GPCR dimerization, several high-resolution X-ray structures of class A GPCR dimers have been reported. The structure of the CXCR4 homodimer revealed a small interface between TM helices V and VI (Wu et al., 2010), whereas MOR dimerization occurred through a four-helix bundle formed by TM helices V and VI (Manglik et al., 2012). The  $\kappa$ -opioid receptor was crystallized as a parallel dimer interface formed by TM helices I, II, and VIII (Wu et al., 2012), and the  $\beta$ 1-AR displayed two dimer interfaces between TM I and TM II and between TM IV and TM V (Huang et al., 2013). A high-resolution

structure of PAR1 bound to the antagonist vorapaxar was also recently reported (Zhang et al., 2012), but it did not reveal any dimer formation. Similar to X-ray structures of GPCR dimers, it will be important to confirm that PAR4 dimer interface mapped to TM IV (De la Fuente et al., 2012) is not an anomaly by using multiple different approaches. Besides PAR4, virtually nothing is known about how PAR dimer formation is regulated. Because of the transient nature of class A GPCR dimers reported using more sophisticated methods (Dorsch et al., 2009; Fonseca and Lambert, 2009), we speculate that PAR dimers are also transient in nature but in some instances may be stabilized by interaction with adaptor proteins and/or by compartmentalization within specific membrane microdomains.

## IX. Conclusions

In the present review, we discuss numerous studies that provide evidence for PAR dimerization (Table 1). In many instances, dimerization of PARs has been implicated in enhancing the activation of an adjacent PAR. The cofactoring and dimerization of PARs have been shown to facilitate efficient G protein activation (Nakanishi-Matsui et al., 2000), allosterically modulate G protein coupling specificity (McLaughlin et al., 2007), and/or promote coupling to distinct effectors such as  $\beta$ -arrestins (Lin and Trejo, 2013). Despite substantial documentation of PAR dimerization, the mechanisms that govern receptor dimer formation and regulation of signaling responses are not known. Similar to other GPCR dimers, some PAR-PAR interactions are probably in dynamic equilibrium between monomers and dimers of varying stability, but may differ depending on the dimer pair. The capacity of PARs to form dimers may be affected by receptor activation, association with adaptor proteins, and/or localization in distinct membrane microdomains. PAR post-translational modifications may also make important contributions to dimer formation. The finding that PAR2 expression increases dimer formation with PAR1 (Kaneider et al., 2007; Lin and Trejo, 2013) indicates that, in certain pathologic conditions, PARs may exist as dimers or higher-order oligomers. This has important implications in drug development, since most therapeutics are being developed to target PAR monomers, and it is not clear how such drugs would affect signaling by dimers or oligomers. Thus, further studies are essential to fully understand the biologic significance of PAR dimerization and for the development of therapeutics that could selectively target PAR monomers, homodimers, or heterodimeric complexes.

### Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lin, Liu, Smith, Trejo.

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