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Signaling and Regulation of Protease-Activated Receptors 1 and 2 (PAR1 and PAR2)

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Signaling and Regulation of Protease-Activated Receptors 1 and 2 (PAR1 and PAR2)

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An Abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
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Abstract

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By Kelly L. McCoy

Unique aspects of PAR1 and PAR2 include their distinctive mechanism of activation, their multiplicity of G protein coupling, their differential signal regulation, and their contribution to remarkably diverse cellular processes. Recent evidence suggests that these closely related receptors regulate different physiological outputs in the same cell, though little is known about their comparative signaling pathways. Here we report that PAR1 and PAR2 couple to overlapping and distinct sets of G proteins to regulate receptor-specific signaling pathways involved in cell migration. We also investigated potential regulatory mechanisms in place to fine-tune PAR1 and PAR2 signaling. Their signaling must be tightly controlled since they are irreversibly activated and stimulate multiple G protein-linked pathways. My studies demonstrate that selective, cell type-specific G protein coupling to PAR1 and/or PAR2 may provide one such level of regulation. An additional level of PAR regulation may come from regulators of G protein signaling (RGS) proteins, which act as GTPase-activating proteins and thereby inhibit G protein signaling. Little is known about RGS regulation of PAR signaling but my data using purified proteins and intact cells suggest that RGS proteins have both distinct and overlapping effects on PAR1 and PAR2, including a capacity to modulate functional readouts of PAR signaling. Together, these studies demonstrate that PAR1 and PAR2 functionally couple to overlapping and distinct G protein-linked functional pathways, and these signaling events are regulated, at least in part, by specific RGS proteins in receptor- and G protein-dependent manners.

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List of Abbreviations

μ-opioid receptor	MOR
Blood-brain barrier	BBB
Bovine serum albumin	BSA
Central nervous system	CNS
Cholecystokinin receptor-2	CCK2R
Dulbecco's modified Eagle medium	DMEM
Enhanced chemiluminescence	ECL
Extracellular-regulated kinase	ERK
Fetal bovine serum	FBS
G protein-coupled receptor	GPCR
G protein-receptor kinase	GRK
GTPase-activating protein	GAP
Guanine nucleotide exchange factor	GEF
Inositol phosphate	InsP
Melanin concentrating hormone receptor 1	MCH1R
Mitogen-activated protein kinase	MAPK
Muscarinic acytelcholine receptor	mAChR
N-methyl-D-aspartic acid	NMDA
Opioid-receptor-like 1	ORL-1
PAR-activating peptide	PAR-AP
Peripheral nervous system	PNS
Pertussis toxin	PTX
Phosphate buffered saline	PBS
Phosphatidyl inositol 4,5-bisphosphate	PIP2
Phosphoinositide	PI
Phosphoinositide-3 kinase	PI3K
Phospholipase C	PLC
Protease-activated receptor	PAR
Protein kinase A	PKA
Protein kinase C	PKC
Regulator of G protein signaling	RGS
Transient receptor potential	TRP
Tris buffered saline	TBS

CHAPTER 1: Introduction[1]

[1] A version of this chapter has been published elsewhere: McCoy KL and Hepler J R (2009) Regulators of G Protein Signaling (RGS) Proteins As Central Components of G Protein-Coupled Receptor Signaling Complexes. *Progress in Molecular Biology and Translational Science* **86**:49-74.

1.1. Cardiovascular Disease and Stroke

1.1.1. Overview

In the United States, heart disease is the leading cause of death and stroke is the third most common cause of death (Lloyd-Jones et al., 2010). Currently, more than one in every three Americans is living with a cardiovascular disease, which includes high blood pressure, coronary heart disease, heart failure, stroke, or congenital heart defects. Combined, these diseases are expected to cost the United States $503.2 billion in 2010 (Lloyd-Jones et al., 2010).

1.1.2. Stroke prevention in individuals living with cardiovascular disease

Several types of cardiovascular disease are major risk factors for stroke. For example, atherosclerosis, atrial fibrillation, and heart failure all increase a person's chance of having a stroke. Also, stroke and cardiovascular disease have many overlapping risk factors, including obesity, high low-density lipoprotein (LDL) levels, hypertension, smoking, diabetes, and physical activity.

People with cardiovascular risk factors for stroke may take anticoagulant medications to prevent blood clots from forming and decrease their risk of having a stroke. Anticoagulants function by blocking steps in the coagulation cascade, which is a series of enzyme reactions that are activated when coagulation factors in the circulation contact tissue factor and ultimately leads to blood clot formation (Coughlin, 2005). Vitamin K antagonists, including warfarin, are widely prescribed in the United States, and function by blocking vitamin K, an essential cofactor for many coagulation factors.

Although the efficacy of warfarin has been proven for decades, tens of thousands of American die from fatal hemorrhaging as a result of its use each year (Cundiff, 2009). Another commonly used anticoagulant is heparin, which was discovered in 1916 and is one of the oldest drugs that still is widely prescribed today. Heparin activates antithrombin, an inhibitor of thrombin. Thrombin is involved in the final step of the coagulation cascade, which consists of thrombin cleaving fibrinogen to fibrin, which makes up the fibrous portion of a blood clot. As such, thrombin is the main effector protease of this enzyme cascade, and by blocking its activity with heparin, blood clot formation is less likely to occur.

However, similar to warfarin, heparin and its low molecular weight derivatives have been associated with potentially fatal bleeding events (Rosenberg, 1975). Due to the limitations of currently available anticoagulation medications, there is a need to discover and implement newer, safer therapeutics, and this area is under active investigation by many large pharmaceutical companies. For example, direct thrombin inhibitors such as bivalirudin and argatroban have been FDA-approved and a third inhibitor, dabigatran, may soon receive FDA-approval and will potentially replace the older, drugs that carry potentially life-threatening side effects.

Another area of active investigation is assessing the inhibition of the thrombin receptor, which also is called protease-activated receptor-1 (PAR1). Thrombin is formed when factor Va catalyzes the conversion of prothrombin to thrombin near the end of the coagulation cascade. This cascade occurs near cells, and therefore, newly formed thrombin is spatially positioned to act on cell surfaces (Coughlin, 2005). PAR1 is located within cell membranes and has been implicated in platelet activation, an essential step in

platelet aggregation that contributes to blood clot formation (Coughlin, 2005). Therefore, antagonists of this receptor may also be useful in preventing blood clots and stroke. These drugs currently are being tested in clinical trials, and may eventually be used to prevent clot formation without increasing the risk of bleeding (Macaulay et al., 2010).

1.2. Protease-Activated Receptors

1.2.1. Overview

The first member of the protease-activated receptor (PAR) family was discovered as a receptor for thrombin in 1991 (Vu et al., 1991a). As such, the thrombin receptor, which is now referred to as PAR1, is best known for its role in blood coagulation, hemostasis and thrombosis (Coughlin, 2005). A more profound understanding of this receptor and the cloning of three more PARs (PAR2-4) (Nystedt et al., 1994;Ishihara et al., 1997;Xu et al., 1998) has implicated these receptors in strikingly diverse pathophysiological processes including platelet activation, stroke, reactive gliosis, pain, inflammation, and cancer metastasis (Ossovskaya and Bunnett, 2004).

PARs are expressed in many overlapping and distinct tissues. Throughout the body, PARs have a wide distribution pattern and are present in endothelial cells, platelets, smooth muscle cells, skin cells, neutrophils, leukocytes, neurons, and glia (reviewed in (Macfarlane et al., 2001;Coughlin, 2005;Bunnett, 2006;Traynelis and Trejo, 2007)). PAR1, PAR3 and PAR4 are mainly expressed in vascular tissues. Within these tissues, the important role of PARs in platelet activation, blood coagulation, and maintaining hemostasis are well characterized. PAR2 also is found in vascular cells but is highly

expressed in gastrointestinal and bronchial smooth muscle cells as well. Other functions of PARs in these diverse tissues include recruiting immune cells to injury sites, stimulating the release of cytokines and other inflammatory mediators, disrupting endothelial cell barriers, spurring reactive gliosis in the central nervous system, and inducing hyperalgesia in the peripheral nervous system (reviewed in (Macfarlane et al., 2001;Coughlin, 2005;Bunnett, 2006;Traynelis and Trejo, 2007)). Notably, the majority of the studies of PARs in the nervous system have focused on PAR1 and PAR2, which are found in both the central nervous system (i.e., in neurons and glia) as well as in the peripheral nervous system (i.e., in sensory neurons) (Traynelis and Trejo, 2007). However, the precise roles that these receptors play in these diverse pathophysiological processes are not yet fully understood. Because PARs are highly expressed in many different cells and since these receptors have been implicated in an array of physiological processes, understanding the pharmacological properties that differentiate these receptors from other GPCRs is an important step in understanding the molecular details that underlie these processes.

Unique aspects of PARs include their distinctive mechanism of activation, their multiplicity of G protein coupling, their differential signal regulation, and their contribution to remarkably diverse cellular processes. These characteristics of PAR activation and signaling are listed in Table 1-1 and are depicted for PAR1 and PAR2 in Figure 1-1. Irreversible cleavage of PAR N-termini by serine proteases initiates G protein-linked signal transduction. All of the PARs that have been reported to couple to G proteins have the unusual capacity to promiscuously activate multiple families of G proteins (e.g., $G_{q/11}$, $G_{12/13}$, and/or $G_{i/o}$). In doing so, PAR activation has been associated

with phospholipase C (PLC) activation, intracellular calcium mobilization, mitogen-activated protein kinase (MAPK) signaling, Rho GTPase activation through activation of Rho guanine nucleotide exchange factors (RhoGEFs), and other signaling pathways. Given that PARs activate such an array of signaling cascades and that they are widely expressed in many different tissues, these receptors have been associated with diverse physiological functions including platelet activation, cellular proliferation, inflammation, endothelial cell barrier disruption, smooth muscle relaxation and contraction, immune cell migration, and others (reviewed in (Macfarlane et al., 2001;Traynelis and Trejo, 2007)).

	PAR1	PAR2	PAR3	PAR4
Agonists	Thrombin Factor Xa Trypsin Plasmin APC	Trypsin Tissue factor Factor VIIa Factor Xa Tryptase Proteinase 3	Thrombin	Thrombin Trypsin CapathesinG
Activating peptides	SFLLRN TFLLR	SLIGRL LIGRLO	None	GYPGQV AYPGKF
Cleavage residue	Arg41	Arg34	Lys38	Arg47
Hirudin-like domain?	Yes	No	Yes	No
G protein coupling	$G_{q/11}$, $G_{i/o}$, $G_{12/13}$	$G_{q/11}$, maybe $G_{12/13}$, $G_{i/o}$	None reported	$G_{q/11}$, $G_{12/13}$

APC, activated protein C; Arg, arginine; Lys, lysine

Table 1-1. Activation and G protein signaling of PAR1-4.

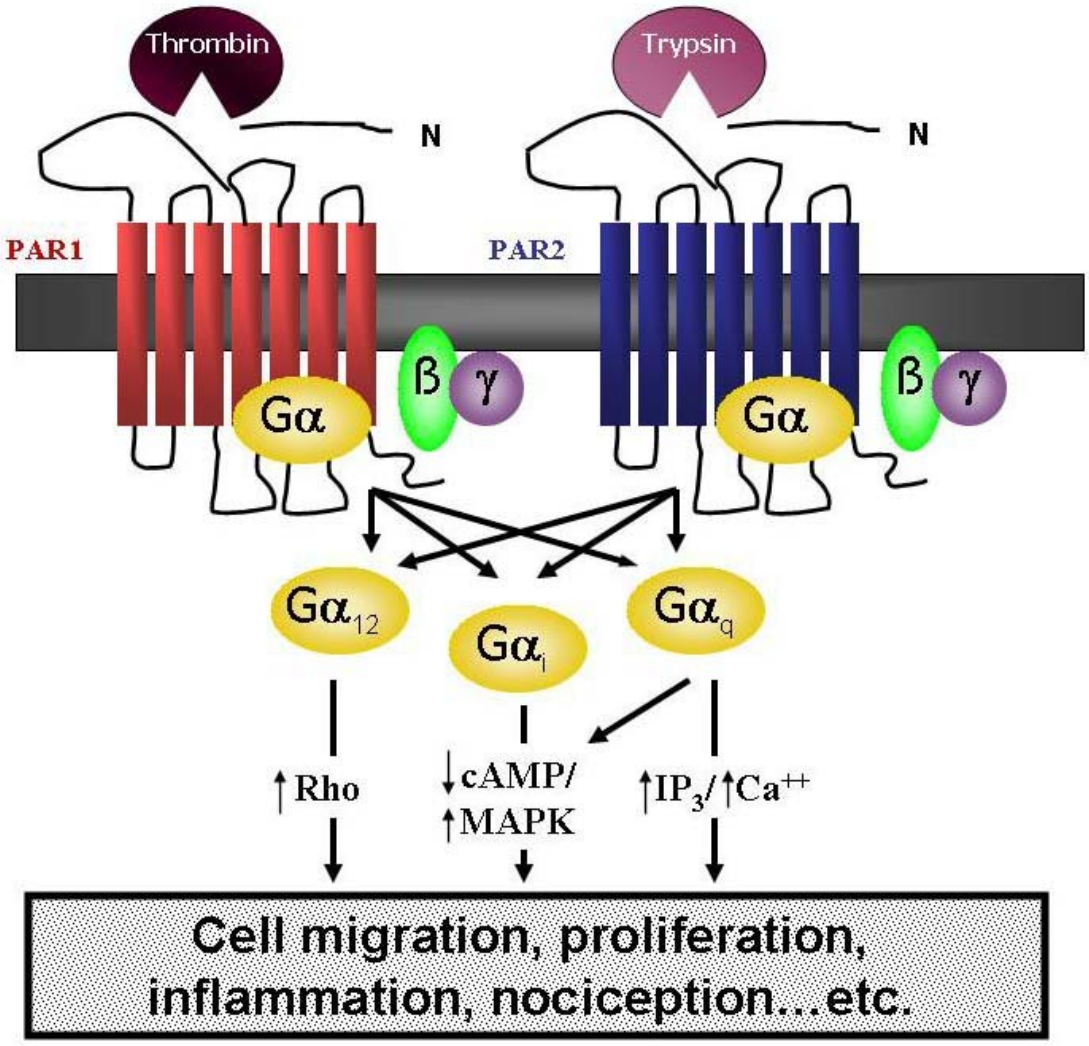

Figure 1-1. Activation and signaling mechanisms of PAR1 and PAR2.

1.2.2 Conventional GPCR Activation and Signaling

PARs are members of the largest family of cell surface receptors, the G-protein-coupled receptors (GPCRs). Approximately 1% of the mammalian genome is composed of genes encoding GPCRs, and in some cells, up to 5% of the total protein content is made up of these receptors (reviewed in (Hermans, 2003)). There are six subfamilies of GPCRs, denoted A-F, and PARs are part of the largest group, the class A subfamily (Lagerstrom and Schioth, 2008). Class A GPCRs are a heterogeneous group of more than 670 full-length receptors. Receptors within this subfamily typically have short N termini and no conserved domains. However, PARs, which have N-terminal cleavage sites and protease-binding domains, are notable exceptions to this rule.

Conventional GPCRs become activated when an agonist binds to the receptor, causing it to undergo a conformational change, thereby promoting its interaction with linked heterotrimeric G proteins. As their name implies, heterotrimeric G proteins are a complex of three subunits, Gα, Gβ, and Gγ, when they are in their resting state (i.e., when the Gα subunit is GDP-bound). Typically, receptors couple to only one G protein but evidence for GPCR linking to multiple G proteins also exists (Hermans, 2003;Riobo and Manning, 2005;Katanaev and Chornomorets, 2007). When receptors are activated by their cognate agonists, they relay the extracellular signal inside the cell to their bound G protein. Upon doing so, the GDP molecule on the Gα subunit is exchanged for GTP. Once GTP-bound, Gα subunits dissociate from the Gβγ complex, and both components are then free to interact with effector proteins and stimulate downstream signaling cascades. The duration of the signaling event is determined by the lifetime of the GTP bound to the Gα subunit that, in turn, is dictated by the intrinsic GTPase activity of the

Gα. In some cases, Gα GTPase activity may be accelerated when a Gα interacts with its effector protein (Ross and Berstein, 1993). Upon activation, G proteins have diverse physiological outputs depending on which family they belong to. In general, G_s activates adenylyl cyclase, $G_{q/11}$ mobilizes intracellular calcium through PLCβ, $G_{i/o}$ inhibits adenylyl cyclase and stimulates MAPK signaling, and $G_{12/13}$ activates Rho signaling through RhoGEF activation.

1.2.3. PAR Activation and Signaling

1.2.3.1. PAR activation—Unlike canonical GPCRs, PARs are not activated by agonists binding and provoking them to undergo conformation changes. Rather, they are enzymatically activated when their N-termini are proteolytically cleaved by serine proteases (Figure 1-1). These enzymes have important functions in biological processes, notably in blood coagulation and wound repair. Thrombin is the prototypical PAR1 agonist but it also activates PAR3 and PAR4. Trypsin is the prototypical activator of PAR2, which is the only PAR that cannot be activated by thrombin. Other serine proteases also have been shown to activate PARs. PAR1 can be activated by coagulation factor Xa, trypsin, plasmin and activated protein C. PAR2 is the only PAR that cannot be activated by thrombin but it can be activated by tissue factor, factor VIIa, factor Xa, and mast cell tryptase (Bunnett, 2006;Traynelis and Trejo, 2007). PAR3 and PAR4 activation has primarily been attributed to thrombin; however, cathepsin G and trypsin also have been reported to be an endogenous PAR4 activator (Sambrano et al., 2000;Bunnett, 2006).

The mechanisms by which serine proteases activate PARs have been intensely studied and well-characterized. In the case of PAR1, thrombin binds to a hirudin-like domain contained between amino acid residues 51 and 63 in the receptor's N-terminus (Vu et al., 1991b). Once bound to PAR1, thrombin cleaves a peptide bond located after the receptor's Arg41 residue. In doing so, a new PAR1 N-terminus is generated, revealing a six amino acid tethered ligand, SFLLRN, that intramolecularly interacts with the second extracellular loop of PAR1, thereby activating the receptor (Chen et al., 1994). Similar to the PAR1 mechanism of activation, PAR2 is cleaved by its prototypical agonist, trypsin, at the receptor's N-terminal Arg34 amino acid residue. The tethered peptide unmasked by this cleavage event, SLIGRL, is the intramolecular ligand for PAR2. When PAR3 was cloned, its cleavage site was found to be located after the receptor's Lys38 residue, which unmasks a tethered ligand composed of the amino acids TFRGAP. Similar to PAR1, the N-terminus of PAR3 also contains a hirudin-like binding sequence for thrombin, FEEFP, located just downstream of its cleavage site (Ishihara et al., 1997), which allows thrombin to bind to the receptor and cleave it. The PAR4 thrombin cleavage site is located after its Arg47 residue, revealing its activating peptide sequence, GYPGQV. Unlike PAR1 and PAR3, PAR4 has no hirudin-like binding site for thrombin, which prevents low concentrations of thrombin from activating it since the receptor has no way to sustain an interaction with thrombin long enough for it to be cleaved (Xu et al., 1998). Interestingly, PAR3, which potentially sits adjacent to PAR4 since both receptors are expressed in many of the same cells, including murine platelets (Ishihara et al., 1997), has a high affinity for thrombin due to its hirudin-like domain. However, PAR3 has never been shown to initiate intracellular signaling. Therefore, it is thought to act as a cofactor for

PAR4 by bringing thrombin in close proximity to it, which promotes PAR4 cleavage and subsequent activation (Nakanishi-Matsui et al., 2000).

Synthetic activating peptides corresponding to the tethered ligand amino acid sequences revealed following proteolytic cleavage of PARs (i.e., SFLLRN for PAR1, SLIGRL for PAR2, and GYPGQV for PAR4) and derivatives of these sequences are commonly used in experimental settings to selectively activate PAR1, PAR2, or PAR4, respectively. Interestingly, the peptide sequence that corresponds to PAR3's tethered ligand, TFRGAP is inactive (Ishihara et al., 1997).

1.2.3.2. G protein signaling linked to PAR activation—Unlike typical GPCRs, which couple to one family of heterotrimeric G proteins, three members of the PAR family, PAR1, PAR2, and PAR4, have been reported to activate multiple G proteins (Hung et al., 1992;Offermanns et al., 1994;Post et al., 1996;Schultheiss et al., 1997;Faruqi et al., 2000). Due to its unique role as a cofactor for PAR4 signaling in murine models, PAR3 coupling to G proteins and linked signaling pathways remains to be characterized. When it was cloned, it was shown to activate InsP signaling in human bone marrow cells and mouse megakaryocytes (Ishihara et al., 1997), but whether this signal was initiated due to its interaction with a G protein or to its role as a cofactor for PAR4 is unknown.

Evidence for PAR/G protein interactions were first demonstrated in cells known to be thrombin responsive, CCL-39 cells, which are Chinese hamster lung fibroblasts. Upon activation with thrombin or the PAR1 activating peptide, measurable levels of InsPs, indicative of $G_{q/11}$-mediated activation of PLC, and reduced cAMP levels, likely

due to $G_{i/o}$-mediated inhibition of adenylyl cyclase were detected (Hung et al., 1992). To ensure that PAR1 was actually mediating these downstream effects, the investigators mutated the PAR1 cleavage recognition sequence, LDPR, to the sequence recognized by the enterokinase cleavage receptor, DDDDK. Using a cell line that is known to be unresponsive to enterokinase, the mutant PAR1 receptor gained the capacity to induce InsP production and adenylyl cyclase inhibition in response to stimulation with entrokinase (Hung et al., 1992). These findings provided the first evidence that PARs couple to at least 2 different G proteins.

A few years later, a requirement for G_{12} in PAR-stimulated gene expression and DNA synthesis was reported in 1321N1 astrocytoma cells and COS-7 cells (Aragay et al., 1995;Post et al., 1996), thereby potentially linking PAR activation to a third family of G proteins. In the astrocytoma cells, PAR1 stimulation led to Ras-dependent, AP-1-mediated transcriptional activation and DNA replication (Aragay et al., 1995). This effect was abrogated by both a G_{12}-directed inhibitory antibody and a dominant negative Ras construct, thereby implicating G_{12} and Ras in these processes (Aragay et al., 1995).

An initial screen for direct PAR1/ G protein interactions confirmed that G_{i2} and $G_{q/11}$ co-immunoprecipitate (co-IP) with PAR1 in human neuroblastoma cells (Ogino et al., 1996) but interactions with the $G_{12/13}$ family were not investigated. Together, these initial studies (and others published during the same timeframe) provided the first evidence that PARs couple to multiple G proteins and linked signaling pathways.

Although many of the initial studies on PAR-stimulated intracellular signaling focused on investigating effects of thrombin (i.e., through PAR1, PAR3, and PAR4) and not trypsin (i.e., through PAR2), more recent efforts have provided a better understanding

of PAR2 and its signaling capacity. Both trypsin and the PAR2 activating peptide (PAR2-AP, LIGRLO) increase InsP production and mobilize calcium in kidney and intestinal epithelial cells stably expressing PAR2, suggestive of $G_{q/11}$ coupling (Bohm et al., 1996). Early reports also showed that PAR2-mediated increases intracellular calcium signaling have a PTX-sensitive (i.e., $G_{i/o}$-mediated) component in *Xenopus laevis (X. laevis)* oocytes (Schultheiss et al., 1997). In addition, early western blot analyses revealed that trypsin activates MAPK signaling, including c-Raf activation and ERK1/2 phosphorylation in rat aortic smooth muscle cells and bovine pulmonary arterial fibroblasts (Belham et al., 1996), which may be a consequence of either $G_{q/11}$ or $G_{i/o}$ coupling. PAR2 coupling to $G_{12/13}$ is not as well-characterized, although, PAR2 activation has been associated with rho signaling pathways in human umbilical vein endothelial cells (HUVEC) and in alveolar type II endothelial (A549) cells (Vouret-Craviari et al., 2003;Yagi et al., 2006). Typically, pathways associated with Rho signaling are attributed to $G_{12/13}$. Despite these findings and many studies that have since confirmed PAR2 signaling through second messenger pathways, no studies have ever reported direct PAR2 coupling to G proteins.

With the studies described above serving as the foundation for what is known about PAR signaling, it is now generally accepted that PARs link to multiple G proteins (Figure 1-1). It is known that PAR1, PAR2 and PAR4 all stimulate $G_{q/11}$-mediated PLC activation, thereby increasing InsP production and intracellular calcium levels, and they all initiate G_{12}-mediated Rho activation (reviewed in (Coughlin, 2005)). Roles for PAR/$G_{i/o}$ signaling are not as well defined since they seem to be cell type-specific and the findings are sometimes contradictory. A link between PAR1 and Gi/o-stimulated MAPK

signaling was first discovered in thrombin-responsive cells, CCL-39 fibroblasts (Kahan et al., 1992). In these cells, thrombin had previously been shown to be an unusually potent mitogen (Perez-Rodriquez et al., 1981). Therefore, Kahan and colleagues used kinase experiments and ^{3}H-thymidine incorporation techniques to correlate the mitogenic effects of thrombin with the activity of p44 MAPK, which also is referred to as ERK1 (Kahan et al., 1992). The mitogenic activity of thrombin also was found to be PTX-sensitive in these experiments. Later studies confirmed the involvement of p42/44 MAPKs in thrombin-stimulated mitogenesis by using p42/44 proteins containing point mutations in their phosphorylation sites (Pages et al., 1993). In cells expressing these mutants, ^{3}H-thymidine incorporation experiments found that thrombin-mediated cell proliferation was greatly reduced and a collagenase promoter assay with a chloramphenicol acetyltransferase reporter showed that gene transcription also was decreased (Pages et al., 1993). To further resolve the mechanism underlying PAR1-mediated MAPK activation, crosstalk with tyrosine kinase pathways—Ras in particular—were tested. Using dominant negative mutant proteins, Ras was shown to be necessary for thrombin stimulated ERK phosphorylation (as demonstrated in western blotting experiments) (Ellis et al., 1999). In these experiments, a non-specific tyrosine kinase inhibitor, genistein, also blocked MAPK signaling and cell proliferation, thereby confirming the role of tyrosine kinase/Ras pathway in thrombin-and PAR- induced MAPK activation. Currently, it is generally accepted that depending on the cell type being studied, PAR1 and possibly PAR2, inhibit adenylyl cyclase and activate pertussis toxin (PTX)-sensitive (i.e., $G_{i/o}$-activated) MAPK signaling resulting in the phosphorylation of ERK1/2 (reviewed in (Traynelis and Trejo, 2007)). $G_{i/o}$-linked signaling pathways also have been implicated in PAR1- but not

PAR4-mediated platelet activation (Voss et al., 2007). Therefore, although many reports have shown that PAR1 clearly links to $G_{i/o}$-mediated pathways, the evidence for PAR2 and PAR4 coupling to $G_{i/o}$ is less clear and further studies on this topic are warranted.

1.2.4. PARs in Physiological Processes

1.2.4.1. PARs as mediators of thrombin's actions in coagulation—Given that PARs promiscuously couple to several different G proteins and linked signaling pathways, it is not surprising that these receptors have been implicated in diverse functions throughout the body. Most notably, PARs are well-appreciated for their role as mediators of thrombin's actions in platelet activation and the coagulation cascade (Coughlin, 2005). In platelets, thrombin has been shown to activate PAR1 at low concentrations to stimulate PAR4 at high concentrations, thereby triggering platelet activation (reviewed in (Coughlin, 2005)). Intracellular signaling pathways that have been linked to in this process include PAR1- and PAR4-mediated activation of PLC and subsequent increases in intracellular calcium (Vaidyula and Rao, 2003;Holinstat et al., 2006) as well as PAR1-induced $G_{i/o}$-mediated PI3K signaling (Voss et al., 2007). Once activated, platelets change shape, and then produce and secrete factors that mediate platelet aggregation and blood coagulation (reviewed in (Coughlin, 2005)). Other than their role in platelet activation, PARs also have been extensively studied in endothelial cells where they contribute to blood vessel formation, disrupting endothelial cell barriers, and maintaining hemostasis (reviewed in (Macfarlane et al., 2001;Ossovskaya and Bunnett, 2004;Coughlin, 2005;Traynelis and Trejo, 2007)). Because many studies and recent reviews have focused on the role of PARs in platelet activation, hemostasis, and

thrombosis, this overview of PAR physiology now moves away from similar discussion and instead touches on the role of PARs in the nervous system.

1.2.4.2. Roles of PARs in the nervous system—In addition to their well-characterized roles in hemostasis and blood coagulation, PARs also are expressed throughout the peripheral nervous system (PNS) and central nervous system (CNS). In the periphery, PAR1 and PAR2 are both expressed by sensory neurons where emerging roles for these receptors in inflammation and pain are currently being characterized (Bunnett, 2006). The expression of PARs and their functions in the CNS are better understood. Interestingly, all four PARs have been found in various regions of the brain (Striggow et al., 2001). PAR1 is found mainly in the CA2 and CA3 regions of the hippocampus, and also is expressed in the hypothalamus, thalamus, striatum, cortex, and amygdala at low levels. PAR2 and PAR3 also are highly expressed in the hippocampus, and are found in every layer of the cortex, hypothalamus, thalamus, striatum, amygdala, and the medial habenular nucleus as well. Similar to the other PARs, PAR4 also is primarily expressed in the hippocampus, as well as in the cortex, hypothalamus, thalamus, and amygdala. (Striggow et al., 2001).

The expression of PARs in the CNS changes during development and upon damage to the brain tissue. By using in situ hybridization techniques, Niclou and colleagues found that PAR1 mRNA is widely expressed throughout the developing rat brain (Niclou et al., 1998). In adults, the expression pattern is different with a smaller overall distribution but high PAR1 mRNA levels found in dopaminergic neurons, thalamic and brainstem nuclei, the olfactory bulb, and Purkinje cells (Niclou et al., 1998).

PAR1 mRNA also has been identified in astrocytes, as demonstrated by its colocalization with glial fibrillary acidic protein (GFAP) (Weinstein et al., 1995). These studies also demonstrated that prothrombin and PAR1 have overlapping and distinct expression patterns through cRNA hybridization techniques (Weinstein et al., 1995). Together, these findings suggest that PARs and their activators may have physiological roles in the nervous system.

The PAR agonists, thrombin and other serine proteases, also have been found in the brain. Here, these enzymes have been implicated in synaptic plasticity (Pang et al., 2004), repair and recovery processes following ischemic stroke (reviewed in (Xi et al., 2003a)), Alzheimer's Disease (Akiyama et al., 1992) (Suo et al., 2003), and Parkinson's Disease (Hamill et al., 2007). Thrombin has been shown to be endogenously expressed in brain tissues (Deschepper et al., 1991), and it is possible that it also is produced there. Its precursor, prothrombin, has been found in both neuronal and glial cells, at the mRNA (PCR, Northern Blotting, and in situ hybridization) level (Dihanich et al., 1991;Weinstein et al., 1995). However, whether the conversion of prothrombin to thrombin actually occurs in CNS tissues *in vivo* remains unknown.

In addition to the presence of endogenous PAR activators in the brain, various pathophysiological conditions such as a traumatic CNS injury and stroke can lead to the infiltration of serine proteases into brain tissue. Hemorrhagic stroke is a particular type of cerebrovascular insult in which blood vessel rupture results in the breakdown of the blood-brain barrier (BBB) and the infiltration of blood-derived proteases into brain tissue. Since all four PARs are expressed in both glia and neurons (reviewed in (Macfarlane et al., 2001;Traynelis and Trejo, 2007)), this leakage of serine proteases provides PAR

activators with direct access to the receptors. In this context, PARs potentially serve neuromodulatory roles following stroke and the resulting ischemia. With regard to stroke, accumulating evidence suggests that PAR1 and PAR2 in particular, may play crucial roles in reactive gliosis following injury to the CNS (Striggow et al., 2000;Junge et al., 2003;Xi et al., 2003a;Junge et al., 2004;Olson et al., 2004;Nicole et al., 2005;Park et al., 2006). Specifically, they are thought to influence astrogliosis, which contributes to glial scarring and to the subsequent rebuilding of the BBB (Faulkner et al., 2004). Conflicting reports have implicated PAR1 specifically in both neurodegeneration and neuroprotection, depending on the concentration of the activating protease (reviewed in (Xi et al., 2003a)). Therefore, whether the effects of PARs are more beneficial or harmful to recovering brain tissue remains unresolved. Furthermore, the molecular details underlying the function of PARs in astrocytes remain incompletely characterized.

As in other cells, PARs activate various G protein-linked signaling pathways in astrocytes that underlie the functions of serine proteases and PARs in the brain. For example, PARs have long been known to induce proliferative responses in astrocytes. The role for PAR1 in astrocyte proliferation has been particularly well-studied. Mechanisms implicated in MAPK-induced astrocyte proliferation include the following: activation of tyrosine kinases by PAR1 (Grabham and Cunningham, 1995), $G_{i/o}$ stimulation of protein kinase C (PKC) (Debeir et al., 1996), $G_{i/o}$ -mediated phophoinositide-3 kinase (PI3K) activation with simultaneous PLC activation and intracellular calcium mobilization through $G_{q/11}$ (Wang et al., 2002b), and stimulation of Rho kinase activity and cyclin D1 (Nicole et al., 2005). Similar studies with PAR2 have

shown that like PAR1, it also stimulates MAPK signaling and ERK1/2 phosphorylation and intracellular calcium signaling to induce astrocyte proliferation (Wang et al., 2002a).

Other than having proliferative functions in astrocytes, PARs also are known to cause morphological changes (i.e., thickening and lengthening of cellular processes) following injury. Under normal physiological conditions, astrocytes exist in a star-like (stellate) shape. However, following cerebrovascular insults, astrocytes become reactive and lose their stellate morphology. Accumulating data suggests that PAR1 and PAR2 may be involved in mediating such morphological changes in these (and other) cells (Wang and Reiser, 2003;Park et al., 2006). In cultured astrocytes, forskolin and isoproterenol stimulate cAMP formation, which, in turn, induces astrocyte stellation. Interestingly, PAR1 reverses this morphological change, as shown using light microscopy, presumably by inhibiting increases cAMP production through $G_{i/o}$-mediated inhibition of adenylyl cyclase (Cavanaugh et al., 1990;Beecher et al., 1994). Other PAR1-activated signaling pathways involving Rho activation (Suidan et al., 1997) and $G_{12/13}$-induced cytoskeletal changes, including cell rounding (Majumdar et al., 1999), have been implicated as modulators of astrocyte morphology as well. Less is known about the role of PAR2 in this process but Park and colleagues recently showed that in cultured astrocytes, trypsin activation of PAR2 mediates stellation reversal through a calcium and PKC-dependent pathway (Park et al., 2006).

In addition to inducing astrocyte proliferation and morphology, other novel roles for PARs in the nervous system have been described in recent years. For example, in the brain, PAR1 has been reported to potentiate N-methyl-D-aspartic acid (NMDA) receptor signaling in a calcium-dependent manner. Upon activation of PAR1, astrocytes have been

shown to release glutamate, which promotes subsequent activation of NMDA receptors on the surface of proximal neurons (Lee et al., 2007). These findings suggest that PARs may play a role in synaptic transmission, which is consistent with recent reports that have implicated PARs in cognitive processes such as learning and memory (Almonte et al., 2007).

Inflammatory and pain responses within the nervous system may also involve PARs. Using selective PAR-APs, PAR knockout mice, antagonists of PARs, and antibodies that prevent activation of PARs, it has become evident that PARs and their activating proteases may be involved in inflammation and pain processes in many different tissues (Bunnett, 2006). In the vasculature, PAR1 is a well-known stimulator of inflammatory reactions at sites of tissue injury. In doing so, it recruits immune cells to wound sites, thereby contributing to tissue repair by, in part, mediating swelling and inflammation (Suo et al., 2004). Similarly, in the CNS, PAR1 has been shown to activate microglial cells upon injury to the brain (Suo et al., 2002). Microglial cells then migrate to the site of injury where they mediate neuroinflammatory reactions by secreting chemokines and cytokines, potentially to the detriment of CNS tissues (Hamill et al., 2005). The molecular details underlying the inflammatory roles of PARs in the CNS remain incompletely understood and further studies are needed to fully characterize these processes.

Intriguingly, findings outside the CNS, in sensory neurons, also have linked PAR activation and signaling to neurogenic inflammation and pain (Vergnolle et al., 2003). Some of these effects have been attributed to the interaction between PAR2 and transient receptor potential (TRP) channels, which are both expressed in dorsal root ganglion

neurons (Amadesi et al., 2004;Dai et al., 2004;Amadesi et al., 2006). The association between this receptor and channel was identified when PAR2 activation was shown to potentiate TRPV1 channel signaling, thereby modulating neurogenic inflammatory responses, causing edema, and inducing hyperalgesia (Amadesi et al., 2004;Dai et al., 2004;Amadesi et al., 2006;Grant et al., 2007). The mechanisms underlying these actions of PAR2 remain incompletely resolved but may involve PKCε, PKA, and/or PLC pathways that in turn regulate TRP channel functioning (Amadesi et al., 2004;Dai et al., 2004;Amadesi et al., 2006;Grant et al., 2007).

1.2.5. Regulation of PAR Signaling

Given that PARs have been implicated in strikingly diverse pathophysiological processes, are irreversible activated, and promiscuously signal through multiple G protein-linked pathways, these receptors require tight regulation. Typically, the duration of signals initiated by activated GPCRs are limited by receptor desensitization, which involves receptor phosphorylation by G protein-receptor kinases (GRKs) and binding of arrestins to these phosphorylation sites, thereby halting G protein signaling (Lohse, 1993;Krupnick and Benovic, 1998). In turn, arrestins also mediate receptor internalization through their interactions with internalization machinery, which includes clathrin and the clathrin adaptor protein-2 (Ferguson, 2001). Upon internalization, GPCRs are dephosphorylated and eventually returned to the cell membrane where they are once again poised to be activated and trigger intracellular signaling. PAR2 uses this classic paradigm of desensitization but its internalization mechanisms remain incompletely understood (Ricks and Trejo, 2009). The internalization of PAR1 is different. Its rapid

desensitization leads to internalization but unlike conventional GPCRs, PAR1 is not recycled back to the cell membrane; rather, it is sorted to lysosomes, where it is degraded (Hoxie et al., 1993;Trejo and Coughlin, 1999). The most likely explanation for the destruction of PAR1 in lysosomes is due to its cleaved N-terminus being absent, which would lead to its constitutive activation upon being recycled back to the plasma membrane (Trejo et al., 1998;Trejo and Coughlin, 1999). Details underlying the desensitization of PAR1 are not fully known but intriguingly, arrestins are not required for the process (Chen et al., 2004) like they are for PAR2 (Stalheim et al., 2005;Ricks and Trejo, 2009). Less is known about the desensitization and internalization of PAR3 and PAR4. Nothing is known about PAR3 and phosphorylation of PAR4 has not been shown directly but is most likely important for determining signal duration and promoting receptor internalization (Shapiro et al., 2000;Covic et al., 2000).

Other than desensitization and internalization, additional regulatory mechanisms of PARs may exist but have not been identified. For this dissertation, we sought to identify additional regulatory mechanisms of PAR1 and PAR2 in particular, given that they are known to be expressed in similar cells and tissues, signal through similar G proteins and linked pathways, yet tend to have divergent physiological effects. In doing so, we tested the capacity of regulator of G protein signaling (RGS) proteins, described below, to modulate PAR signaling.

1.3. RGS Protein Overview

A primary function of RGS proteins is to regulate the lifetime of G protein signaling events. Agonist activation of a GPCR triggers the exchange of GDP for GTP on a bound Gα, thereby stimulating the protein to initiate a downstream signaling cascade. The duration of the signaling event is determined by the lifetime of the GTP bound to the Gα subunit that, in turn, is dictated by the intrinsic GTPase activity of the Gα. In some cases, Gα GTPase activity may be accelerated when a Gα interacts with its effector protein (Hepler et al., 1997;Yan et al., 1997). However, in most cases, RGS proteins serve in this capacity as GTPase-activating proteins, or GAPs, for active Gα subunits to limit their signaling. In a cellular context, RGS proteins serve to fine tune GPCR and G protein signal transduction.

RGS proteins are both modulators and integrators of receptor and G protein signaling (Hollinger and Hepler, 2002). The RGS family has more than 30 members, all of which share a conserved 120 amino acid RGS domain that defines the family and confers the capacity to bind one or more active Gα–GTP subunits of heterotrimeric G proteins (De et al., 2000;Ross and Wilkie, 2000;Hollinger and Hepler, 2002). Early recognition of Gα/RGS interactions provided an appreciation for the important role of RGS proteins in cellular signaling (Ross and Wilkie, 2000). As a consequence of an RGS binding to a G protein subunit, signal duration was limited, which shed light on how RGS proteins are mechanistically involved in GPCR and G protein signaling. Recent studies suggest models whereby GPCRs act as docking platforms for G proteins and functionally related binding partners, including RGS proteins (Brady and Limbird, 2002;Hall and Lefkowitz, 2002;Neitzel and Hepler, 2006;Lutz et al., 2007;Shankaranarayanan et al.,

2008). Together, these multi-protein complexes share a common goal of targeted signal transduction. Below, we will summarize evidence that details the important role of RGS proteins in these GPCR/G protein complexes.

1.3.1. RGS protein structure determines function

Apart from their shared RGS domain, RGS proteins have diverse tertiary structures and functions that vary widely. The 37 identified proteins that contain RGS domains or RGS-like domains have been divided into subfamilies according to the shared sequence identities within these domains. Two nomenclatures have emerged for classifying RGS proteins, either non-descript alphabetical (subfamily A-H, etc) or, alternatively, abbreviations signifying a representative family member (e.g. the RZ subfamily, represented by RGSZ and the R4 family represented by RGS4) (De et al., 2000;Ross and Wilkie, 2000). Members of the A/RZ and B/R4 subfamilies are the smallest RGS proteins and consist of RGS domains flanked by small but variable N- and C-terminal regions. Because these proteins consist of little more than an RGS domain, their primary function is to bind active Gα-GTP and serve as GAPs, though evidence for other diverse signaling functions of these small RGS proteins has emerged (Tinker, 2006). By contrast, members of the C/R7, D/R12, E/RA, F/GEF, G/GRK, and H/SNX subfamilies are large, multi-domain proteins that range in size from 60 to 160kDa and have assorted functions that are not limited to modulating GPCR and G protein signal transduction (Siderovski and Willard, 2005;Willars, 2006).

The GAP activity of RGS proteins is contained within the RGS domain. Like some other GAPs, RGS proteins are not responsible for the actual hydrolysis of the GTP

molecule but induce a change in the active Gα-GTP complex which creates a much more favorable conformation for the complex to act as its own efficient hydrolase (Ross and Wilkie, 2000). However, unlike GAPs for monomeric Ras-like GTPases, RGS proteins utilize different amino acids that do not directly contribute to GTP hydrolysis (Ross and Wilkie, 2000). RGS domains also have the capacity to serve as binding sites for Gα and as effector antagonists (Hepler et al., 1997;Yan et al., 1997). In the case of the RGS domains of the G/GRK subfamily (Carman et al., 1999), this is their primary role as these proteins block $G\alpha_{q/11}$ signaling without any apparent GAP activity for Gα. The N- and C-terminal regions flanking the RGS domain also are important, as they provide RGS proteins with the capacity to form protein-protein and membrane interactions. It is through these domains and their interactions that RGS proteins vary widely from one another and gain a large degree of their specificity of function. As we will discuss below, these regions serve as binding sites for specific receptors and effectors.

1.3.2. RGS protein interactions with GPCRs

Compelling evidence from many independent studies now indicates that RGS proteins selectively interact with GPCRs to form functional pairs (Table 1-2). These studies have demonstrated that RGS protein interactions with receptors may be G protein-dependent, G protein-independent, or both—though which of these possibilities applies in individual cases remains to be clearly established. Considerable information is now available regarding how RGS proteins interact with G proteins (Ross and Wilkie, 2000). Early studies using purified proteins in reconstituted systems provided initial evidence that the RGS domain of specific RGS proteins can selectivity bind and regulate preferred

Table 1-2.
Interactions between GPCRs and RGS proteins

Direct RGS/ GPCR interactions

GPCR	RGS	Receptor binding region
α_{1A} adrenergic	RGS2	i3 loop
δ opioid	RGS4	C-terminus
μ opioid	RGS4	C-terminus
CCK2	RGS2	C-terminus
CXCR2	RGS12	C-terminus
M1 mAChR	RGS2, RGS8	i3 loop
MCH1	RGS8	i3 loop
ORL1	RGS19 (GAIP)	unknown

RGS/ GPCR interactions mediated by intermediate proteins

GPCR	RGS	Intermediate protein
α_{1B} adrenergic	RGS2, RGS4	spinophilin
μ opioid	RGS9-2	spinophilin, beta-arrestin-2
D2 dopamine	RGS19 (GAIP)	GIPC
M1-mAChR	RGS8	spinophilin

GPCR	RGS
δ opioid	RGS9
μ opioid	RGS1, RGS2, RGS4, RGS9, RGS10, RGS14, RGSZ1, RGSZ2
β2 Adrenergic	RGS2
5-HT1A	RGS4, RGS10, RGSZ1
5-HT2A	RGS2, RGS7
AT1A angiotensin II	RGS2, RGS5
D2 dopamine	RGS9-2
D3 dopamine	RGS19 (GAIP)
Endothelin-1 (ET-1)	RGS3, RGS4
GNRHR	RGS2, RGS3, RGS4
LPA	PDZrhoGEF
M2 mAChR	RGS4
M3 mAChR	RGS2, RGS3, RGS4
S1P1	RGS2, RGS3
S1P2	RGS1-3
S1P3	RGS1, RGS3, RGS4
Substance P	RGS8
Thrombin	LARG

Gα subunits. For example, members of the F/GEF subfamily exhibit high binding selectivity for $G\alpha_{12}$ and $G\alpha_{13}$, and members of the C/R7 and D/R12 subfamilies selectively bind to members of the $G\alpha_{i/o}$ family. By contrast, certain members of the B/R4 subfamily (RGS1-5, 8, 13, 16, and 18) have been shown to non-selectively bind to Gα subunits of the $G\alpha_{q/11}$ and $G\alpha_{i/o}$ subfamilies. Among these RGS proteins, RGS2 exhibits a strong apparent specificity for $G\alpha_{q/11}$ (Heximer et al., 1997), though this specificity may be receptor and/or cell type-dependent (Ingi et al., 1998;Heximer et al., 1999). The preference of RGS2 for binding to $G\alpha_{q/11}$ over other Gα subunits is determined by only a few defined amino acids in the RGS/Gα interface. Likewise, specificity of F/GEF RGS proteins for $G_{12/13}$ also is defined by specific amino acids. Taken together, these findings and others [reviewed in (Tinker, 2006)] clearly indicate that some level of signaling specificity is built into the RGS/Gα interaction.

Although some RGS proteins selectively interact with only certain Gα subunits, many others do not, and this apparent "promiscuity" raised the question of exactly how RGS/G protein selectivity is determined in a cellular environment. In the absence of cellular and molecular mechanisms with the capacity to dictate RGS/Gα selectivity, chaotic signaling would ensue. The first clue that such mechanisms exist came from studies on RGS regulation of receptor signaling in pancreatic acinar cells (Xu et al., 1999). Introduction of RGS1, RGS4, and RGS16 into these cells inhibited calcium signaling by $G_{q/11}$-linked by muscarinic acetylcholine receptors (mAChRs) with different potencies. However, these same RGS proteins inhibited cholecystokinin (CCK) receptor calcium signaling (also mediated by $G_{q/11}$) with a much lower (30-100 fold) potency or not at all (Xu et al., 1999). In stark contrast, RGS2 did not exhibit the same selectivity for

inhibition of CCK-calcium signaling but, instead, it blocked signaling by both muscarinic and CCK receptors in this system (Xu et al., 1999). In summary, while each of these RGS proteins had been shown to bind and inhibit $G_{q/11}$ signaling in isolation, their striking selectivity for inhibition of $G_{q/11}$ signaling depended upon their linked receptor when they were in a cellular context. In other words, RGS regulation of G protein signaling appeared to be dictated by the receptor, not the G protein. These studies provided the first indication that RGS proteins and receptors form preferred functional pairs to differentially regulate cellular signaling. In doing so, such GPCR/RGS pairs (shown in Table 1-2) could impart specificity and order to what otherwise could be chaotic signaling in cells. Based on these findings, a number of studies have focused on understanding cellular and molecular mechanisms underlying RGS interactions with receptors.

1.3.2.1. GPCRs interact directly with RGS proteins—GPCRs contain seven transmembrane-spanning domains, an extracellular N-terminus, three extracellular loops, three intracellular loops, and an intracellular C-terminus. Many signaling and regulatory proteins, most notably G proteins, GRK's and arrestins, have been shown to have precise binding sites on particular receptors' intracellular loops and C-tails (reviewed in (Ferguson, 2007;Moore et al., 2007)). Recent data supports that in other cases, interactions between GPCRs and proteins may be indirect and occur through intermediate scaffolding proteins [reviewed in (Hall and Lefkowitz, 2002)]. RGS proteins have been shown to modulate receptors in both manners—directly and indirectly. Considerable evidence now suggests that, at least for some GPCR/ RGS functional pairs, specific regions on the GPCR and RGS protein are responsible for dictating the direct binding that

occurs between these two proteins, though no consensus domains shared among receptors have been defined so far.

Early evidence suggested that the N-terminal portion of some RGS proteins might be responsible for selective receptor binding (Zeng et al., 1998). When the N-terminal region of RGS4 was truncated, leaving only the RGS domain intact, the potency of inhibition of receptor and Gq/11-stimulated calcium signaling by the truncated protein was 10,000-fold decreased compared to that of full-length RGS4 in pancreatic acinar cells (Zeng et al., 1998). These studies demonstrated a requirement for the N-terminus of RGS4 in determining RGS4 regulation of GPCR signaling. Independent of this work, the N-terminus of RGS12 was shown to directly interact with the C-tail of the interleukin-8 receptor, CXCR2 (Snow et al., 1998). RGS12 and the CXCR2 GPCR have complimentary PDZ domain and binding motifs, respectively, which facilitate the direct interaction that occurs at the C-terminus of the receptor. The physiological significance of this interaction remains to be demonstrated in cells since only the isolated receptor C tail was shown to interact with the PDZ domain of RGS12. Besides RGS12, the only other RGS protein that contains a PDZ domain is a splice variant of RGS3, which has been shown to interact directly with the PDZ binding motif for the ephrin-B receptor, a non-GPCR (Lu et al., 2001). While PDZ domains are not a general mechanism for RGS/GPCR interaction, these findings did suggest that RGS proteins can directly interact with GPCRs.

Other studies have demonstrated that direct RGS/GPCR interactions occur independent of a PDZ domain. The first study that documented such an interaction showed that RGS2, but not RGS16, binds directly to the third intracellular (i3) loop of the

$G_{q/11}$-coupled M1 mAChR (Bernstein et al., 2004a). RGS2 formed a stable complex with the i3 loop of M1 mAChR and Gqα indicating that RGS2 can serve as a bridge to bind both receptor and G protein simultaneously. The N-terminus of the RGS protein was reported to be responsible for binding to the receptor while the RGS domain of the protein bound to active, but not inactive $G\alpha_q$. Furthermore, phosphatidyl inositol 4,5-bisphosphate (PIP2) hydrolysis triggered by activation of the M1 mAChR was significantly decreased in the presence of purified RGS2, and this inhibition was dependent on the N-terminus of RGS2. By contrast, RGS2 did not bind to the i3 loops of either the Gi/o-linked M2 or M4 mAChRs. These findings supported the notion that this RGS2/M1 mAChR interaction is direct, selective and receptor-dependent, and that the N-terminus of RGS2 and the i3 loop of the receptor define the complex interface (Bernstein et al., 2004a). In follow-up studies (Hague et al., 2005), RGS2 also exhibited selectivity for specific adrenergic receptors. Specifically, RGS2 was shown to bind directly to the i3 loop of the α_{1A}-adrenergic receptor (α_{1A}-AR) but not the α_{1B}-AR. This interaction was demonstrated using purified protein pull-downs of the i3 loop of the receptor and the full-length RGS protein and was supported in cells by receptor-mediated recruitment of GFP-tagged RGS2 from the cytosol/nucleus to the plasma membrane by the α_{1A}-AR but not the α_{1B}-AR. Three discrete amino acids within the i3 loop of α_{1A}-AR were identified that were shown to be necessary for RGS recruitment to the receptor. When substituted with the corresponding amino acids from the i3 loop of the α_{1B}-ARs, α_{1A}-AR no longer bound RGS2, and RGS2 no longer modulated mutant receptor signaling in cells (Hague et al., 2005).

Consistent with these reports are others showing RGS protein binding to receptor i3 loop, specifically RGS8 binding to the melanin concentrating hormone receptor 1 (MCH1R) (Miyamoto-Matsubara et al., 2008) and to the M1-mAChR (Itoh et al., 2006). In the latter case, earlier studies had shown that RGS8 selectively modulated M1 mAChR signaling (Saitoh et al., 2002). In follow-up work, to examine mechanism, the authors found that RGS8 bound directly to the i3 loop of the M1 mAChR, that this interaction was mediated by a specific sequence (MPRR) in the N-terminus of RGS8, and that this binding was responsible for RGS8 modulation of receptor signaling (Table 1-2) (Itoh et al., 2006;Fujii et al., 2008a). This same group also examined RGS8 interactions with the MCH1R (Miyamoto-Matsubara et al., 2008). Both RGS8 and the MCH1R are highly expressed in the brain, thus indicating that they may physiologically interact in a normal cellular environment. RGS8 was shown to directly associate with the i3 loop of the MCH1R *in vitro*, similar to what has been observed for RGS8 and RGS2 modulation of M1 mAChR and of RGS2 modulation of α1A-AR, as discussed above. Co-localization of these proteins at the plasma membrane in HEK-293T and the attenuation of receptor-mediated calcium mobilization in the presence of RGS8 also were demonstrated in this study (Miyamoto-Matsubara et al., 2008). Together, these studies demonstrated direct interactions between GPCRs and RGS proteins, and defined an important role for the N-terminus of the RGS protein and for the i3 loop of the receptor as contact sites and determinants for selective GPCR/RGS protein interactions.

Recent studies have shown that RGS4 also interacts directly with certain GPCRs at regions other than the receptor i3 loop, most notably the C-tail. RGS4 was reported to bind directly to the C-termini of both the μ- and δ-opioid receptors in a complex with Giα

(Table 1-2) (Georgoussi et al., 2006), the first demonstration of an RGS protein directly interacting with the C-tails of receptors. In this study, RGS4 was reported to block μ-opioid receptor (MOR)-mediated inhibition of forskolin-stimulated adenylyl cyclase. However, these effects of RGS4 were not observed upon activation of the δ opioid receptor, suggesting that they are receptor-dependent (Georgoussi et al., 2006). Independent of these findings, RGS2 recently was shown to bind to the C-terminus of the cholecystokinin receptor-2 (CCK2R) (Langer et al., 2009). When activated with agonist, the CCK2R binds to discrete residues that lie within the N-terminus of RGS2. The residues on the CCK2R responsible for this interaction were shown to be located on its C-tail. An increased affinity for the binding of RGS2 was observed when two specific CCK2R amino acid residues, S434 and T439, were phosphorylated. The functional role of RGS2 in CCK2R signaling was demonstrated by its involvement in reducing CCK2R-mediated InsP production. In contrast, CCK2R-mediated signaling was reported to be insensitive to RGS8, also a member of the B/R4 family (Langer et al., 2009).

Still other studies have further confirmed an important role for the N-terminus of RGS protein in GPCR/RGS complex formation, without defining the involved receptor region. The opioid-receptor-like 1 (ORL1) receptor was shown to preferentially bind RGS19 (GAIP), while the μ, δ, and κ opioid receptors exhibit a greatly decreased affinity for this RGS protein (Xie et al., 2005). Results from this study showed that an N-terminally truncated form of RGS19 (GAIP) did not bind to the receptor, offering yet another example where the N-terminal region of the protein is necessary for this interaction (Xie et al., 2005). RGS4 also was shown to exhibit a range of affinities for

these opioid receptors, binding with highest affinity for the MOR and with the least affinity for the ORL1 receptor (Xie et al., 2005).

In summary, this collective body of work with various GPCRs (M1-AChR, α1-AR, CCK2, MCHR-1, MOR, δ-OR, ORL-1) and various RGS proteins (RGS2, RGS8, RGS4, RGS19) provide compelling evidence that certain RGS proteins directly and selectively interact with certain receptors to form preferred functional pairs.

1.3.2.3. Indirect GPCR/ RGS Protein Interactions—Growing evidence now indicates that, in some cases, RGS proteins also can functionally interact with specific GPCR's indirectly with the assistance of an intermediate scaffolding protein. The first such report showed that, following agonist activation, the D_2R recruits RGS19 (GAIP) to the plasma membrane (Jeanneteau et al., 2004). The authors demonstrated that this recruitment required a scaffold protein, GIPC (GAIP-interacting protein, C terminus), in order to occur. GIPC also was shown to be necessary for RGS19 to modulate D_2R-mediated inhibition of forskolin-stimulated cAMP accumulation. Although no other examples of GIPC bridging GPCRs to RGS proteins have been reported to date, these results provide the first evidence that some RGS proteins require scaffolding proteins in order for them to bind certain receptors and to function effectively (Jeanneteau et al., 2004).

Considerable recent attention has focused on the role of different scaffolding proteins in mediating GPCR/RGS interactions. Spinophilin, a large (~90 kDa) multifunctional scaffolding protein, has been shown to facilitate indirect RGS protein interactions with GPCRs. Previous work had established that this protein binds the i3

loops of a number of receptors including the D2R and the α2 adrenergic receptors (Smith et al., 1999;Richman et al., 2001;Brady et al., 2003). A subsequent study reported that spinophilin is involved in GPCR/RGS functional coupling (Wang et al., 2005). In this study, spinophilin was shown to directly interact with the N-terminus of RGS2 and to bind RGS1, RGS4, RGS16, and RGS19/GAIP as well. This interaction also was shown to have functional consequences since RGS2 modulation of adrenergic receptor signaling was enhanced in the presence of spinophilin. When co-expressed, RGS2 and spinophilin block the receptor-activated calcium-activated chloride current in *X. laevis* oocytes. These data indicate that RGS2, spinophilin and α1B-adrenergic receptors form stable ternary complexes that allow them to signal optimally in cells (Wang et al., 2005).

Separate studies suggest that certain opioid receptors also may use spinophilin to functionally interact with RGS proteins. Initial reports showed that the striatum-specific splice variant of RGS9, RGS9-2, blocks signaling by the μ-opioid receptor (as does the retina-specific RGS9-1) (Psifogeorgou et al., 2007;Xie et al., 2007). The functional effects of RGS9-2 on the μ-opioid receptor are to delay its agonist-induced internalization. Importantly, this study also showed that the MOR/ RGS9-2 complex can be co-immunoprecipitated out of PC12 cell lysates suggesting that these proteins form a stable complex. However, this interaction recently was shown to result from the formation of a multi-protein complex that includes the μ-opioid receptor, spinophilin, the GRK2, RGS9-2, and the $G\alpha_{i1}$ subunit (Charlton et al., 2008). Yet another report showed a different GPCR/RGS interaction to be modulated by spinophilin. As discussed above, RGS8 was shown previously to bind the i3 loop of the M1 mAChR (Itoh et al., 2006). A more recent follow-up report indicates an unexpectedly complicated interaction between

RGS/spinophilin and the receptor. The authors show that spinophilin binds to RGS8 at the same N-terminal residues of RGS8 (MPRR) that binds the M1 mAChR i3 loop. In the presence of spinophilin, RGS8 binding to the M1 mAChR is decreased but, its inhibition of receptor signaling is enhanced (Fujii et al., 2008b).

Indirect evidence also supports a role for spinophilin in mediating RGS protein regulation of adrenergic receptor signaling, in this case modulation of NMDA receptors in neuronal cortex derived from mice lacking the spinophilin gene and protein (Liu et al., 2006). In prefrontal cortical neurons, activating the α_1-AR (linked to $G_{q/11}$) and the α_2-AR (linked to $G_{i/o}$) results in a net decrease in the NMDA receptor excitatory post-synaptic current amplitude and whole-cell NMDA receptor current amplitude. The effects of α_1-AR on NMDA receptors were shown to be dependent on inositol phosphate and calcium, whereas the effects of α_2-AR on NMDA receptors relied on PKA and downstream ERK signaling. RGS2 and RGS4 each were tested for their capacity to negatively regulate the effect of these receptors on NMDA receptor signaling. Both RGS2 and RGS4 inhibited α_1-AR-regulated NMDA receptor currents, but only RGS4 had the capacity to block α_2-AR regulation of NMDA receptor currents. Of note, in brain slices from spinophilin knockout mice, α_1-AR regulation of the NMDA receptors was not observed but the effect of RGS4 on α_2-AR signaling was unaffected. These data suggest that the effects of the two adrenergic receptors on NMDA receptors are differentially regulated by RGS proteins (Liu et al., 2006) and that spinophilin mediates RGS2 actions.

In summary, considerable evidence now indicates that certain RGS proteins can form stable functional complexes with preferred GPCRs. These interactions can occur either through direct contact between N-terminus of the RGS protein and the receptor i3

loop and/or C-tail, or indirectly, though the assistance of an intermediate scaffolding protein such as GIPC or spinophilin.

While considerable evidence has emerged to indicate that RGS proteins and certain GPCRs can form stable complexes and preferred functional pairs (outlined above), there also is some evidence to the contrary. One study demonstrated that RGS2 and RGS4 fail to exhibit selectivity for inhibition of signaling by various $G_{q/11}$-linked muscarinic (M1, M3 and M5) receptors when controlled for protein expression (Bodenstein et al., 2007). A separate study also suggests that members of the B/R4 subfamily of RGS proteins do not selectively inhibit signaling by different $G_{q/11}$-linked GPCRs (Karakoula et al., 2008). This study showed that RGS2, RGS3, and RGS4 do not discriminate between binding to M3 mAChR receptor and the gonadotropin-releasing hormone receptor (GNRHR) (Karakoula et al., 2008). These examples of failures of RGS proteins to discriminate among receptors may be a result of the specific RGS/GPCR pairs examined or of the specific cellular systems used in these studies. It also is reasonable to propose that, in some cases, RGS proteins do not selectively interact with certain GPCRs, but inhibit receptor signaling by recognizing the linked G protein (shared in many cases among receptors), or the receptor/G protein complex, rather than the receptor itself.

1.4. Rationale and Objectives for this Dissertation

Since PARs are involved in diverse pathophysiological processes and therefore are potential future drug targets, gaining insight into their signaling and regulation is

important. Therefore, the goal of this dissertation is to provide a more complete understanding of these receptors, with a specific focus on PAR1 and PAR2. The vast majority of work in the PAR field has been devoted to these two receptors, which has laid the foundation for our hypothesis and specific aims.

PAR1 and PAR2 are expressed in many of the same tissues. Both receptors are irreversibly activated and have been reported to couple to the same G proteins and linked signaling pathways. However, sometimes PAR1 and PAR2 have the same effects in the same tissues (i.e., stimulating astrocyte proliferation) and sometimes they have opposing actions (i.e., having differential effects on pain and inflammation in the CNS and PNS). As such, these receptors may have remarkably different signaling and cellular responses despite being very closely related receptors that are expressed in the same cells. Gaining a detailed understanding of PAR signaling and regulation will provide insight into how these promiscuous receptors activate multiple G proteins and how they are regulated. Therefore, the working hypothesis for this work is that PAR1 and PAR2 have both shared and distinct signaling profiles that differentially regulate diverse physiological functions, particularly within in the CNS.

To meet these goals, I first sought to define differences in PAR1 and PAR2 G protein coupling, signal transduction, and functional outcomes, as described in Chapter 2. In doing so, I employed biochemical and molecular biology techniques, including receptor mutagenesis, co-immunoprecipitation binding studies and functional studies to measure ERK1/2 phosphorylation, InsP production, calcium signaling, and RhoA activation. In these studies, I have determined that PAR1 and PAR2 do exhibit distinct and overlapping signaling profiles in the cell lines tested (e.g., COS-7 and Neu7 cells). In

Chapter 3, I present findings which suggest that different families of G proteins interact with PAR1 through different receptor binding sites, thereby uncovering a potential mechanism that explains how receptors couple to multiple G proteins. Using similar techniques, I also aimed to determine whether PARs are regulated by RGS proteins, as described in Chapter 4. Nothing is currently known about RGS regulation of PARs. Therefore, our findings that RGS proteins selectively regulate PAR1 and/or PAR2 in receptor- and G protein-dependent manners are entirely novel.

For the vast majority of these studies, we used recombinant protein expression in the COS-7 cell line to address our aims. However, for some of these studies, we moved into more physiologically relevant cells, including a Neu7 astrocyte cell line and primary astrocyte cultures from mice. In doing so, we have found that PAR1 and PAR2 both trigger Neu7 cell migration but rely on overlapping and distinct signaling pathways to do so. In addition, we have used astrocytes from PAR1 knockout mice to partially explain how PAR1 couples to multiple G proteins. Taken together, these findings have highlighted potentially important mechanisms of PAR signaling in the brain.

CHAPTER 2: PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology[2]

[2] This chapter has been accepted for publication: McCoy KL, Traynelis SF, and Hepler JR (2010) PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. *Mol Pharm.* In press.

2.1. Introduction

Protease-activated receptors (PARs) are a family of four G protein-coupled receptors (GPCRs) that are irreversibly activated through proteolytic cleavage of their N-termini by serine proteases (e.g., thrombin, trypsin, plasmin and others). This cleavage creates new extracellular N-termini, which serve as tethered ligands that intramolecularly activate the receptors and initiate complex intracellular signaling events (Macfarlane et al., 2001;Traynelis and Trejo, 2007). PAR1 was first discovered as a receptor for thrombin (Vu et al., 1991a). As such, it is best known for its role in the cardiovascular system's coagulation cascade and hemostatic mechanisms (Coughlin, 2005). A broader understanding of PAR1 and the cloning of three additional PARs (PAR2-4) (Nystedt et al., 1994;Ishihara et al., 1997;Xu et al., 1998) has implicated them in strikingly diverse pathophysiological functions including stroke, inflammation, reactive gliosis, and cancer (Ossovskaya and Bunnett, 2004).

With regard to the role of PARs in stroke, mounting evidence implicates PAR1 and PAR2 in reactive gliosis following head injury and/or hemorrhagic stroke, which lead to the breakdown of the BBB of the CNS ((Traynelis and Trejo, 2007) and references therein)). Since PARs are expressed in both glia and neurons, as well as in many other cells (Macfarlane et al., 2001;Ossovskaya and Bunnett, 2004), this leakage of serine proteases into the CNS provides PAR activators with direct access to their receptors following stroke and ischemia. PARs are thought to influence astrogliosis, which contributes to glial scarring and to the subsequent rebuilding of the BBB (Nishino et al.,

1993;Pindon et al., 2000;Nicole et al., 2005). Conflicting reports have implicated PAR1 specifically in both neurodegeneration and neuroprotection, depending on the concentration of the activating protease ((Traynelis and Trejo, 2007;Hamill et al., 2009) and references therein). Whether these effects are more beneficial or harmful to recovering brain tissue remains unresolved. Furthermore, the molecular details underlying the function of PARs in these cells are not fully elucidated.

PAR1 and PAR2 often are expressed in the same cells. In mediating their physiological effects, these closely related receptors have been reported to activate multiple G protein-linked signaling pathways including MAPK, PLC, and intracellular calcium (Dery et al., 1998;Macfarlane et al., 2001;Traynelis and Trejo, 2007). PAR1 appears to functionally couple to one or more of the $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ subfamilies (Macfarlane et al., 2001;Traynelis and Trejo, 2007), and a previous screen for direct PAR1 binding partners found that G_{i2} and $G_{q/11}$ both co-IP with PAR1 in human neuroblastoma cells (Ogino et al., 1996). Several studies also have suggested that activating PAR2 triggers responses traditionally mediated by $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ (Macfarlane et al., 2001;Traynelis and Trejo, 2007). However, a comprehensive understanding of the G protein signaling pathways stimulated by PAR1 and PAR2 in the same cell is lacking.

In the present study, we sought to define the G protein coupling and signaling profiles of PAR1 and PAR2 in the same cellular context and to identify differences in their physiological roles. Using both ectopic cellular systems expressing recombinant proteins (COS-7 kidney cells lacking functional PAR readouts) and cells of neuronal origin that natively express PARs (Neu7 astroglia), we have found that PAR1 and PAR2

couple to overlapping and distinct sets of G proteins and linked signaling pathways to modulate different cellular responses. In doing so, we have highlighted previously unappreciated differences between these two closely related receptors.

2.2. Experimental Procedures

2.2.1. Materials

Materials were obtained from the following sources: Anti-FLAG M2 affinity gel and anti-FLAG M2 monoclonal antibody-peroxidase conjugate, bovine serum albumin (BSA), isoproterenol, U73122, L-(-)-Norepinephrine, penicillin, and streptomycin from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum from Atlanta Biologicals (Atlanta, GA); trypsin, Dulbecco's modified Eagle's medium (DMEM) from Cellgro (Herndon, VA); Lipofectamine 2000 transfection reagent from Invitrogen (Carlsbad, CA); *myo*-[^{3}H]inositol from American Radiolabeled Chemicals, Inc.(St. Louis, MO); RhoA G-LISA™ Activation Assay colorimetric format kit and C3 exoenzyme from Cytoskeleton, Inc. (Denver, CO); cAMP ELISA Kit (colorimetric) from Cell Biolabs, Inc. (San Diego, CA); conjugated goat anti-mouse monoclonal antibody from Rockland Inc. (Gilbertsville, PA); PTX was purchased from List Biologicals (Campbell, CA); p44/42 ERK1/2 (extracellular signal-regulated kinase 1/2) antibody, phospho-p44/42 ERK1/2 antibody, MEK1/2 inhibitor U0126, and bisindolymaleimide (BIS) from Cell Signaling Technology (Beverly, MA); Glu-Glu monoclonal antibody (anti-EE) from Covance, Inc. (Princeton, NJ), anti-$G\alpha_s$, anti-$G\alpha_o$ anti-$G\alpha_{i1}$, anti-$G\alpha_{i2}$, anti-$G\alpha_{i3}$ anti-$G\alpha_{12}$, and anti-$G\alpha_{13}$ antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-$G\alpha_{q/11/14}$

antibody Z811 was kindly provided by Dr. Paul Sternweis (U. Texas Southwestern, Dallas, TX); peroxidase-conjugated goat anti-mouse IgG antisera from Rockland Immunochemicals, Inc. (Gilbertsville, PA), and peroxidase-conjugated goat anti-rabbit was from Bio-Rad (Hercules, CA). The PAR-activating peptides (PAR-APs), TFLLR-NH_2 (TFLLR) and 2-furoyl-LIGRLO-NH_2 (LIGRLO), were synthesized by Dr. Jan Pohl at the Emory University Microchemical Facility (Atlanta, GA).

2.2.2. Methods

PAR1 and PAR2 constructs: Mouse PAR1-FLAG and PAR2 are both in the pcDNA3.1 vector. A C-terminal FLAG epitope tag was added to PAR2 by PCR amplification of BamHI-XhoI fragment that contained the FLAG sequence. An antisense primer was designed to eliminate the stop codon of the PAR2 sequence and introduce the FLAG sequence with a new C-terminal stop codon. The antisense primer was 5'-CTCGAGTTACTTGTCATCGTCGTCCTTGTAGTCGTAGGAGGTTTTAACAC-3' and was used in combination with either the sense primer 5'-CGGGGATCCATGCGAAGTCTCAGCCTGGCG-3' to generate a BamHI-XhoI fragment from the existing pcDNA3.1 sequence.

RGS protein constructs: p115-RGS and GRK2-RGS, truncated RGS proteins used as selective G protein pathway inhibitors, were kindly provided by Dr. T. Kendall Harden (UNC-Chapel Hill, Chapel Hill, NC) and were created as previously described (Hains et al., 2004).

Cell culture and transfections – COS-7 (ATCC® Number CRL-1651™) and Neu7 (a generous gift from Dr. Isobel Scarisbrick, Rochester, MN) cells were propagated in

DMEM with sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, 100 μg/mL streptomycin and 100U/mL penicillin at 37°C in a humidified atmosphere with 5% CO_2. Subculturing of confluent plates was done at a ratio of 1:10 for transfection. COS-7 cells were transfected according to Lipofectamine 2000® transfection reagent protocol and cells were used for experimentation 24-48 h after transfection.

Immunoblot Analysis—Nitrocellulose membranes were blocked in blocking buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5% milk, 0.5% Tween 20, 0.02% sodium azide) at room temperature for 1 h and subsequently incubated in a primary antibody dilution for 3 h at room temperature or overnight at 4°C. Dilutions differed for each antibody and are listed here: anti-FLAG 1:1000, anti-p44/42 ERK1/2 1:300 and anti-phospho p44/42 1:1000 in Tris-buffered saline + 0.1% Tween 20 (TBST) with 5% BSA; anti-$G\alpha_q$ family Z811 1:2000, anti-$G\alpha_o$ 1:200, anti-$G\alpha_{i1}$ 1:150; anti-$G\alpha_{i2}$ 1:150; anti-$G\alpha_{i3}$ 1:150; anti-$G\alpha_{12}$ 1:200; anti-$G\alpha_{13}$ 1:200, and anti-Gβ 1:150 in blocking buffer. Membranes were washed three times with TBST and then probed with horseradish peroxidase-conjugated secondary antisera for 1 h at room temperature. For secondary antibodies the dilutions were: goat anti-rabbit IgG 1:25,000 in TBST and goat anti-mouse IgG 1:20,000 in TBST. The protein bands were visualized using enhanced chemiluminescence (ECL) and exposed to film.

Measurement of [^{3}H]InsP formation – Levels of [^{3}H]InsP accumulation were determined in confluent 12-well plates. Untransfected Neu7 cells or COS-7 cells transiently transfected with PARs alone or in combination with either the $G_{q/11}$-pathway inhibitor GRK2-RGS, or the $G_{12/13}$ pathway inhibitor p115-RGS were metabolically labeled with *myo*-[^{3}H]inositol in serum-free media for 18-24 h. Due to difficulty

transfecting Neu7 cells, pharmacological inhibitors of PLC signaling (U73122) or Rho signaling (C3 toxin) were added during the last 30 min or 4 h of serum starvation, respectively. After pre-labeling, medium containing *myo*-[^{3}H]inositol was removed and incubation buffer (DMEM buffered with 25mM HEPES, pH 8.0, and containing 10 mM $LiCl_2$) was added to each well for 20 min. Cells were incubated with PAR-APs for 5 min. Cells were then solubilized with 20 mM formic acid, neutralized with 0.7 M NH_4OH, and centrifuged for 5 min at 10,000 x g at 4^{o}C. [^{3}H]InsPs were separated by anion exchange chromatography (AG 1-X8 Dowex, Bio-Rad) using increasing amounts of ammonium formate. Samples were subjected to anion exchange chromatography to isolate [^{3}H]InsPs, which were quantified by scintillation counting and expressed as mean ± S.E.M.

Two-electrode voltage clamp recordings from Xenopus laevis oocytes: Oocytes were harvested from *X. laevis* were defolliculated and maintained in 1x Barth's culture solution at 16ºC. Stage V-VI oocytes were either injected with 5ng PAR1 or PAR2 cRNA, which was synthesized from cDNA according to the manufacturer's specifications (Ambion, TX). Recordings were performed 4-5 days after injections. The recording solution contained (in mM) 60 NaCl, 38 KCl, 2.3 $CaCl_2$, 1 $MgCl_2$, and 6 HEPES. The pH was adjusted to 7.4 with NaOH. Patch pipettes with tip diameters of 1-2 µm were used as electrodes and filled with 300 mM KCl. Current responses were recorded at a holding potential of -40 mV. Data was acquired and voltage was controlled with a two-electrode voltage-clamp amplifier (OC-725; Warner Instruments, Hamden, CT). The PAR-APs diluted in 1x Barth's to final concentrations of 30µM TFLLR and 10µM LIGRLO, respectively, were used to elicit the $I_{Cl(Ca)}$.

Measurement of ERK1/2 phosphorylation: After serum starvation in the absence or presence of pharmacological inhibitors (PTX overnight, C3 toxin for 4 hours, U73122 for 30 minutes, and BIS for 30 minutes), untransfected Neu7 cells or COS-7 cells separately transfected with PAR1 or PAR2 were stimulated with the PAR-APs for 2-5 min, harvested, sonicated, boiled in sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 13.5%) and transferred to nitrocellulose membranes. Membranes were blocked and washed once in TBST + 5% BSA followed by overnight incubation with p44/42 ERK1/2 and phospho- p44/42 ERK1/2 antibodies at 4ºC. Membranes were washed with TBST and incubated for 1 h with HRP-conjugated goat anti-rabbit IgG. The membranes were again washed and protein bands were detected by ECL. Densitometry was performed using Image J software (NIH website), and samples were normalized by dividing phospho-ERK densitometry units by total ERK Densitometry units and expressing these numbers as a percent of maximal ERK phosphorylation. Two-way ANOVA analyses were performed using SigmaStat software (Aspire Software International; Ashburn, VA).

Measurement of RhoA activation: The GTP-bound form of RhoA was measured using the absorbance-based RhoA Activation G-LISA™ kit (Cytoskeleton, Inc., Denver, CO) according to the manufacturer's protocol. Before using the kit's components, Neu7 cells or transiently transfected COS-7 cells were serum-starved overnight and then treated for 2 min with the PAR-APs in the presence or absence of the rho inhibitor, C3 toxin or the transfected G_{12}-pathway inhibitor, p115-RGS. The absorbance from the G-LISA™ plate was read by a spectrophotometer at a wavelength of 490nm.

Co-Immunoprecipitation of PAR/G protein complexes—COS-7 cells were transfected in 15 cm plates with a total of 40μg of DNA per plate (20μg of receptor + 20μg G protein; empty vector was used in place of either component, receptor or G protein, for the controls) for 18-24 h. The following day, cells were washed in PBS and harvested in 0.5 mL of Tris Buffer (50mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl, 1 mM EDTA, and a protease inhibitor pellet), and sonicated. In experiments with agonist, PAR-APs or norepinephrine were added to lysates for 30 min. *n*-Dodecyl-β-D-maltoside (DβM; Calbiochem) was added to a final concentration of 2%. Membrane proteins were extracted with 2% DβM for 3 h, rotating end-over-end at 4°C, and debris was pelleted by ultracentrifugation (100,000 x *g*, 4°C, 30 min). An aliquot of the lysate was kept to be run as "input" on gel. Remaining cytosol was incubated overnight at 4°C with anti-FLAG M2 affinity gel, rotating end-over-end. The following day, the anti-FLAG resin was pelleted and washed three times with Tris Buffer containing 0.2% DβM. The resin then was resuspended in 2X Laemmli Sample Buffer (100mM Tris, pH 6.8, 0.5% SDS, 20% glycerol, 0.5% β-mercaptoethanol, 0.004% bromophenol blue). Following recovery by centrifugation, entire supernatants were loaded onto 11% polyacrylamide gels for SDS-PAGE separation. Samples for immunoblot analysis were transferred to nitrocellulose membranes, and immunoblotting was carried out as described.

Measurement of cAMP inhibition: cAMP inhibition was measured using the absorbance-based cAMP ELISA kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's protocol. Before using the kit's components, transiently transfected 12-well plates of COS-7 or untransfected Neu7 cells were plated overnight and then treated

for 2 min with isoproterenol, PAR-APs, and the phosphodiesterase inhibitor IBMX in the presence or absence of PTX. The absorbance from the ELISA plate was read by a spectrophotometer at a wavelength of 450nm.

Wound-scratch test to measure migration: Migration of Neu7 cells was measured using a wound-scratch test. Briefly, cells were grown to confluence in 6-well plates and the cell monolayer was "wounded" by using a 0.5-10μL pipette tip to scratch a line across the monolayer. Immediately after wounding, cell media was replaced with serum-free media containing vehicle, 100μM TFLLR, or 200μM LIGRLO in the presence or absence of 100 ng/mL PTX or 10μM U0126. Pictures were taken with an Olympus IX51 light microscope at time 0 and 24 h after agonist addition. Quantification of the cell migration images was achieved using ImageJ software (NIH website). The total area of the "wound" was highlighted and quantified and cell migration was determined by subtracting the cell-free area from the total area covered by cells (expressed as a percent of total area of the wound). Statistical *T* tests were performed on figures obtained from analyzing two different images for each condition. Graphpad Software (Graphpad Software, Inc.) was used to perform statistical analysis.

Measurement of [^{3}H] Thymidine Incorporation: Proliferation of Neu-7 cells was measured as previously described (Sorensen et al., 2003). Briefly, cells were plated and serum starved for 24 h in the absence or presence of PTX. Cells were then challenged with agonist (vehicle, TFLLR or LIGRLO) for 24 h. During the final 2 h of stimulation, [^{3}H]thymidine was added to a final concentration of 1 μCi/mL. Cells were washed in ice-cold PBS and then 20% trichloroacetic acid was added for 30 min at 4°C. Cells were

again washed in PBS, and the acid-insoluble material was lysed in 0.1 N NaOH/1% SDS. [^{3}H]Thymidine in lysates was measured by scintillation counting.

2.3. Results

2.3.1. PAR1 and PAR2 link to multiple G protein-regulated pathways

PAR1 and PAR2 have both been reported to activate signaling pathways regulated by $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$. To define which signaling pathways PAR1 and PAR2 are linked to in a defined biological system, we screened various cell lines to identify a model system that did not respond to either of the specific PAR-activating proteins (PAR-APs; i.e., TFLLR for PAR1 or LIGRLO for PAR2). Previous studies have reported that COS-7 cells express undetectable (or very low) levels of PARs (Ishihara et al., 1997;Blackhart et al., 2000), and showed that COS-7 cells do not activate inositol phosphate or calcium signaling in response to stimulation with TFLLR, thrombin, trypsin, or other proteases. Consistent with these reports, we found that our COS-7 cells did not respond to either peptide in various signaling assays (as shown in basal and vector controls, Figs. 2-1, 2-4C-D, 2-5) and that these cells could be readily transfected to express recombinant receptors and G proteins. Over many repeated experiments, we found that both PAR1 and PAR2 proteins consistently express well when transfected into in COS-7 cells (Figure 2-1A). A caveat to our experiments is that quantitatively measuring active PARs is technically difficult due to the limited range of experimental tools that are available for

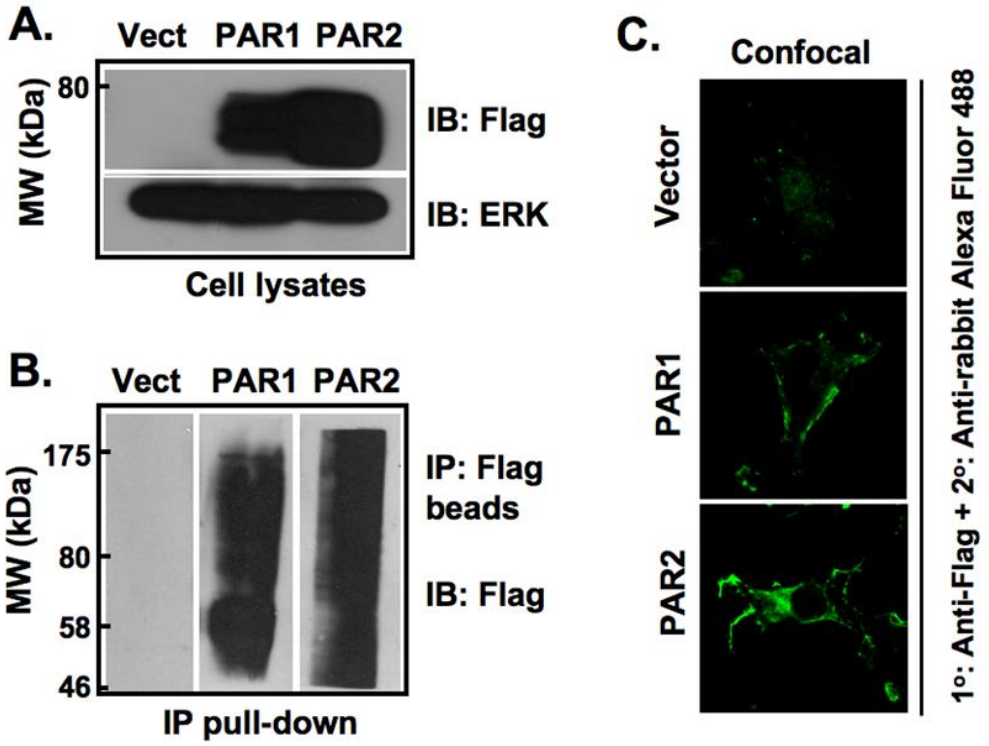

Figure 2-1. PAR1 and PAR2 express at relatively similar levels and are found at the plasma membrane and in the cytosol. A) Western blots were performed in COS-7 cells separately transfected with either PAR1 or PAR2 cDNA. Cells were harvested, subjected to SDS-PAGE, and immunoblotted with an anti-FLAG antibody. B) Immunoprecipitation techniques were performed as described and PAR1 and PAR2 were recovered with an antibody to their FLAG epitopes. Recovered material was subjected to SDS-PAGE and immunoblotted with an anti-FLAG antibody. C) Slides of PAR1- and PAR2-transfected COS-7 cells were fixed and immunostained with an anti-FLAG primary antibody and Alexa Fluor 488 rabbit IgG secondary antibody. Cells were analyzed by confocal microscopy.

studying these receptors. However, fluorescence imaging of FLAG-tagged PAR1 and PAR2 by confocal microscopy (Figure 2-1C) shows that a substantial portion of total expressed receptors localize at the plasma membrane, and other studies (Figures 2-1 through 2-5) confirm that some fraction of these receptors is functional. Both PAR1 and PAR2 can be recovered by anti-FLAG antibodies covalently coupled to agarose beads and detected by immunoblot analysis (Figure 2-1B). Both receptors are readily recovered and migrate on SDS-PAGE as a prominent smear (as is the case with many GPCRs). However, quantification of active receptors remains challenging, and we can only make qualitative statements about PAR amounts and recovery. With these limitations in mind, we initiated experiments using expressed PAR1 and PAR2 with specific G proteins in COS-7 cells to compare PAR1 and PAR2 signaling.

Depending on the cell type being studied, both PAR1 and PAR2 are reported to activate one or more isoforms of phospholipase C (PLC) to initiate PIP hydrolysis and InsP signaling (Hung et al., 1992;Dery et al., 1998;Hains et al., 2006). To determine whether PAR1 and PAR2 stimulated PLC activity in COS-7 cells, we measured accumulation of radiolabeled InsPs in cells transfected with either PAR1 or PAR2 in response to each PAR-AP--TFLLR or LIGRLO (Figure 2-2A). Consistent with previous reports, both receptors stimulated measurable InsP production whereas control cells transfected with the empty pcDNA3.1 vector did not (Figure 2-2A).

We also examined whether PAR1 and PAR2 stimulate calcium mobilization. The amphibian *X. laevis* oocytes express calcium-activated chloride currents that provide a simple and sensitive readout of $G_{q/11}$-simulated mobilization of intracellular calcium

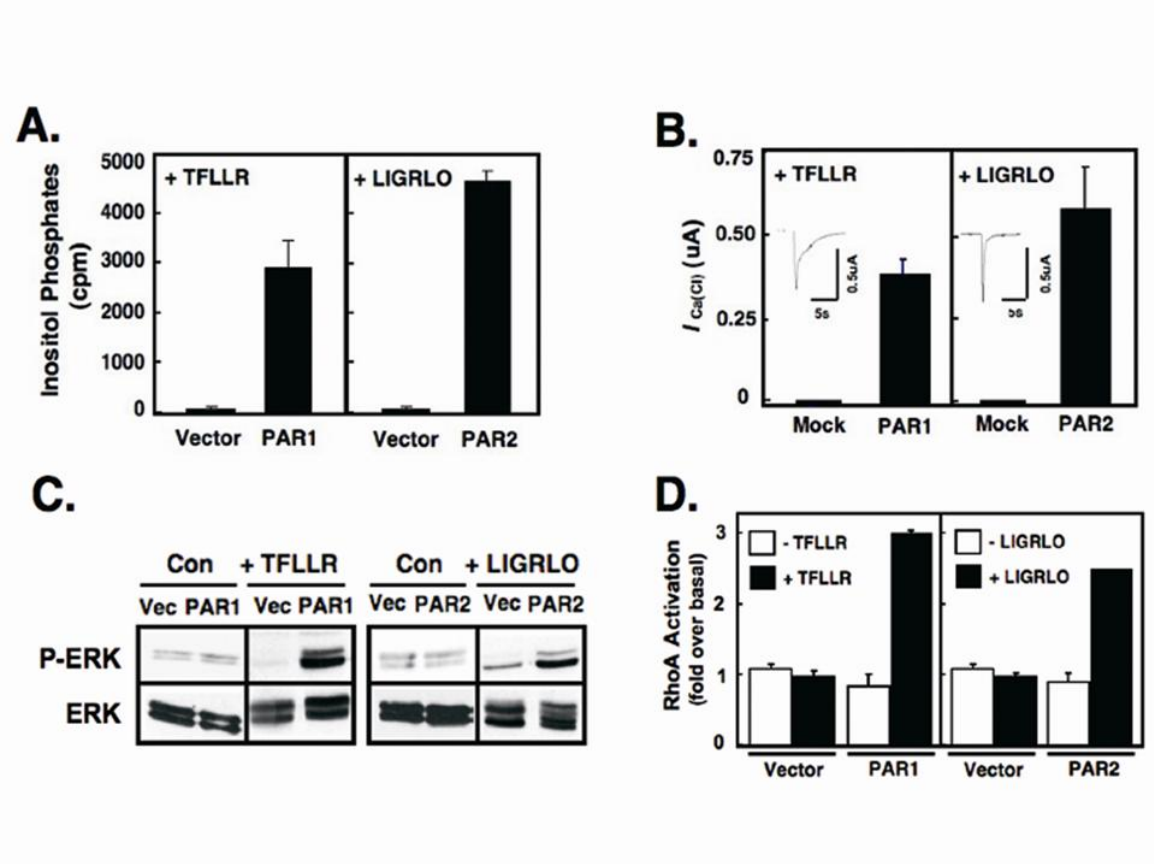

Figure 2-2. PAR1 and PAR2 activate multiple G protein-regulated signaling responses. A) [^{3}H]Inositol phosphate accumulation in intact COS-7 cells were transfected with the indicated PAR cDNA as described under "Materials and Methods." After a 5 h transfection period, cells were metabolically labeled overnight with 4 μCi/mL *myo*-[^{3}H]inositol in serum-free media. Following a 20 min incubation at 37°C in 10 mM $LiCl_2$, cells were either left unstimulated or were activated with 30μM TFLLR or 10μM LIGRLO. To stop the reaction, cells were solubilized with 20 mM formic acid, and lysates were neutralized with 0.7 M NH_4OH. [^{3}H]InsPs fractions were separated by anion exchange chromatography, and total [^{3}H]InsP content was assessed by liquid scintillation spectrometry. Data are presented as the average of total InsPs from 3 different experiments (mean cpm + S.E.M; each point performed in triplicate). B) 5 ng of PAR1 or

PAR2 cRNA was injected into *X. laevis* oocytes, which were maintained in 1x Barth's solution. 4-5 days after injection, oocyte $I_{Ca(Cl)}$ measurements were obtained in response to stimulation by either 30μM TFLLR or 10μM LIGRLO using a two-electrode voltage clamp, as described. Data is expressed as the mean change in $I_{Ca(Cl)}$ + S.E.M. (n>11 oocytes). C) Vector alone, PAR1 or PAR2 were separately transfected into COS-7 cells. Cells were either unstimulated or stimulated with, 30μM TFLLR or 10μM LIGRLO, as indicated, for 2 min. Immunoblots were performed with either phospho-ERK1/2 or total ERK1/2 antibodies followed by a goat-anti rabbit secondary antibody or with an HRP-conjugated anti-HA antibody and detected by ECL. D) PAR-mediated RhoA activation was measured using a RhoA G-LISA™ Assay kit. First, PAR cDNA was separately transfected into COS-7 cells for 5 h before the media was replaced with serum-free media overnight. The following day, cells were either left unstimulated or were activated with 30μM TFLLR or 10μM LIGRLO for 2 min before cell lysis. After following the manufacturer's protocol, the absorbance of each well was read with a spectrophotometer wavelength of 490nm.

(Oron et al., 1985;Dascal and Cohen, 1987;Nystedt et al., 1994;Mannaioni et al., 2008). We found that oocytes injected with PAR1 or PAR2 cRNA and stimulated with the appropriate PAR-AP increase the activity of calcium-activated chloride channels. At a holding potential of -40 mV, separate activation of PAR1 and PAR2 evokes an inward current characteristic of the calcium-activated chloride channel, indicating that both PAR1 and PAR2 mobilize intracellular calcium in response to InsP production. Using mock-injected oocytes as controls, we found that these cells did not evoke an inward current in response to stimulation with PAR-APs, as expected (Figure 2-2B).

PARs also have been reported to activate MAPK pathways and stimulate ERK1/2 phosphorylation (Kramer et al., 1995;DeFea et al., 2000). Various G proteins (G_s, $G_{q/11}$, $G_{i/o}$) initiate signaling pathways that converge on ERK1/2 (DeFea et al., 2000;Ramachandran et al., 2009), and it is well established that $G_{i/o}$-linked pathways activate ERK1/2 phosphorylation by release of Gβγ, in a PTX-sensitive manner (Gerhardt et al., 1999). Our lab and others have shown that MAPK signaling stimulated by PARs contributes to the proliferation of a number of different cell types including astrocytes (Wang et al., 2002b;Sorensen et al., 2003). Here we confirm that in COS-7 cells expressing recombinant PARs, ERK1/2 phosphorylation is elicited by each of their receptor-specific PAR-APs. No response to agonist stimulation occurs with either of the PAR-APs when cells are transfected with vector alone (Figure 2-2C).

A third G protein-linked pathway that is reported to be activated by PARs is Rho signaling, which is known to be mediated primarily through the $G_{12/13}$ family (Offermanns et al., 1994;Aragay et al., 1995;Post et al., 1996) but also can be activated through $G_{q/11}$ stimulation of p63RhoGEF (Lutz et al., 2005). Previous studies have shown

that PAR1 and PAR2 activation of Rho triggers cellular responses including cellular proliferation, migration, and morphological changes, including platelet shape change, neurite retraction, and growth cone collapse (Klages et al., 1999;Citro et al., 2007;Nurnberg et al., 2008). To determine whether PAR1 and PAR2 also activate this pathway in COS-7 cells, we employed a chemiluminescence-based ELISA Rho assay system that relies on the Rho-binding domain of Rho effector proteins to detect formation of Rho-GTP from cell lysates. We found that the levels of activated RhoA-GTP is increased approximately 3- and 2.5-fold over basal, respectively, following stimulation of PAR1 or PAR2 with the appropriate PAR-AP (Figure 2-2D). Taken together, these findings indicate that both PAR1 and PAR2 functionally couple to multiple G protein regulated pathways in COS-7 cells.

2.3.2. PAR1 and PAR2 form stable complexes with both overlapping and distinct sets of G proteins

Although functional PAR coupling to $G_{q/11}$-, $G_{i/o}$-, and $G_{12/13}$-linked signaling pathways has been reported previously (and confirmed here), only very limited information is available regarding direct PAR complex formation with individual G protein family members. Therefore, we screened members of each of these candidate G protein subfamilies ($G_{q/11}$, $G_{i/o}$ and $G_{12/13}$) for their capacities to form a stable complex (i.e., recovered by co-IP) with PAR1 or with PAR2 (Figure 2-3). Carboxy-terminally FLAG-tagged PAR1 or PAR2 and individual Gα protein subunits were each independently co-expressed as PAR/G protein pairs in COS-7 cells. The FLAG-tagged α_{1A}-adrenergic receptor (α_{1A}-AR), which is known to be $G_{q/11}$-linked, was compared in

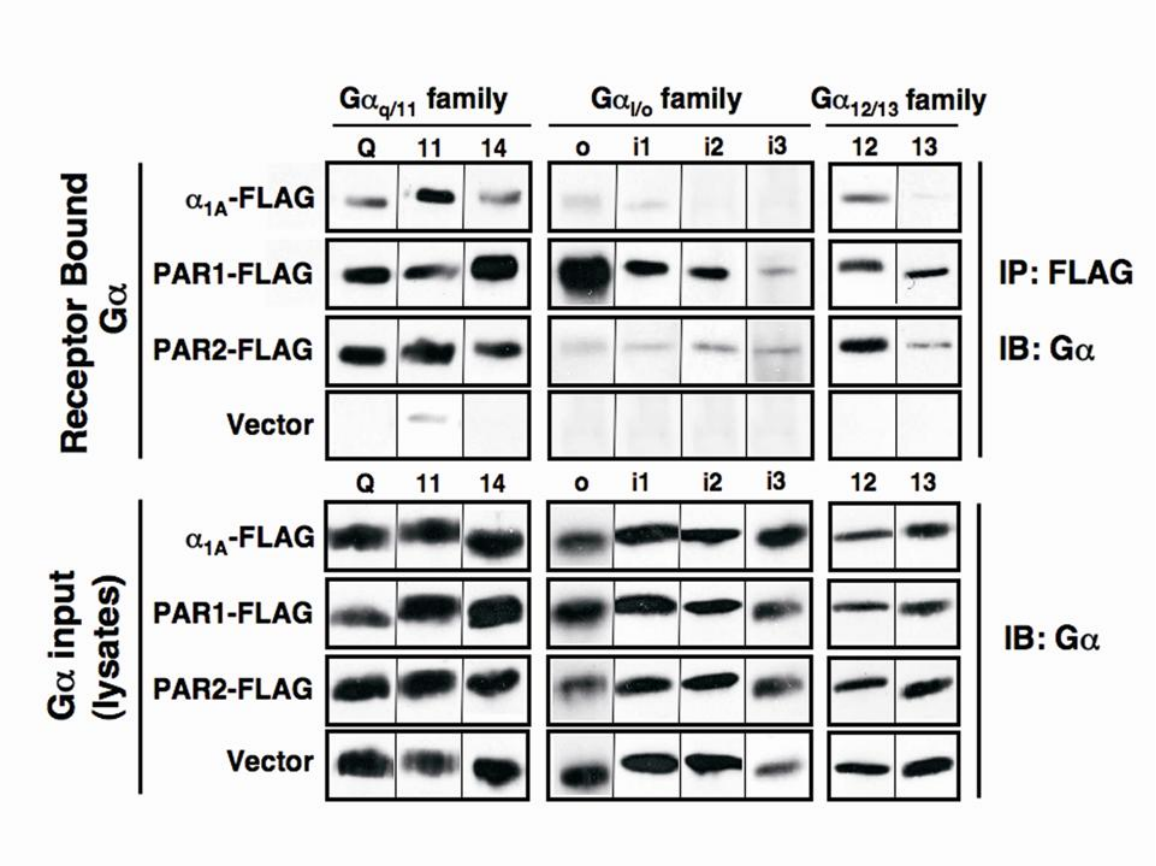

Figure 2-3. PAR1 and PAR2 form stable complexes with distinct sets of G proteins. Twenty-four hours after co-transfection with separate receptor/G protein pairs and controls (as indicated), cells were lysed, harvested, and sonicated in Tris Buffer. Proteins were extracted from membranes with 2% DβM (3 h, 4°C) and IP'ed overnight at 4°C with anti-FLAG affinity gel. Immunoprecipitates were resolved by SDS-PAGE (11% polyacrylamide). Proteins were immunoblotted and visualized with ECL. *Top panel,* Western blot analysis of IP'ed G proteins with corresponding G protein-specific antibodies. *Bottom panel,* Western blot analysis of cell lysates (input) with corresponding G protein-specific antibodies. Results are representative of at least three separate experiments.

parallel with the PARs as a control. In addition, α_2-AR, a G_s linked receptor, was also evaluated for its capacity to bind to $G\alpha_s$, $G\alpha_{11}$, $G\alpha_o$ and $G\alpha_{12}$ (Figure 2-4). Anti-FLAG agarose beads were used to recover the receptor/G protein complexes (as in Figure 2-1B), and samples were analyzed for the presence of the G protein in the recovered material (IP, Figure 2-3 top) and in the lysate (input, Figure 2-3 bottom). We found that PAR1 and PAR2 couple to overlapping and distinct sets of G proteins. Little or no detectable G proteins are recovered when only the individual G proteins and control vector are transfected into cells in the absence of receptor expression (Figure 2-3, bottom row, top panel). All of the tested $G\alpha_{q/11}$ family members ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$) and the $G\alpha_{12}$ family members ($G\alpha_{12}$, $G\alpha_{13}$) formed a stable complex with PAR1 and PAR2, as well as with α_{1A}-AR; each of these G protein subunits bound to similar extents to both PAR1 and PAR2, which were recovered at comparable levels (Figure 2-1B). In stark contrast, all of the $G\alpha_{i/o}$ subunits (except $G\alpha_{i3}$) bound to PAR1, but only weakly or not at all to PAR2 or to α_{1A}-AR. Of note, much more of the $G\alpha_o$ subunit appears to have bound to PAR1 than any other $G\alpha$ subunits tested (Figure 2-3). Whether this binding reflects a more robust coupling is uncertain since the $G\alpha$-specific antibodies differ in their relative staining intensities. Therefore, we can only make qualitative statements about PAR/G protein coupling from these data.

To further test the specificity of these apparent interactions, we compared PAR1/G protein coupling with the G_s-coupled β_2-AR (Figure 2-4). As expected, β_2-AR bound to $G\alpha_s$ but *not* to $G\alpha_o$ or $G\alpha_{12}$, whereas PAR1 bound to $G\alpha_o$, $G\alpha_{11}$ and $G\alpha_{12}$ (as before) but *not* to $G\alpha_s$. We also observe a small amount of $G\alpha_{11}$ that co-eluted with β_2-AR. Since β_2-AR is not reported to activate $G_{q/11}$-linked pathways, we believe this

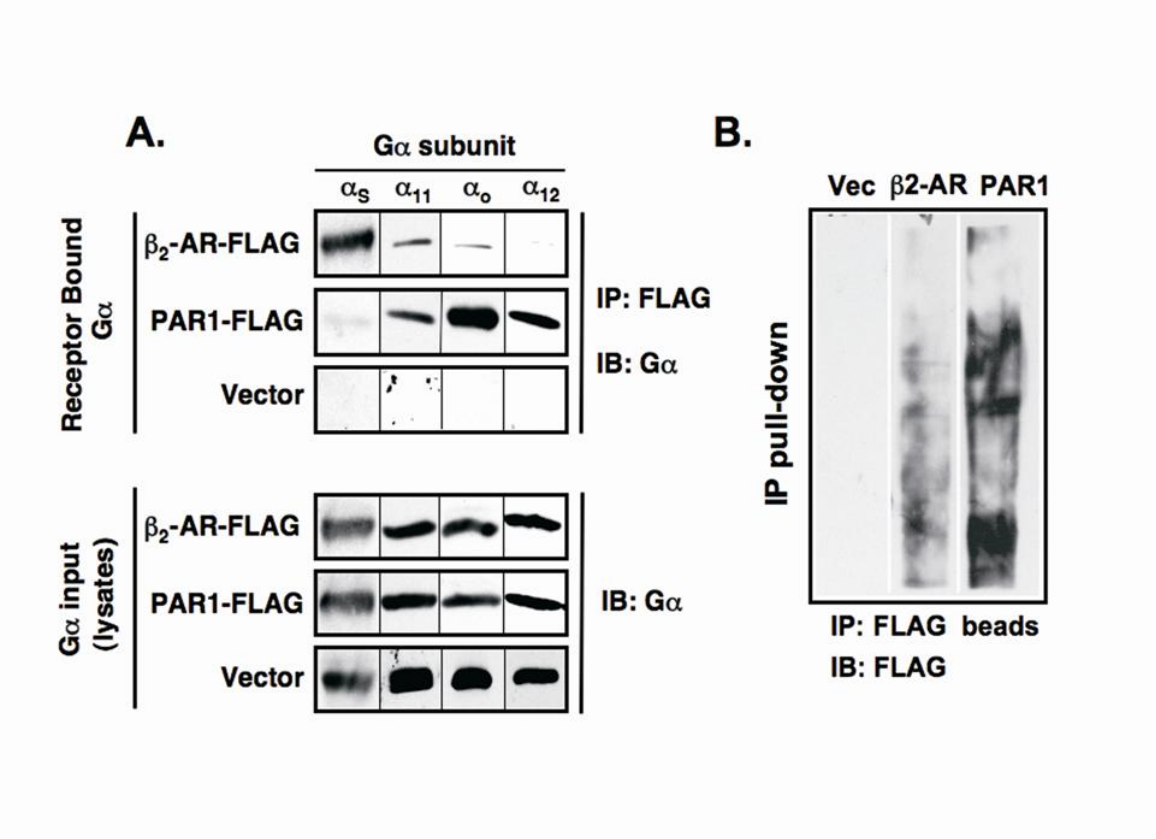

Figure 2-4. The β2 adrenergic receptor (β2-AR) co-immunoprecipitates with distinct G proteins. Following a 24 h co-transfection of receptor and G protein pairs, cells were lysed, harvested, and sonicated in Tris Buffer. Proteins were extracted from membranes and immunoprecipitated overnight with anti-FLAG affinity gel. Immunoprecipitates were resolved by SDS-PAGE. Proteins were immunoblotted and visualized with ECL.

interaction (possibly non-specific) does not reflect functional coupling. Apart from this observation, all of the PAR/G protein complexes we identified seem real and reflect previous reports of functional coupling. To our knowledge, these data are the first to demonstrate stable interactions between PARs and a wide variety of Gα proteins and identifies clear differences between PAR1 and PAR2 G protein coupling. Of particular note, PAR1, but not PAR2 couples to specific $G_{i/o}$ family members.

2.3.3. PAR1 and PAR2 form stable complexes with G protein heterotrimers

In our screens for receptor/G protein pairs, no agonist was added to the cells to either promote or disrupt the complexes. Therefore, we examined the effects of PAR-APs and activating guanine nucleotide on the formation and stability of PAR/Gα complexes. Furthermore, we tested whether PARs interacted with G protein heterotrimers (Gαβγ) as determined by the presence of Gα in the recovered complex. Protein complexes were recovered from COS-7 cell lysates expressing PAR/G proteins as described above (Figure 2-3). Specifically, we examined the effects of agonist and activating nucleotide (GTPγS) on PAR1 and PAR2 interactions with either G_{11} or G_o in cell lysates. COS-7 cell lysates containing both membranes and cytosol were incubated either alone or in the presence of agonist and 10 μM GTPγS for 30 min. Following co-IP, we found that PAR1 was recovered in complex with both G_{11} and G_o, and PAR2 with only G_{11} (Figure 2-5), as before. Of note, endogenous Gβ (and likely Gγ, though not tested) subunits also were present in the recovered complexes, presumably in a heterotrimeric complex with recombinant Gα. Somewhat surprisingly, no differences in PAR/G_o or PAR/G_{11} complexes were elicited by addition of PAR-APs and GTPγS (Figure 2-5).

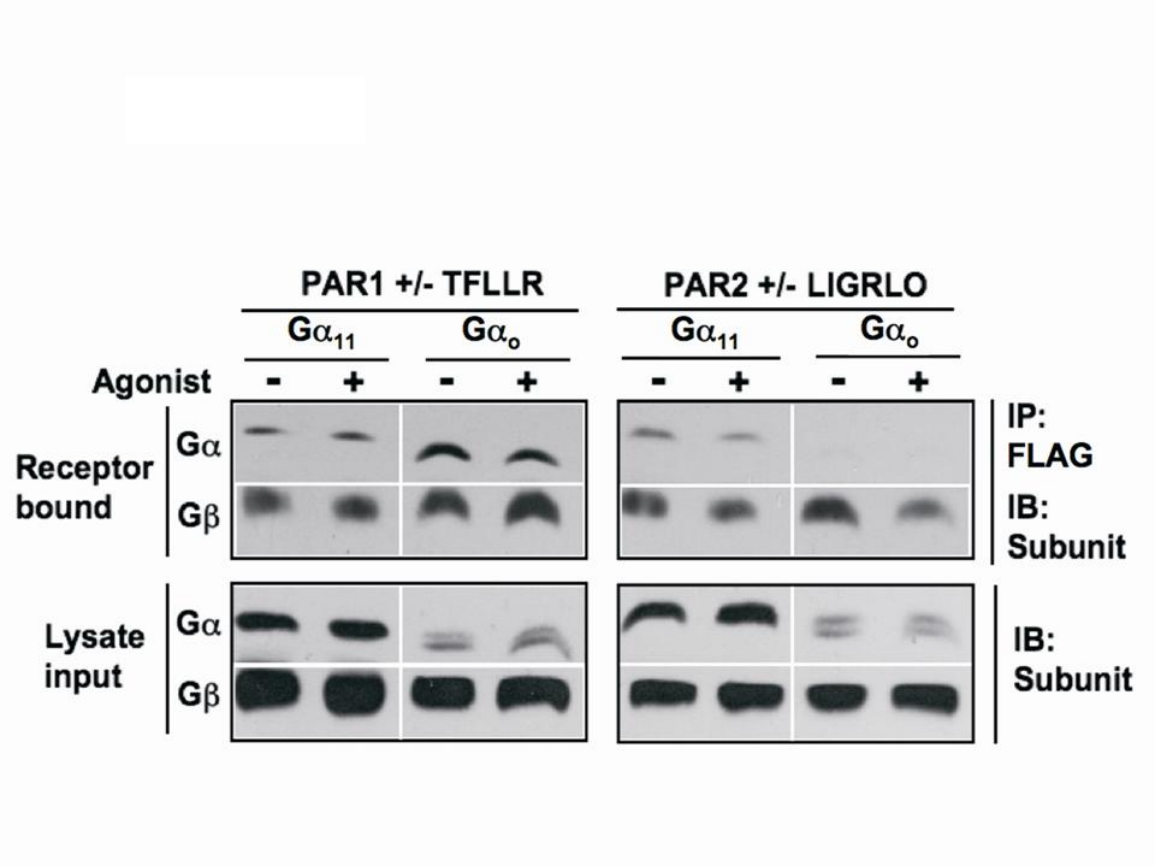

Figure 2-5. PAR1 and PAR2 form stable complexes with G protein heterotrimers. Co-IP studies were performed as described but for these experiments; either $G\alpha_o$ or $G\alpha_{11}$ was co-transfected with PAR1 or PAR2 and pulled down in the presence of GTPγS, in the presence and absence of agonist. Here, we have also used a pan-Gβ antibody to detect the presence of endogenous Gβ in the receptor/Gα complex. *Top panel,* Western blot analysis of IP'ed G proteins with corresponding G protein-specific antibodies. *Bottom panel,* Western blot analysis of cell lysates (input) with corresponding G protein-specific antibodies. Antibodies to $G\alpha_o$ and to $G\alpha_{11}$ were mixed in one tube to blot the entire membrane at once. The same goat anti-rabbit secondary antibody was then used, and proteins were visualized using ECL.

2.3.4. PAR1 selectively couples to $G_{i/o}$ signaling pathways

Thus far, our findings have identified a difference between PAR1 and PAR2 interactions with $G_{i/o}$ family members. Because we showed that PAR1 but not PAR2 physically couples with $G\alpha_{i/o}$ subunits, we investigated whether there were functional differences in PAR activation of $G_{i/o}$-mediated intracellular signaling pathways in COS-7 cells. To do so, we tested the role of PARs in the $G_{i/o}$-mediated inhibition of α_1-AR-induced cAMP accumulation and in the $G_{i/o}$-mediated stimulation of ERK1/2 phosphorylation (Figure 2-6). Measurements of cellular cAMP were performed in COS-7 cells transiently expressing either PAR1 or PAR2 following stimulation with isoproterenol alone or in combination with either PAR-AP. PTX-sensitivity also was determined as a measure of $G_{i/o}$ involvement. In cells expressing either PAR1 or PAR2, isoproterenol elicited high levels of cAMP production, which indicates that the β-AR is also present in these cells. When cells were stimulated in parallel with TFLLR, cellular cAMP levels were significantly reduced by 20-25% (p = 0.012; Figure 2-6A), and this inhibition is reversed by pretreatment of cells with PTX. By contrast, LIGRLO does not reduce isoproterenol-stimulated cAMP production in PAR2-expressing COS-7 cells, nor is this response affected by PTX (Figure 2-6B).

Activation of $G_{i/o}$-linked pathways also stimulates MAPK signaling. Therefore, we also measured ERK1/2 phosphorylation experiments in COS-7 cells expressing either PAR1 or PAR2, in the presence or absence of PTX treatment. Preliminary studies indicated that both PAR1 and PAR2 maximally stimulated ERK1/2 phosphorylation following a 2 min activation with the appropriate PAR-AP (data not shown). Cells expressing either PAR1 or PAR2 were pretreated with increasing concentrations of PTX

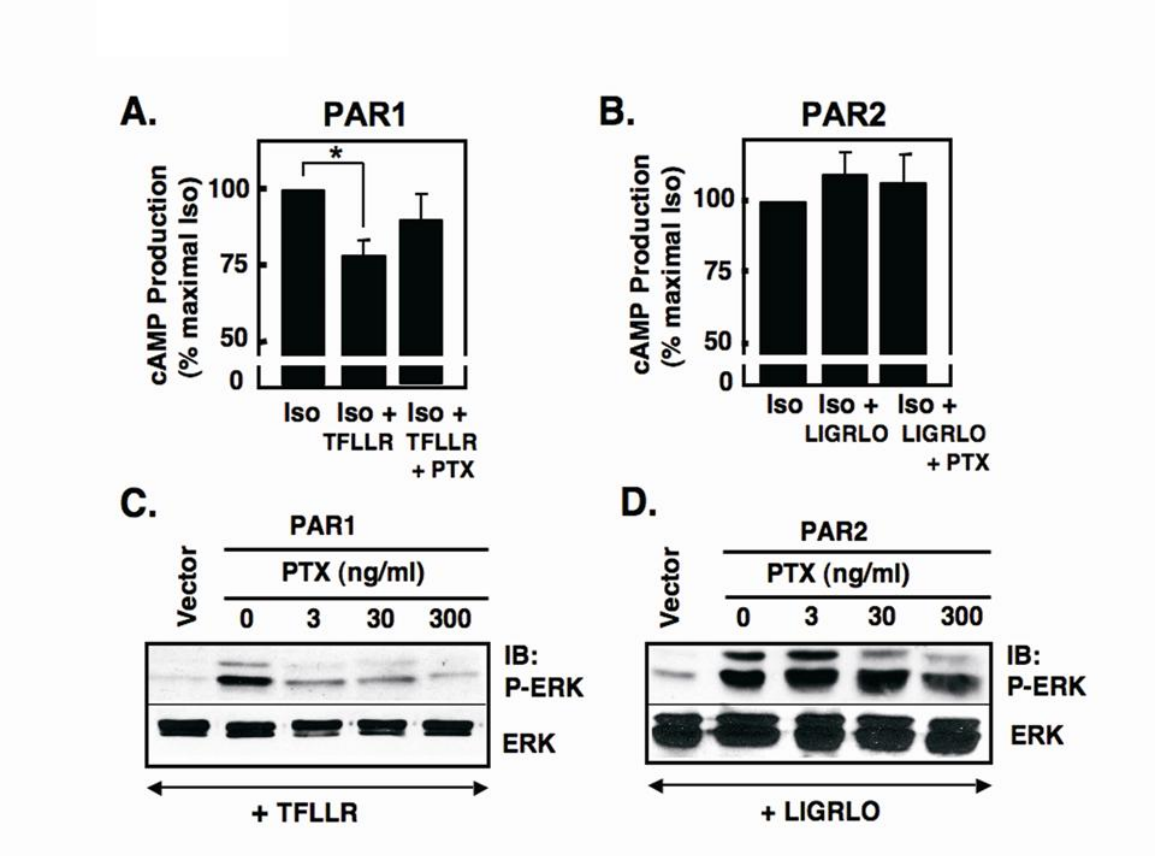

Figure 2-6. PAR1, but not PAR2, inhibits the accumulation of cAMP and stimulates ERK1/2 phosphorylation in a PTX-sensitive manner. The inhibition of cAMP accumulation and stimulated ERK1/2 phosphorylation were measured in COS-7 cells over-expressing PAR1 or PAR2. A,B) All PAR-expressing COS-7 cells were stimulated with 10μM isoproterenol in the presence of 100μM IBMX. Some cells also were activated with 30μM TFLLR or 10μM LIGRLO for 2 min in the presence and absence of 100 ng/mL PTX. Lysates were added to a 96-well ELISA plate, provided in the cAMP assay kit (Cell Biolabs). After following the manufacturer's protocol, cAMP levels were measured using a spectrophotometer. Results are expressed as the average + S.E.M. of 3 different experiments. C,D) COS-7 cells expressing PAR1 or PAR2 were serum-starved overnight and stimulated with 30μM TFLLR or 10μM LIGRLO for 2 min in the presence

and absence of 100 ng/mL PTX. Cells were lysed and harvested in 2X Laemelli buffer, sonicated and subjected to SDS-PAGE. Western blots were performed with phospho-ERK1/2 and total ERK1/2 antibodies. Protein bands were detected by ECL.

overnight, and then stimulated with PAR-APs. Of note, the PAR1-induced ERK1/2 phosphorylation response was reduced to control levels (cells transfected with vector but stimulated with PAR-AP) by PTX pretreatment whereas the ERK1/2 phosphorylation elicited by PAR2 remained unchanged (Figure 2-6C-D). For both PAR1 and PAR2, total ERK1/2 levels remained the same for all conditions. Taken together, our data showing PTX-sensitivity of TFLLR effects on cAMP accumulation and ERK1/2 phosphorylation indicate that PAR1 signaling responses in COS-7 cells rely, in part, on $G_{i/o}$ activation, whereas the parallel PAR2-mediated signaling responses do not. Our findings here with functional assays are consistent with our biochemical data above (Figure 2-3), and together these findings show that PAR1, but not PAR2, forms a stable functional complex with $G_{i/o}$ proteins to selectively activate linked pathways in COS-7 cells.

2.3.5. PAR1 and PAR2 both utilize $G_{q/11}$ and $G_{12/13}$ to activate PLC and Rho, respectively

Besides PAR1-$G_{i/o}$ interactions, our findings (Figure 2-3) also show that both PAR1 and PAR2 complex with $G_{q/11}$ and $G_{12/13}$ family members and activate pathways linked to these G proteins (Figure 2-2). Therefore, we investigated whether PAR1 and PAR2 activated inositol lipid and RhoA signaling by employing inhibitors of select G proteins in COS-7 cells. For these studies, we utilized GRK2-RGS and p115-RGS, which bind directly to and specifically inhibit signaling by $G_{q/11}$ and $G_{12/13}$, respectively (Hains et al., 2006). COS-7 cells were separately transfected with either PAR1 or PAR2 alone or together with either GRK2-RGS or p115-RGS. Cells then were challenged with the appropriate PAR-AP and either InsP accumulation or active RhoA-GTP was measured as before (Figure 2-2). RhoA activation was measured in cells expressing PAR1 or PAR2

alone or in combination with p115-RGS. Whereas the PAR1-AP and PAR2-AP both stimulated RhoA activation two-fold over basal, this response was reduced to basal levels in the presence of p115-RGS (Figure 2-7A), indicating that RhoA activation by PARs relies on $G_{12/13}$ activation (in these cells using these methods). By contrast, activation of InsPs by PAR1 and by PAR2 in COS-7 cells appears to be mediated by $G_{q/11}$ (Figure 2-7B). We found that both of the PAR-APs stimulated maximal InsPs in the presence or absence of p115-RGS (Figure 2-7B). Since both $G_{q/11}$ and $G_{12/13}$ stimulate inositol lipid signaling by distinct PLC iosforms (PLC-β and PLC-ε, respectively), we tested inhibitors of both G proteins. The PAR-activated responses were reduced by approximately 85% and 65% of maximal InsP production, respectively, in cells that expressed GRK2-RGS (Figure 2-7B) suggesting that both PAR1- and PAR2-directed InsP production in COS-7 cells is mediated predominantly by $G_{q/11}$ (and likely PLC-β) and not by $G_{12/13}$ (and PLC-ε) under these experimental conditions.

2.3.6. PAR-stimulated cAMP, PLC and RhoA signaling in Neu7 cells

Up to this point, we have compared PAR1 and PAR2 coupling to G proteins by examining recombinant proteins exogenously expressed in cells that express undetectable levels of functional PARs (COS-7 cells). These studies (Figs. 2-1 through 2-7) have been valuable in identifying both similarities and differences between these two closely related receptors. However, in order to confirm the physiological relevance of these observations, we deemed it necessary to determine whether these differences in PAR/G protein coupling and signaling are maintained in cells that endogenously express these proteins.

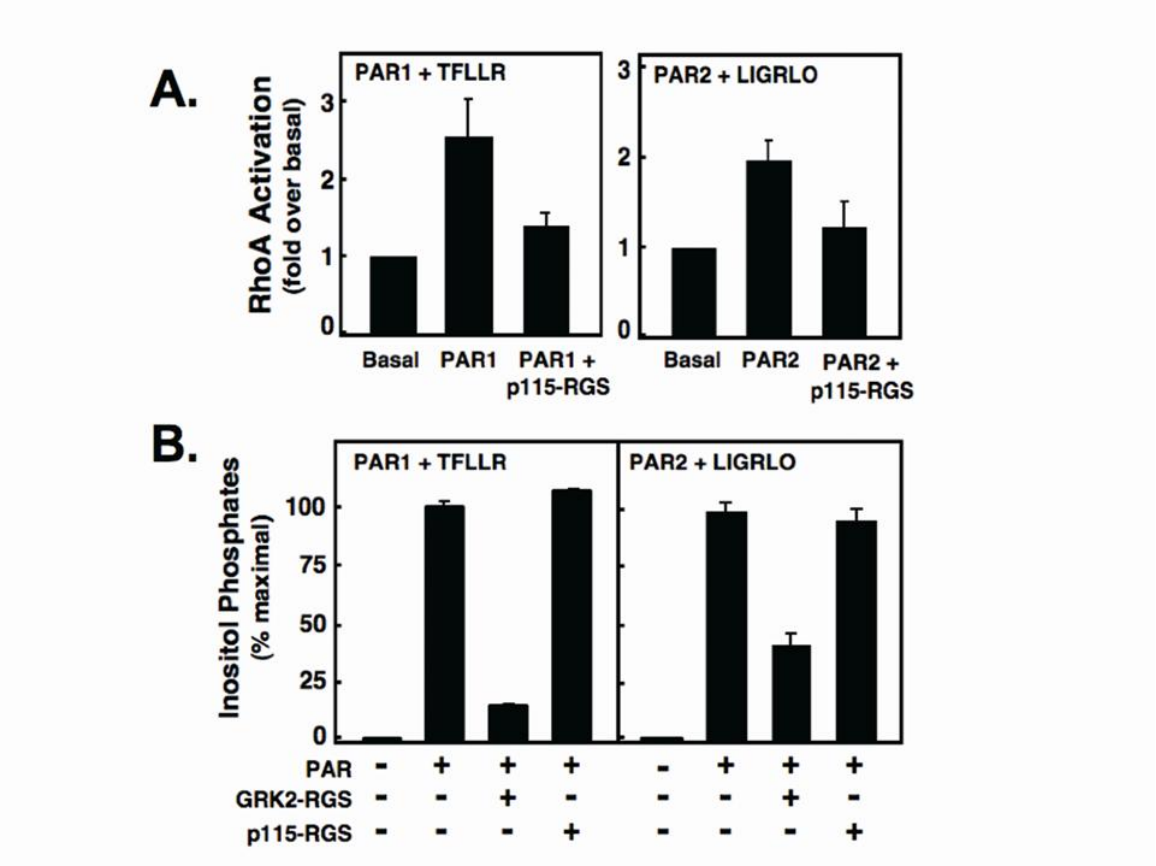

Figure 2-7. PAR1 and PAR2 both utilize $G_{q/11}$ to activate PLC-β signaling and $G_{12/13}$ to activate Rho. A) PAR-mediated RhoA activation was measured using a RhoA G-LISA™ Assay kit as described in Figure 2-2. PARs were transfected either alone or in combination with p115-RGS cDNA into COS-7 cells, serum-starved overnight, and stimulated with 30μM TFLLR or 10μM LIGRLO for 2 min before cell lysis. Lysates were added to the ELISA plate supplied in the G- LISA™ Assay kit and the manufacturer's protocol was followed. The absorbance of each well was read with a spectrophotometer wavelength of 490nm. Data are presented as the average RhoA activation from three different experiments (fold over basal + S.E.M.; each point performed in duplicate). B) As described for Figure 2-2, [^{3}H]InsP accumulation in intact

COS-7 cells were transfected with the indicated PAR alone or in the presence of the specific G protein inhibitor (GRK2-RGS or p115-RGS), pre-labeled with 4 μCi/ml *myo*-[^{3}H]inositol, incubated with $LiCl_2$, and activated with 30μM TFLLR or 10μM LIGRLO for 30 min. After solubilization, lysates were neutralized and separated by anion exchange chromatography. Data are presented as the average of total InsPs from three different experiments (% maximal InsPs + S.E.M; each point performed in triplicate).

For this purpose, we obtained Neu7 astrocytes, a cell line reported to express both native PAR1 and PAR2 (Vandell et al., 2008).

We first tested whether endogenous PAR1 and PAR2 both activate the same G protein signaling pathways in Neu7 cells as we observed with recombinant proteins in COS-7 cells (Figure 2-8). Since these cells do not transfect well, we employed PTX and selective pharmacological inhibitors of PLCβ (U73122) and RhoA (C3 toxin) to dissect the involved downstream signaling pathways. Cellular cAMP levels were measured in Neu7 cells following stimulation of an endogenous α-AR with isoproterenol alone or in combination with either TFLLR or LIGRLO. As shown in Figure 2-8A, isoproterenol stimulated cAMP production. Upon simultaneous activation with isoproterenol and TFLLR, cellular cAMP levels were reduced by nearly 40% (p =0.035), and this inhibition is reversed in the presence of PTX. Conversely, LIGRLO in the presence or absence of PTX had no effect on cAMP production in Neu7 cells (Figure 2-8A). TFLLR- or LIGRLO-stimulated InsP accumulation or RhoA-GTP formation also was measured as before (Figure 2-2 and Figure 2-7). We found that both of the PAR-APs stimulated InsPs in the presence or absence of C3 toxin (Figure 2-8B), suggesting no role for $G_{12/13}$-linked Rho pathways. However, this PAR-activated response was reduced to approximately basal levels of InsP production in cells treated with U73122 (Figure 2-8B), indicating that both PAR1- and PAR2-mediated InsP production in Neu7 cells is activated by a $G_{q/11}$-PLC pathway under these conditions. Conversely, PAR1 and PAR2 activation of RhoA in Neu7 cells (Figure 2-8C) is likely mediated by $G_{12/13}$-RhoA pathways since both PAR-APs activated RhoA. This activation was reversed to near basal levels in the presence of C3 toxin (Figure 2-8C).

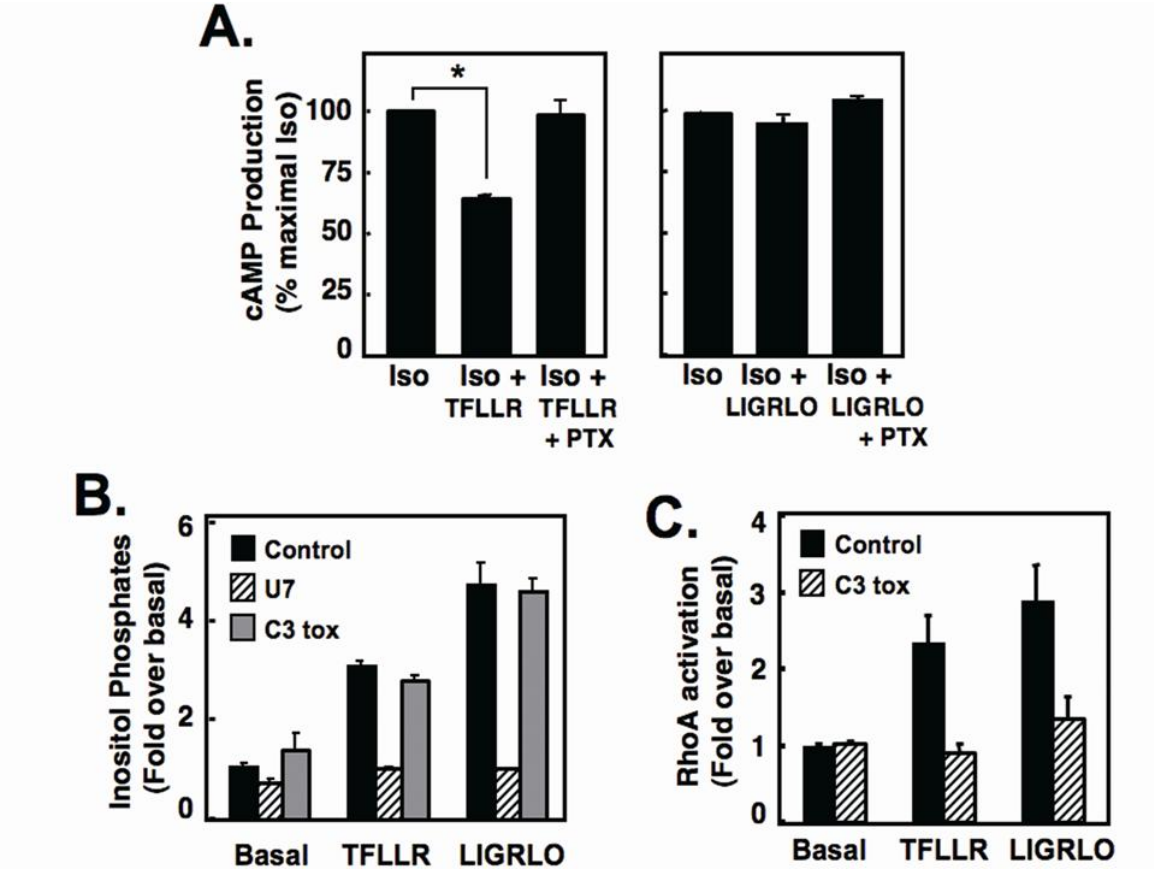

Figure 2-8. PAR1 and PAR2 both utilize $G_{q/11}$-linked pathways to activate inositol phosphate signaling and $G_{12/13}$-linked pathways to activate RhoA in Neu7 cells. A) [^{3}H]InsP accumulation was measured in Neu7 cells in the presence and absence of pharmacological inhibitors of PLC (10μM U73122; added 30 min prior to stimulation) or Rho (1 μg/mL C3 toxin; added 4 h prior to stimulation) signaling. Cells were stimulated with 100μM TFLLR or 10μM LIGRLO for 30 min before solubilization. Then lysates were neutralized and separated by anion exchange chromatography. Data are presented from three different experiments (fold over basal InsPs + S.E.M; each point performed in triplicate). B) Similar to Figures. 2-2 and 2-7, PAR-mediated RhoA activation in Neu7 cells was measured using a RhoA G-LISA™ Assay kit. Cells were serum-starved

overnight, and during the final 4 hours of stimulation, 1 μg/mL C3 toxin was added to appropriate wells. Cells were then stimulated with 100μM TFLLR or 200μM LIGRLO for 2 min before cell lysis. Lysates were placed in the G-LISA™ plate and the manufacturer's protocol was followed. The absorbance of each well was read with a spectrophotometer wavelength of 490nm. Data are presented as the average RhoA activation from three different experiments (fold over basal + S.E.M.; each point performed in duplicate).

2.3.7. PAR1 and PAR2 utilize overlapping and distinct G protein pathways to stimulate ERK1/2 phosphorylation in Neu7

Because our studies in COS-7 cells indicate that PAR1 selectively couples to $G_{i/o}$ to activate ERK1/2 signaling (Figures 2-2 through 2-6), and PAR1 inhibition of cAMP production in Neu7 cells is PTX-sensitive, we sought to determine whether PAR1 activation of ERK1/2 in Neu7 cells relied on $G_{i/o}$ signaling as well (Figure 2-9). Neu7 cells were treated with varying concentrations of PTX (0-300 ng/mL) overnight and then separately stimulated with the PAR-APs. Cells were harvested and levels of ERK1/2 phosphorylation, normalized to total ERK levels, were measured by immunoblot analysis (Figure 2-9A) and quantified by densitometry (Figure 2-9B). PTX treatment inhibited TFLLR-stimulated ERK1/2 phosphorylation in Neu7 cells (greater than 50%) when compared to the effects of LIGRLO. This inhibition was statistically significant (Figure 2-9B; $p < 0.001$) across all PTX concentrations tested, independent of the concentration of toxin used. By contrast, PTX had no effect on LIGRLO-directed ERK signaling. These findings with endogenous proteins in native cells are consistent with our studies in COS-7 cells (Figures 2-2 through 2-6), which show that PAR1, but not PAR2, forms a functional complex with $G_{i/o}$ family members, and that PAR1, but not PAR2 relies on $G_{i/o}$ to stimulate ERK1/2 phosphorylation.

To determine the mechanism whereby PAR2 elicits ERK1/2 phosphorylation, we employed inhibitors of various other signaling pathways known to be involved in ERK1/2 signaling. Neu7 cells were treated with PAR-APs together with either no inhibitor, the selective PKC inhibitor BIS, the selective PLC□ inhibitor U73122, or the Rho inhibitor C3 toxin. Cells were harvested and ERK1/2 phosphorylation levels were assessed through

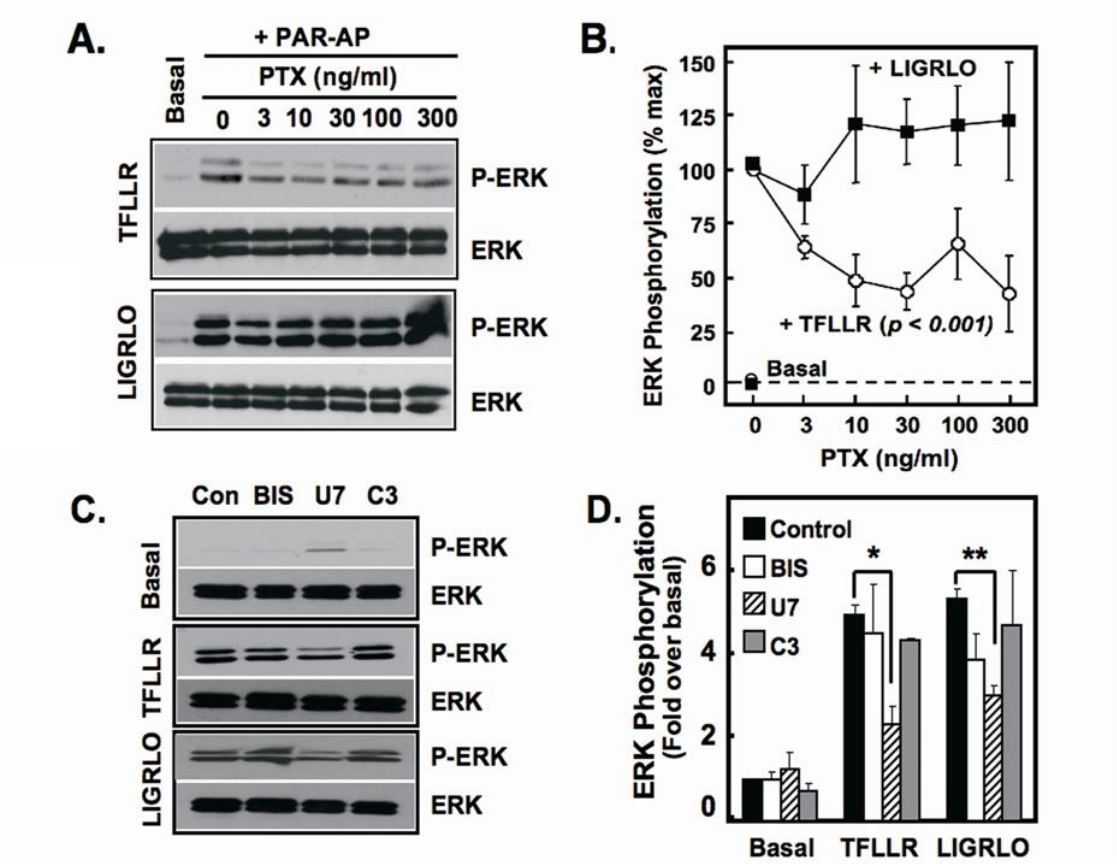

Figure 2-9. PARs stimulate ERK1/2 phosphorylation in Neu7 cells. A-B) Neu7 cells were serum-starved overnight in a range of PTX concentrations (0-300 ng/mL), and stimulated with either nothing, 100μM TFLLR or 200μM LIGRLO, as indicated. Densitometry was performed on three independent experiments and phospho-ERK1/2 levels were normalized to total ERK levels. C) Neu7 cells were serum-starved overnight. Prior to stimulation with either 100μM TFLLR or 200μM LIGRLO, inhibitors to PKC (1μM BIS; 30 min) PLC (10μM U73122; 30 min), or Rho (1 μg/mL C3 toxin, 4 h) were added to the serum-free media. Densitometry was performed on three independent experiments and phospho-ERK1/2 levels were normalized to total ERK levels. All immunoblots were performed with either phospho-ERK1/2 or total ERK1/2 antibodies and protein bands were detected by ECL.

immunoblot analyses followed by densitometry (Figure 2-9C-D). Pretreatment of cells with the PLC inhibitor, U73122, but not inhibitors of PKC or Rho signaling, reduced TFLLR- and LIGRLO-stimulated ERK1/2 phosphorylation levels nearly half (p <0.05 and p <0.01, respectively; Figure 2-9C), suggesting that PAR1 and PAR2 both (partially) stimulate ERK1/2 signaling through PLC-mediated pathways (Figure 2-9C-D). However, as shown above, $G_{i/o}$-mediated pathways also contribute to ERK1/2 phosphorylation mediated by PAR1 but not by PAR2 (Figure 2-9A-B).

2.3.8. PAR1, but not PAR2, influences Neu7 cell migration via a PTX-sensitive $G_{i/o}$ pathway

ERK1/2 pathways regulate cell growth, proliferation, and migration among other cellular processes. To provide a physiological readout of the activation of $G_{i/o}$-linked pathways by PARs, we tested whether PAR-APs modulated cellular migration of Neu7 cells as measured by a wound-scratch assay (Figure 2-10). For these experiments, cells were plated and grown to 100% confluence, after which a scratch across the monolayer was introduced resulting in a space devoid of cells. In this assay, migration of cells into the empty space after 24 h in response to agonist is a measure of cell migration. Cells were placed in serum-free media containing either vehicle, TFLLR, or LIGRLO in the presence or absence of PTX or the ERK (MEK1/2) inhibitor U0126. In the absence of serum or PAR-APs (control), Neu7 astrocytes exhibited some migration into the empty space after 24 h, consistent with basal movement of these cells. TFLLR and LIGRLO both stimulated clearly evident migration compared to control cells, nearly filling the space (Figure 2-10A-B). However, following PTX treatment, only TFLLR-directed Neu7

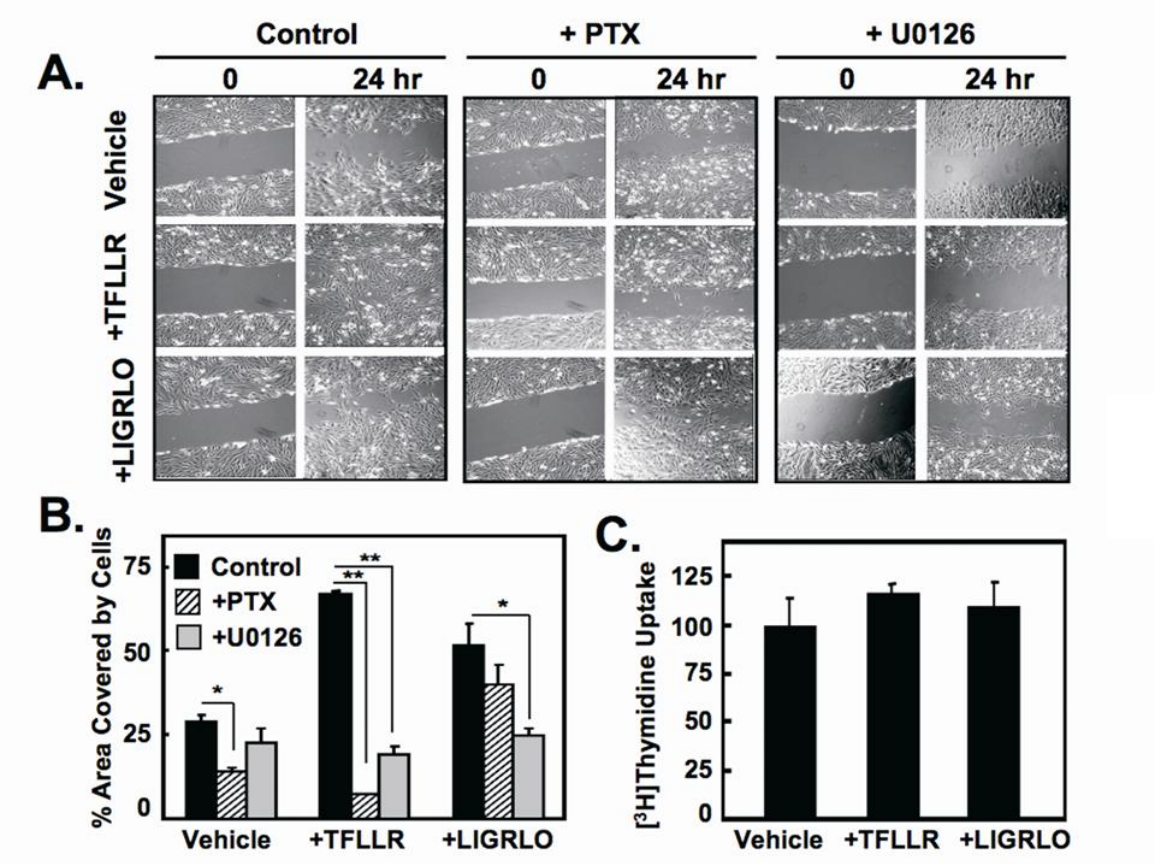

Figure 2-10. PAR1 and PAR2, stimulation of Neu7 cell migration involves ERK-mediated pathways but only PAR1-induced migration is PTX-sensitive A) Neu7 cells were "wounded" with a 10µL pipette tip that was dragged across each monolayer of a 6-well plate. Cells were then serum-starved in the presence and absence of 100 ng/mL PTX or 10µM U0126 and then treated with either vehicle, 100µM TFLLR, or 200µM LIGRLO for an additional 24 h. Pictures were taken with an Olympus IX51 light microscope after 0 h and 24 h of agonist addition. Images shown are representative of three different experiments. B) Cell migration into the wounded area from the images in (A) and also from a different set of similar images was quantified using ImageJ software. For each condition pairings, the cell-free areas were subtracted from the total area of the wound to

obtain the area covered by cells. This number was then divided by the total area value to obtain a percent value. C. Confluent Neu7 cells were serum-starved for 24 h prior to treatment with either vehicle, 100μM TFLLR, or 200μM LIGRLO for an additional 24 h. [^{3}H]thymidine was added to the cells for the final 2 h of the experiment and was recovered in the acid-insoluble material at the end of the experiment. Data are reported as the average of four different experiments (% max TFLLR stimulation + S.E.M.).

cell migration is significantly blocked (p =0.03) whereas cell migration associated with LIGRLO or vehicle treatment was unaffected (Figure 2-10A-B). We believe that the presence of PAR-AP-stimulated cells in the wounded area is indicative of migration and not cellular proliferation because Neu7 cells grown and treated identically failed to incorporate [^{3}H]thymidine into new DNA synthesis, a measure of cellular proliferation (Fig 2-10C).

Interestingly, the MEK inhibitor U0126 significantly blocks cell migration by PAR1 (p =0.003) and PAR2 (p =0.04), respectively, indicating that both receptors rely on ERK1/2 signaling pathways to promote cell migration. To further characterize the mechanism by which PAR2 induces cell migration, we attempted to perform the same wound-scratch experiments in the presence of the PLC inhibitor (U73122) that blocks PAR2-mediated ERK1/2 phosphorylation (Figure 2-9C-D). However, after 24 h, very few cells treated with U73122 remained adhered to the plate, indicating that long-term treatment with this inhibitor is toxic to Neu7 cells, thereby limiting our capacity to measure PLC-effects on PAR-mediated cell migration in Neu7 cells.

2.4. Discussion

Although much has been learned about PAR1 signaling in recent years, substantially less is known about PAR2 signaling. Furthermore, only one study has compared PAR1- and PAR2-directed G protein signaling in the same cells (olfactory sensory neurons of the olfactory bulb) (Olianas et al., 2007). Here we compared PAR1

and PAR2 signaling in COS-7 cells that express undetectable levels of these PAR receptors, and also in Neu7 astrocytes that natively express both receptors. Our key findings indicate the following: 1) PAR1 and PAR2 couple to both overlapping and distinct sets of G proteins; 2) PAR1 but not PAR2 links to G_o and G_i family members; 3) Receptor/G protein complex formation is stable even in the presence of activating ligand and nucleotide; 4) $G_{i/o}$ contributes to PAR1- but not PAR2-directed effects on cellular ERK1/2 and cAMP signaling in both COS-7 cells and Neu7 cells; 5) PAR1, but not PAR2 relies partly on a PTX-sensitive $G_{i/o}$ signaling pathway to stimulate ERK1/2 signaling and cell migration in Neu7 cells; and 6) both PAR1 and PAR2 rely partly on $G_{q/11}$-PLC signaling pathways to stimulate ERK1/2 signaling and cell migration in Neu7 cells. We will discuss each of these findings.

2.4.1. PAR1 and PAR2 both couple to multiple overlapping sets of G proteins

Our findings indicate that PAR1 and PAR2 both couple, to similar extents, to $G_{q/11}$ family members (G_q, G_{11} and G_{14}), $G_{12/13}$ family members (G_{12} and G_{13}) and to the downstream signaling pathways activated by these G proteins. These signaling pathways include InsP production, calcium signaling, and RhoA activation. In COS-7 cells, the former signaling response likely is due to activation of PLC-β, but not PLC-ε, since a direct and selective inhibitor of $G_{12/13}$ did not affect InsP accumulation. Our findings also suggest that in COS-7 cells, PAR1 and PAR2 activation of RhoA is mediated by $G_{12/13}$ since a direct and selective inhibitor of $G_{12/13}$ reduced RhoA activation to near basal levels in response to activation of either receptor. Which G protein signaling pathway PARs choose to utilize in order to activate either InsP/calcium and/or RhoA likely is cell-

specific since cross-talk between these G protein-linked pathways is known to occur (Kelley et al., 2004;Hains et al., 2006;Citro et al., 2007).

2.4.2. PAR1, but not PAR2, couples to G_o and also to G_i family members

Our results indicate that PAR1, but not PAR2, is coupled to G_o and to G_i family members (G_{i1} and G_{i2}). In our studies, we assessed receptor/G protein complex formation, inhibition of adenylyl cyclase-directed cAMP production, and PTX-sensitive ERK1/2 activation. These findings are consistent with previous reports indicating that PAR1-directed PI3K signaling and platelet activation is mediated by PTX-sensitive $G_{i/o}$ signaling (Voss et al., 2007), that PAR1 pre-assembles with G_{i1} in bioluminescence resonance energy transfer studies (Ayoub et al., 2007), and that G_o mediates PAR1-directed intracellular calcium signaling and cytoskeletal rearrangements in endothelial cells (Vanhauwe et al., 2002). Significantly, our findings suggest that, at least in the cells examined in these studies, PAR2 does not couple to G_o or to G_i family members. This difference in G protein coupling could have profound consequences for the physiological responses of cells that express both PAR1 and PAR2.

Our findings raise the important mechanistic question of how PAR's couple to multiple distinct G proteins. The intracellular loops 2, 3 and 4 of PAR1 have been implicated in receptor-G protein coupling (Verrall et al., 1997;Swift et al., 2006). These loops are relatively small and are not likely to couple to three or more G proteins simultaneously due to steric hindrance alone. One possibility is that different populations of PARs may link to distinct G proteins depending on receptor location within the plasma membrane, as is the case with the $S1P_1$ receptor. Like PARs, the $S1P_1$ receptor is a GPCR

that links to multiple G protein signaling pathways (Sorensen et al., 2003;Means et al., 2008). Recent studies show that $S1P_1$ receptor coupling to specific G proteins depends on whether or not the receptor is localized to lipid rafts (caveolae) (Means et al., 2008). Perhaps PAR-G protein coupling also depends on receptor localization within specialized microdomains of the plasma membrane. A separate question centers on whether PARs contain specific recognition sites for each G protein or, alternatively, whether multiple G proteins dock at overlapping recognition sites. Ongoing studies in our laboratory are investigating these two possibilities. We also should note that the agonists we used in our experiments could influence the G protein coupling of the PARs. McLaughlin and colleagues (McLaughlin et al., 2005) have shown that different agonists for the same receptor (PAR1) exhibit a functional selectivity for particular G protein pathways. That is, PAR-APs, rather than endogenous agonists (e.g., thrombin), cause PAR1 to couple much more strongly to $G_{q/11}$ signaling pathways relative to $G_{12/13}$ signaling pathways (McLaughlin et al., 2005). However, this finding does not explain the PAR/G protein complexes we observed that formed *independent* of receptor agonist, and our biochemical data are consistent with PAR/G signaling events we observed in both cells types using the PAR-APs.

2.4.3. PAR1 and PAR2 form complexes with G proteins that are stable in the presence of agonist and nucleotide

We found that PAR1 and PAR2 both form stable complexes with G protein heterotrimers (i.e., $G\alpha_{11}$ plus $G\beta\gamma$ as well as $G\alpha_o$ plus $G\beta\gamma$) that remain intact in cell lysates following addition of agonist and activating nucleotide (e.g., GTPγS). These

findings were unexpected since most established models of GPCR/G protein signaling and many previous reports suggest that agonist and nucleotide activation of GPCRs results in dissociation of the receptor/G protein complex. One possibility is that PAR/G complexes behave differently in broken cell lysates versus whole cells (i.e., missing intact cellular elements that are necessary for uncoupling). Alternatively, these findings also are consistent with more recent reports and proposed models, which suggest that the receptor/G protein complex remains intact following agonist activation. In this new model, receptors serve as signaling platforms that assemble multiple signaling components (e.g., heterotrimeric G proteins, RGS proteins, arrestins, GRKs, effectors) and, following receptor activation, G proteins do not dissociate but instead rearrange *in situ* to initiate signaling (Bunemann et al., 2003;Hein and Bunemann, 2008). Whether these receptor/G protein complexes internalize as a complex is unknown, though sustained coupling following internalization could result in sustained G protein signaling since PARs are constitutively activated following protease cleavage. Sustained PAR/G protein complex formation also is consistent with recent evidence showing that PAR-mediated ERK1/2 activation differs from some other GPCRs (DeFea et al., 2000). In the case of PAR2, ERK1/2 phosphorylation is partially dependent on formation of a stable PAR2/Arrestin2 (Arr2) complex that directs ERK signals away from the nucleus and cellular proliferation. However, uncoupling PAR2 from Arr2 binding results in ERK1/2 signaling that is directed to the nucleus to promote cell proliferation (DeFea et al., 2000). Of note, our findings with ERK activation (Figs. 4 and 7) likely reflect initial PAR2/G protein activation (i.e., 2 min of stimulation) of $G_{q/11}$-PLC-mediated pathways rather than PAR2/Arrestin signaling (under these experimental conditions in Neu7 cells).

2.4.4. $G_{i/o}$ signaling mediates PAR1 but not PAR2 contributions to ERK1/2 signaling and migration in Neu7 astrocytes

We observed that PTX treatment had differential effects on PAR1 and PAR2 signaling and cellular responses in Neu7 cells. Both PAR1 and PAR2 stimulated ERK1/2 phosphorylation and cell migration but only PAR1 effects on MAPK signaling and migration were PTX-sensitive. By contrast, PLC signaling pathways contribute to both PAR1- and PAR2-directed ERK1/2 phosphorylation and Neu7 cell migration. Importantly, cell migration induced by both PARs appears to rely on ERK signaling. The MEK1/2 inhibitor U0126 significantly reduced migration observed when either PAR-AP was used to stimulate migration into the open area of the cell monolayer. Whether this finding is consistent with the mechanism by which PAR2 activates ERK1/2 signaling (i.e., through PLC-mediated pathways) remains unknown. Our attempts to fully characterize the mechanism responsible for PAR2-directed cell migration were unsuccessful since we found that the PLC inhibitor U73122 is extremely toxic to Neu7 cells after 24 h time period required for the studies. Nevertheless, our cell migration data in cells expressing native PARs and G proteins corroborate our observations with recombinant proteins in COS-7 cells–that PAR1 selectively couples to $G_{i/o}$ whereas PAR2 does not. Neu7 cells have been used as a cell culture-based model system to study mechanisms of glial scarring (Fok-Seang et al., 1995). As such, PAR1- and PAR2-directed signaling pathways may interact differentially with those of other CNS-derived factors to modulate cell growth and proliferation involved with glial scarring following head injury, stroke or other insults that compromise the blood-brain barrier.

In summary, we report here that PAR1 and PAR2 activate multiple shared and distinct G protein signaling pathways, and that PAR1, but not PAR2, relies upon G_o and G_i family members to mediate its receptor-specific effects on MAPK signaling and migration. These studies highlight previously unknown G protein signaling mechanisms used by these two closely related receptors, and physiologically relevant differences between them.

Chapter 3: Point mutations in the second intracellular loop of PAR1 selectively disrupt receptor coupling to $G_{q/11}$ but not to $G_{i/o}$ or $G_{12/13}$[3]

[3] This chapter is being prepared to be submitted to *Biochemistry*: McCoy KL, Gyoneva, S, Waters JP, Traynelis SF, Hepler JR (2010) Point mutations in the second intracellular loop of PAR1 selectively disrupt receptor coupling to $G_{q/11}$ but not to $G_{i/o}$ or $G_{12/13}$. *In preparation.*

3.1. Introduction

Protease-activated receptor 1 (PAR1) is a G-protein coupled receptor (GPCR) that was first identified as the thrombin receptor (Vu et al., 1991a). Although it is best known for its role in platelet activation and hemostasis (Coughlin, 2005), PAR1 also is expressed throughout the central nervous system (CNS) and has complex pathophysiological roles within the brain. PAR1 activators are expressed in the brain parenchyma, and the receptor itself is expressed on both neurons and glia (reviewed in (Wang and Reiser, 2003;Ossovskaya and Bunnett, 2004;Traynelis and Trejo, 2007)). Typically, neuronal and astrocytic PAR1 is shielded from proteases by the blood-brain barrier (BBB). However, upon CNS injury and subsequent BBB breakdown, high levels of these proteases infiltrate the brain tissue with consequences that currently are not well understood. In what appears to be a concentration-dependent manner, activation of PAR1 may be either neuroprotective (i.e., by enhancing neuronal or astrocytic survival) or neurodegenerative (i.e., by regulating glutamate excitotoxicity and enhancing seizure sensitivity) (Xi et al., 2003b;Traynelis and Trejo, 2007). The molecular signaling events that underlie these functions of PAR1 in the CNS have not been fully characterized.

PAR1 is one of four protease-activated receptors (PAR1-4) (Vu et al., 1991a;Nystedt et al., 1994;Ishihara et al., 1997;Xu et al., 1998), which are N-terminally cleaved and activated by serine proteases (e.g., thrombin, trypsin, plasmin and others). The newly unmasked extracellular N-termini serve as intramolecular ligands that activate the receptors and cause conformational changes, which allow the receptors to activate

linked G proteins and initiate signaling (Macfarlane et al., 2001;Traynelis and Trejo, 2007).

PAR1 is an unconventional GPCR in that it functionally interacts with $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ subfamilies (Macfarlane et al., 2001;Traynelis and Trejo, 2007;McCoy et al., 2010). In doing so, PAR1 activates multiple effector pathways including mitogen-activated protein kinase (MAPK), phospholipase C (PLC), and intracellular calcium signaling in various cell types including immortalized cell lines (e.g., COS-7 cells) and astrocytes (i.e., primary cultures from rodents) (Dery et al., 1998;Macfarlane et al., 2001;Traynelis and Trejo, 2007). Having the capacity to couple to multiple G proteins is unusual among GPCRs, which makes PAR1/ G protein coupling a particularly important mechanism to investigate. Whether members of different G protein subfamilies bind to PAR1 simultaneously or individually is unknown. Furthermore, whether $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ have distinct or overlapping binding sites on PAR1 has not been explored. Overall, the molecular mechanisms underlying the regulation of G protein coupling by PAR1 have not been elucidated

Several investigators have sought to identify domains on PAR1 responsible for G protein coupling (Verrall et al., 1997;Swift et al., 2006). By studying the signaling properties of chimeric receptors—either the G_s-linked β_2-adrenergic receptor (β_2AR) or the G_i-linked dopamine D_2 receptor (D_2R) containing cytoplasmic portions of PAR1—Verrall and colleagues found that the PAR1 second intracellular loop (PAR1 i2 loop) is responsible for the receptor's coupling to inositol phosphate (InsP) and intracellular calcium signaling (Verrall et al., 1997). Later, Covic and colleagues implicated the PAR1 i2 and i3 loops in PAR1/G protein coupling by using cell-penetrating, membrane-tethered

peptides corresponding to these domains to prevent PAR1 activation of calcium and InsP signaling (Covic et al., 2002). A more recent study by Swift and colleagues used molecular modeling to identify potential domains that are required for G protein coupling to PAR1. The authors suggest that to activate pertussis toxin (PTX)-insensitive (i.e., $G_{q/11}$-mediated) InsP production, PAR1 employs a "7-8-1" mechanism of activation, which requires a network of H-bonds and ionic interactions between the PAR1 transmembrane 7, 8^{th} helix (part of the receptor's cytoplasmic tail), and the PAR1-i1 loop (Swift et al., 2006). Taken together, these three studies have broadly identified multiple PAR1 domains responsible for activation of G protein signaling pathways located within the receptor's intracellular loops and cytoplasmic tail. However, to our knowledge, uncoupling the binding and functioning of one G protein to PAR1 while preserving the binding and functioning of other G proteins to PAR1 has not yet been demonstrated.

In the present study, we investigated G protein binding to PAR1 with the goal of testing: 1) whether all G proteins bind to overlapping sites within the i2 loop and 2) if not, then could we selectively uncoupling PAR coupling to one G protein while preserving PAR coupling to other G proteins. To do so, we used alanine scanning mutagenesis to create a series of 21 individual receptor mutants, each containing a different single point mutation within the PAR1 i2 loop. Individually expressing each of these recombinant mutant receptors in COS-7 cells, which express only low levels of PAR1, and in astrocytes from PAR1-/- mice, we have identified a single amino acid, Arg205, that when mutated, disrupts PAR1 binding and functional coupling to $G_{q/11}$ but not to $G_{i/o}$ or $G_{12/13}$. With these studies, we have uncovered a previously unknown mechanism of PAR1/ G

protein coupling, which suggests that each G protein subfamily likely has distinct binding sites located within the receptors' intracellular loops and/or C-terminus.

3.2. Experimental Procedures

3.2.1. Materials

Materials and reagents used in our studies were purchased from the following sources: QuikChange mutagenesis kit from Stratagene (La Jolla, CA); Anti-FLAG M2 affinity gel, bovine serum albumin (BSA), U73122, penicillin, and streptomycin from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum (FBS) from Atlanta Biologicals (Atlanta, GA); trypsin, Dulbecco's modified Eagle's medium (DMEM) from Cellgro (Herndon, VA); Lipofectamine 2000 from Invitrogen (Carlsbad, CA); *myo*-[^{3}H]inositol from American Radiolabeled Chemicals, Inc.(St. Louis, MO); RhoA G-LISA™ Activation Assay colorimetric format kit and C3 exoenzyme from Cytoskeleton, Inc. (Denver, CO); Pertussis toxin from List Biologicals (Campbell, CA); p44/42 ERK1/2 (extracellular signal-regulated kinase 1/2) antibody and phospho-p44/42 ERK1/2 antibody from Cell Signaling Technology (Beverly, MA); anti-Gα_o and anti-Gα_{12} antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); an anti-G$\alpha_{q/11/14}$ antibody, Z811, was kindly provided by Dr. Paul Sternweis (U. Texas Southwestern, Dallas, TX); and peroxidase-conjugated goat anti-rabbit was from Bio-Rad (Hercules, CA). The PAR1-activating peptide TFLLR-NH_2 (TFLLR) was synthesized by Dr. Jan Pohl at the Emory University Microchemical Facility (Atlanta, GA). Fura-2 etc.

3.2.2. Methods

Alanine scanning mutagenesis—To introduce site-specific mutations into the PAR1-mcherry-FLAG clone, the QuikChange mutagenesis kit was used according to the manufacturer's suggestions. Alanine-scanning mutagenesis was carried out to create a series of 21 mutations in the i2 loop of PAR1. Mutant receptors were generated during separate site-directed mutagenesis reactions with different primer sets, each using the original wtPAR1-mcherry-FLAG clone as a template. To confirm that the appropriate amino acid was changed to an alanine residue, cDNA sequencing was performed by Agencourt (Beverly, MA).

COS-7 cell cultures and transfection—COS-7 (ATCC® Number CRL-1651™) cells were propagated in DMEM with sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, 100 μg/mL streptomycin and 100U/mL penicillin at 37°C in a humidified atmosphere with 5% CO_2. Subculturing of confluent plates was done at a ratio of 1:10 for transfection. COS-7 cells were transfected according to Lipofectamine 2000® transfection reagent protocol and cells were used for experimentation 24-48 h after transfection.

Animals—PAR1-/- and wild-type (wt) mice were created as described (Mannaioni et al., 2008). Briefly, we bred *PAR1+/-* mice, a generous gift from Dr. Shaun Coughlin (University of California, San Francisco, CA), with wt C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME). We bred heterozygous littermates to generate homozygous null mutants and wt controls that were > 99% C57BL/6. All procedures

using animals were approved by the Emory University Institutional Animal Care and Use Committees.

Astrocyte cultures and transfections—Cultured astrocytes were prepared from P0-P3 postnatal mouse cortex. Cells were dissociated into a single-cell suspension by trituration through a Pasteur pipette and plated onto coverslips in 12-well plates coated with 0.05 mg/ml poly-D-lysine and grown in DMEM supplemented with 25mM glucose, 10% heat-inactivated horse serum, 10% FBS, 2 mM glutamine, 10 units/mL penicillin, and 10 μg/ml streptomycin.

Immunoblot Analysis—Nitrocellulose membranes were incubated in blocking buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5% milk, 0.5% Tween 20, 0.02% sodium azide) at room temperature for up to 1 h and then were placed in a dilution of the indicated primary antibody at room temperature for 3 h or overnight at 4°C. Dilutions for each antibody differed: anti-mcherry 1:1000, anti-p44/42 ERK1/2 1:300 and anti-phospho p44/42 1:1000 in Tris-buffered saline + 0.1% Tween 20 (TBST) with 5% BSA; anti-$G\alpha_q$ family Z811 1:1000, anti-$G\alpha_o$ 1:200; anti-$G\alpha_{12}$ 1:200; in blocking buffer. After being washed three times with TBST, membranes were then probed with horseradish peroxidase-conjugated goat-anti-rabbit IgG at 1:25,000 in TBST for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL).

Measurement of [^{3}H]InsP formation—Levels of [^{3}H]inositol phosphate ([^{3}H]InsP) accumulation were determined in confluent plates of COS-7 cells transiently transfected with pcDNA3.1, PAR1, or the PAR1 i2 loop mutants. After a 5 h transfection period, cells were metabolically labeled with *myo*-[^{3}H]inositol in serum-free media for 18-24 h. Prior to experimentation, the pharmacological inhibitor of PLC signaling, U73122, was

added for 30 min in incubation buffer (DMEM buffered with 25mM HEPES, pH 8.0, and containing 10 mM $LiCl_2$), as indicated. Then TFLLR was used to stimulate the cells for 30 min. To stop the reactions, COS-7 cells were solubilized with 20 mM formic acid and subsequently neutralized with 0.7 M NH_4OH. [^{3}H]InsPs were subjected to anion exchange chromatography (AG 1-X8 Dowex, Bio-Rad) to isolate [^{3}H]InsPs, which were quantified by scintillation counting and expressed as mean ± S.E.M.

Measurement of ERK1/2 phosphorylation—After serum starvation in the absence or presence of pertussis toxin (PTX) overnight, COS-7 cells separately transfected with pcDNA3.1, PAR1 or PAR1 mutants were stimulated with TFLLR for 5 min, harvested in sample buffer, sonicated on ice, boiled, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 13.5%) and transferred to nitrocellulose membranes for immunoblotting with p44/42 ERK1/2 and phospho- p44/42 ERK1/2 antibodies.

Measurement of RhoA activation—Activated RhoA was measured with an absorbance-based RhoA Activation G-LISA™ kit (Cytoskeleton, Inc., Denver, CO) by following the manufacturer's protocol. Transfected COS-7 cells were serum-starved overnight and then treated with C3 toxin for 4 h, where indicated. A 30 sec addition of TFLLR was used to elicit the Rho response. The absorbance from the G-LISA™ plate was read by a spectrophotometer at a wavelength of 490nm.

Co-Immunoprecipitation of PAR/G protein complexes—COS-7 cells were transiently transfected for 18-24 h with PAR1/ G protein pairs containing either wt or mutant PAR1 and individual G proteins, as indicated. Unstimulated cells were washed once in PBS and harvested in 0.5 mL of Tris Buffer (50mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl, 1 mM EDTA, plus a protease inhibitor pellet), and sonicated

on ice. The detergent *n*-Dodecyl-β-D-maltoside (DβM; Calbiochem) was added to a final concentration of 2% for 3h rotating end-over-end at 4°C for membrane protein extraction. Debris then was pelleted by ultracentrifugation (100,000 x *g*, 4°C, 30 min). Inputs shown are an aliquot of the lysates from just after the ultracentrifugation step that were kept to run on gel. The remaining cytosol was rotated end-over-end overnight at 4°C with anti-FLAG M2 affinity gel. The anti-FLAG resin was pelleted the following day, washed with Tris Buffer containing 0.2% DβM, and resuspended in 2X Laemmli Sample Buffer. Supernatants were loaded onto 11% polyacrylamide gels for SDS-PAGE separation, transferred to nitrocellulose membranes, and immunoblotting was carried out as described.

Calcium Imaging—Calcium imaging in cultured astrocytes was performed as described (Lee et al., 2007). Briefly, cells were incubated in 5 μM Fura-2-AM in 0.5% pluronic acid (Molecular Probes) for 30 min at room temperature, and coverslips were transferred to a microscope stage for imaging. The external buffer contained (mM): 150 NaCl, 10 Hepes, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 5.5 glucose. Buffer pH was adjusted to 7.3 and osmolarity to 325 mosmol kg−1. Imaging was performed with dual excitation at 340 nm and 380 nm wavelengths using either a MicroMax Camera (Princeton Scientific Instruments, Inc., Monmouth, NJ) or an intensified video camera (PTI), and the two resulting images were used for ratio calculations using AxonImagingWorkbench version 2.2.1 (Axon Instruments).

3.3. Results

3.3.1. Five amino acid residues in the PAR1-i2 loop are important for the receptor's capacity to activate inositol phosphate signaling

It is well-established that PAR1 stimulates phospholipase C (PLC) to initiate phosphatidylinositol (4,5)-bisphosphate hydrolysis and InsP signaling (Hung et al., 1992;Dery et al., 1998;Hains et al., 2006). It also has been reported that the i2 loop of PAR1 confers G_q-like coupling (i.e., InsP signaling) to receptors that exclusively link to G_i or G_s (i.e., D_2R and β_2-AR, respectively) (Verrall et al., 1997). However, the precise sites within this cytoplasmic domain that are important for this signaling event to occur have not been identified. The PAR1 i2 loop contains 21 amino acids. Therefore, we created and screened a series of 21 mutant PAR1 receptors, each with a discrete point mutation of individual amino acids in the receptor's i2 loop, for their capacities to stimulate InsP signaling. These screens were performed by overexpressing the recombinant PAR1 mutants in COS-7 cells. Of the 21 mutants we screened, all but three receptors express at levels detectible by western blotting and comparable to wtPAR1 (Figure 3-1). Therefore, we excluded these three poorly expressing receptors from our remaining studies.

COS-7 cells have been reported to express undetectable levels of PAR1 (Ishihara et al., 1997;Blackhart et al., 2000). We have recently confirmed and expanded on these findings (McCoy et al., 2010). Consistent with these reports, COS-7 cells did not stimulate InsP signaling in response to the selective PAR1 agonist, TFLLR, as is shown in our vector (pcDNA3.1) only controls (Figure 3-1). To determine whether any of the PAR1 i2 loop mutants have reduced capacities to stimulate PLCβ activity in COS-7 cells, we compared levels of radiolabeled InsPs that accumulated in cells transiently transfected

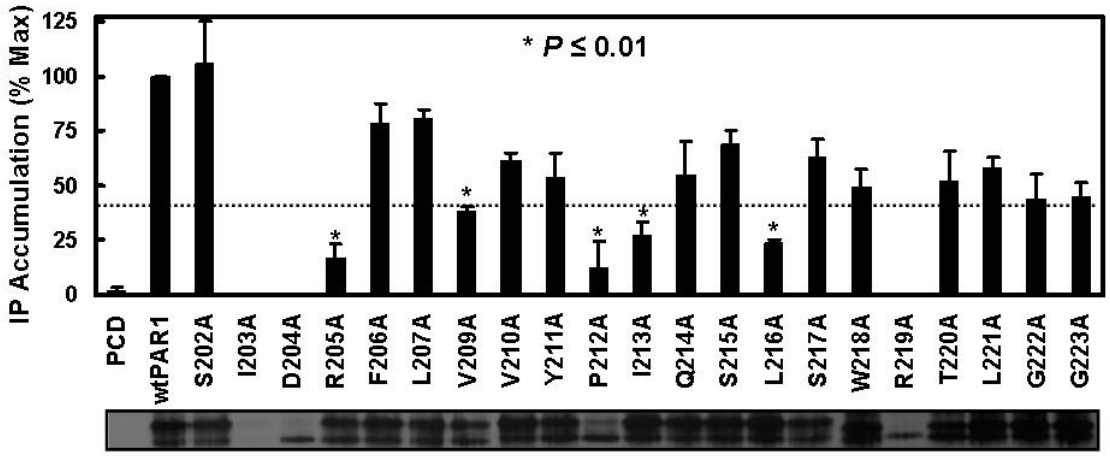

Figure 3-1. PAR1 point mutations differentially impact receptor-activated inositol phosphate signaling. A) COS-7 cells were transfected with either vector alone, wtPAR1, or the indicated PAR1 it loop mutant cDNA. After at least a 5 h transfection period, cells were labeled with 4 μCi/mL *myo*-[^{3}H]inositol in serum-free media overnight. The following day, cells were incubated with $LiCl_2$ and were then activated with 30μM TFLLR for 30 min. To stop the InsP accumulation, cells were solubilized in formic acid, and were subsequently neutralized. After samples were subjected to anion exchange chromatography, total [^{3}H]InsPs were measured using liquid scintillation spectrometry. Data are presented as the percent of maximal InsP accumulation achieved by wtPAR1 (n=3; mean cpm + S.E.M; each point performed in triplicate).

with either wtPAR1 or the mutant receptors and activated with TFLLR (Figure 3-1). From this screen, we identified 5 receptor mutants, R205A, V209A, P212A, I213A, and L216A that elicit significantly reduced ($p \leq 0.01$) levels of InsP signaling relative to wtPAR1. These findings indicate that discrete amino acids within the i2 loop of PAR1 are important for its functional coupling to PLC-mediated InsP signaling in COS-7 cells.

3.3.2. PAR1 mutants that disrupt $G_{q/11}$ coupling do not affect PAR1 coupling to $G_{i/o}$- or $G_{12/13}$

Our recent work showed that PAR1 couples to $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ and signaling pathways linked to these G protein families (McCoy et al., 2010). To explore whether the five amino acids that we identified in our screen (Figure 3-1) are also important for $G_{i/o}$ and $G_{12/13}$ coupling, we tested the capacities of these PAR1 i2 loop mutants to activate $G_{i/o}$- and $G_{12/13}$- linked signaling pathways. As before (Figure 3-1), wtPAR1 and all of the mutant receptors express well and are readily detected upon immunoblotting with the anti-mcherry antibody (Figure 3-2A). As expected, no receptor is present in our pcDNA3.1 control lane (Figure 3-2A). Given the fact that the mutant PAR1 receptors express at similar levels to wtPAR1, we then sought to determine the relative capacities of these mutant receptors to activate RhoA, an effect traditionally attributed to $G_{12/13}$, and to stimulate ERK1/2 phosphorylation, which often is associated with $G_{i/o}$ activation (Figure 3-2C-D). To confirm that the functional readouts of InsP accumulation (from Figure 3-1), Rho signaling, and ERK1/2 phosphorylation reflect true measures of the linked G proteins (i.e., $G_{q/11}$, $G_{12/13}$, and $G_{i/o}$, respectively), we employed selective pharmacological inhibitors of these pathways (i.e., U73122, C3 toxin, and PTX, respectively) to determine

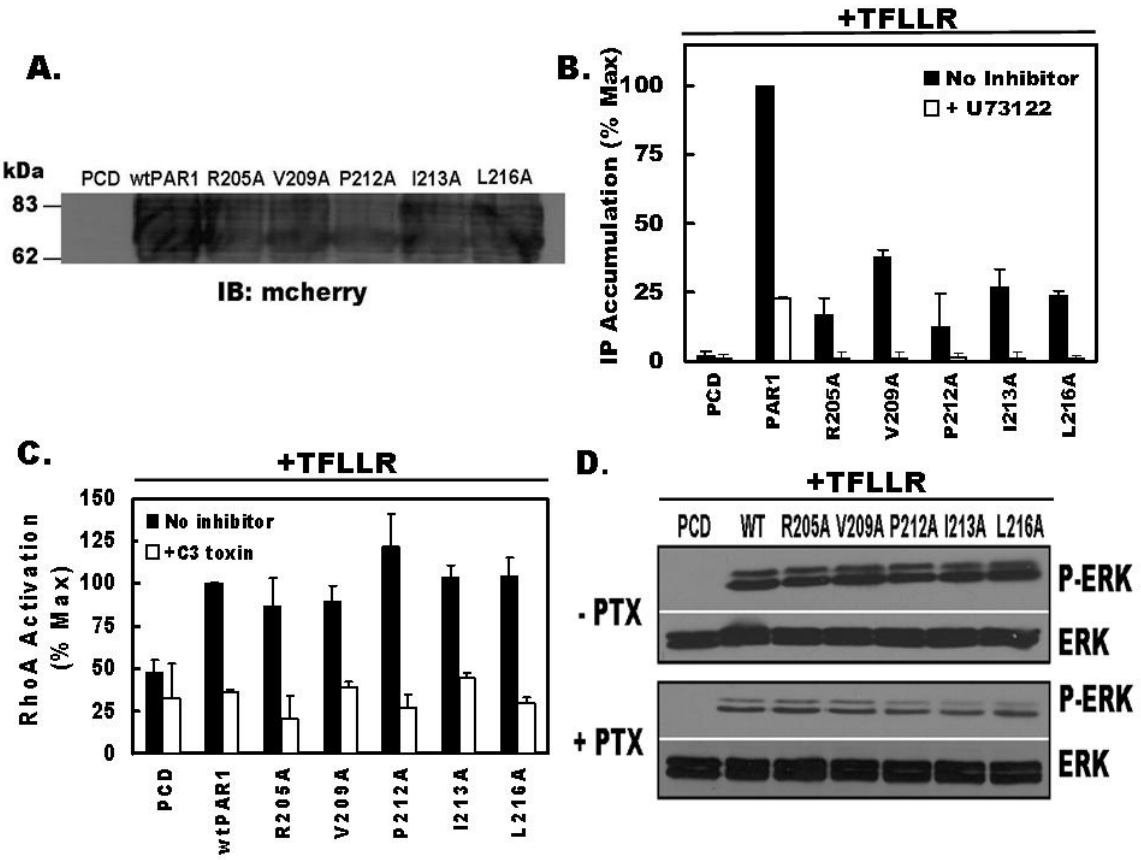

Figure 3-2. PAR1 point mutations have varying effects on G protein-linked signaling pathways. A) After a 24h transfection with vector alone, wtPAR1, or the PAR1 i2 loop mutant cDNA, COS-7 cells were lysed and harvested in sample buffer, sonicated, and subjected to SDS-PAGE. After being transferred to nitrocellulose membranes, immunoblotting was performed with the anti-mcherry antibody. B) InsP accumulation was measured as described for Figure 3-1. Here, samples were also subjected to inhibition with 10μM U73122 for 30 min prior to experimentation, where indicated. DC) PAR1-mediated RhoA activation was measured using a RhoA G-LISA™ Assay kit. First, vector, PAR1, or PAR1 mutant receptor cDNA was separately transfected into COS-7 cells for 5 h before the media was replaced with serum-free media overnight. The

following day, cells were incubated with C3 toxin for 4h, where indicated. They were then activated with 30μM TFLLR for 30 sec before cell lysis. Experimentation was performed according to the manufacturer's protocol, and the absorbances of the wells were read with a spectrophotometer at a wavelength of 490nm. D) Vector alone, PAR1 or the PAR1 i2 loop mutants were separately transfected into COS-7 cells for at least 5 h and then serum-starved overnight in the presence or absence of 30 ng/mL PTX, as indicated. The following day, cells were stimulated with, 30μM TFLLR for 5 min. Immunoblotting was performed with either a phospho-ERK1/2 or total ERK1/2 antibody and then with a goat-anti rabbit secondary antibody. Protein bands were visualized with ECL and exposure to film.

their effects on wtPAR1 and the PAR1 mutant receptors' signaling activities (Figure 3-2B-D).

We first confirmed that activation of inositol lipid signaling by wtPAR1 and the PAR1 i2 loop mutants is mediated by $G_{q/11}$ in COS-7 cells (Figure 3-2B). Because both $G_{q/11}$ and $G_{12/13}$ activate InsP signaling through two different isoforms of PLC, PLC-β and PLC-ε, respectively, we used a known PLC-β inhibitor (U73122) to test whether residual InsP production is mediated through PLC-β. We found that wtPAR1-stimulated InsP production is reduced by nearly 75% and that the five PAR1 i2 loop mutants' signaling is reduced to control levels in the presence of U73122 (Figure 3-2B). These data indicate that wtPAR1 and PAR1 i2 loop mutant receptors stimulate InsP production predominantly through $G_{q/11}$ in COS-7 cells under these experimental conditions.

We next tested whether these mutants couple to $G_{12/13}$. The $G_{12/13}$ family of G proteins is primarily responsible for GPCR-stimulated Rho signaling (Offermanns et al., 1994;Aragay et al., 1995;Post et al., 1996). Previously, we have shown that PAR1 activates $G_{12/13}$-mediated Rho signaling in COS-7 cells (McCoy et al., 2010). Here, we also wanted to determine whether the PAR1 i2 loop mutants had full or reduced capacities to stimulate RhoA activation (i.e., RhoA-GTP formation) in these cells. To do so, we used a Rho G-LISA™ assay kit, which uses the Rho-binding domain of Rho effector proteins to detect the presence of Rho-GTP in cell lysates. We found that when transiently transfected into COS-7 cells and stimulated with TFLLR, all of the tested mutant receptors triggered RhoA activation at levels comparable to that of wtPAR1 when transfected into COS-7 cells (Figure 3-2C). It also is important to note that Rho activation also may occur as a result of $G_{q/11}$ stimulation of p63RhoGEF (Lutz et al., 2005). To

confirm that PAR1 activation of RhoA in COS-7 cells is mediated through $G_{12/13}$, and we used the pharmacological inhibitor, C3 toxin, in our studies. As is indicated in Figure 3-2C, PAR1- and mutant-stimulated activation of RhoA is reduced to vector control levels in the presence of C3 toxin. These results indicate that none of the mutant receptors identified in our InsP screen have reduced capacities to activate $G_{12/13}$-linked Rho signaling. As such, we conclude that none of the five amino acid residues that are mutated to alanine in these mutant receptors are important for $G_{12/13}$ coupling to PAR1 in these cells using these methods.

We also tested these mutants for their capacities to couple to $G_{i/o}$. It is well-established that $G_{i/o}$ activation can stimulate MAP kinase (MAPK) signaling. Therefore, to investigate the capacities of the mutant PAR1 receptors to activate $G_{i/o}$-linked pathways, we measured ERK1/2 phosphorylation. Again, wtPAR1 and the PAR1 i2 loop mutant receptors were transiently transfected into COS-7 cells, which were serum starved overnight in the presence or absence of PTX (as indicated) prior to experimentation. The levels of ERK1/2 phosphorylation shown are a result of a 5 min incubation with TFLLR (Figure 3-2D). We found that, similar to our RhoA activation experiments, all of the tested PAR1 i2 loop mutant receptors retain full capacity to stimulate ERK1/2 phosphorylation. Of note, in the presence of PTX, this response was reduced to similar levels in cells transfected with wtPAR1 and the mutant receptors (Figure 3-2D), as we showed before (McCoy et al., 2010). These data indicate that the five PAR1 mutants tested retain full capacities to signal through $G_{i/o}$-linked MAPK pathways. As such, the five discrete amino acids mutated in the PAR1 i2 loop mutant receptors do not dictate $G_{i/o}$ coupling to PAR1 under these experimental conditions.

3.3.3. Some PAR1 mutant receptors have reduced capacities to bind to G_{11}, but not to G_o, or G_{12}

Now that we have shown that the five identified mutant PAR1 receptors have reduced capacities to functionally initiate $G_{q/11}$ but not $G_{i/o}$- or $G_{12/13}$ -mediated signaling, we also sought to define their relative capacities to form complexes with individual G protein Gα subunits. Using co-immunoprecipitation (co-IP) techniques, we previously showed that PAR1 forms stable complexes with members of $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ G protein subfamilies (McCoy et al., 2010). Here, we used the same technique to evaluate the relative capacities of the PAR1 i2 loop mutant receptors to interact with Gα subunits from these subfamilies ($G\alpha_{11}$, $G\alpha_o$ and $G\alpha_{12}$). Empty vector control (pcDNA3.1) or cDNA encoding FLAG-tagged wtPAR1 or FLAG-tagged PAR1 mutant receptors and individual Gα protein subunits were co-transfected into COS-7 cells as receptor/G protein pairs. Anti-FLAG agarose resin was used to recover the PAR/Gα complexes, and western blotting was performed to detect the receptor and G protein in the recovered sample (Figure 3-3 top). The relative protein expression levels of each of the receptors and G proteins are indicated in the input, or lysate immunoblots (Figure 3-3 bottom). No G proteins are recovered when the individual G proteins were co-expressed and co-IP'ed with the control vector (Figure 3-3, lane 1). By contrast, all of the tested G proteins ($G\alpha_{11}$, $G\alpha_o$ and $G\alpha_{12}$) form stable complexes with wtPAR1 (Figure 3-3, lane 2), as we have shown previously (McCoy et al., 2010). However, two of the tested PAR1 i2 loop mutants, R205A and L216A, have reduced capacities to interact with $G\alpha_{11}$ (Figure 3-3,

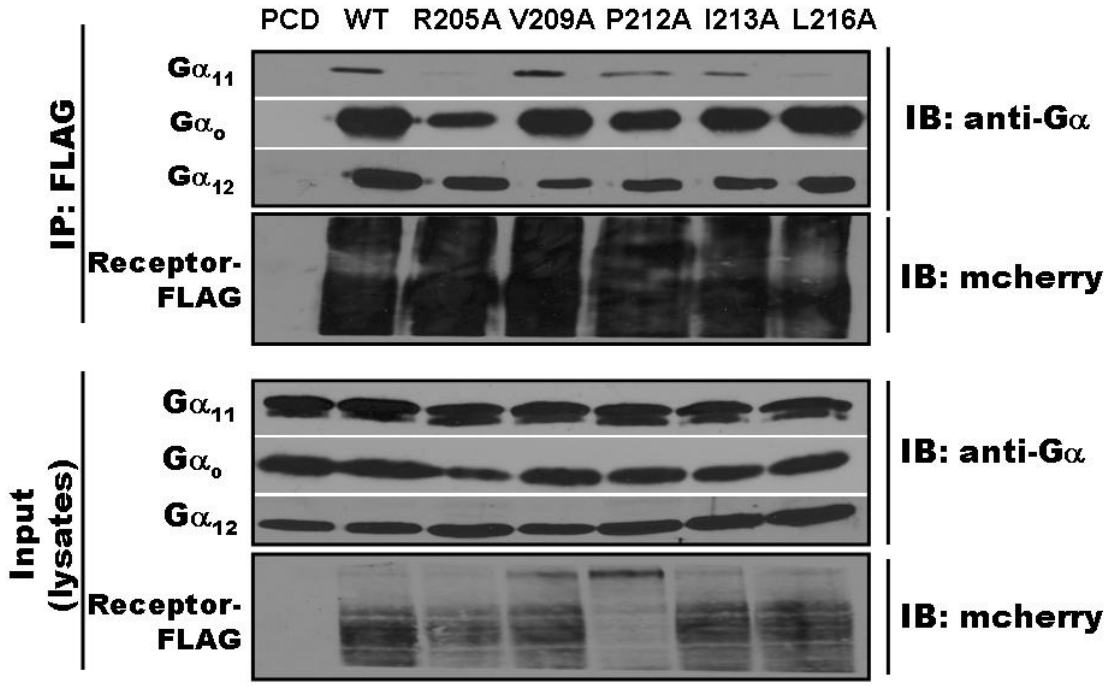

Figure 3-3. PAR1 i2 loop mutants retain full capacities to interact with G_o and G_{12} but not G_{11}. COS-7 cells were co-transfected with separate receptor/G protein pairs and controls (as indicated) for 24h. The following day, cells were lysed, harvested, sonicated in Tris Buffer, and proteins were extracted from membranes with 2% DβM for 3 h at 4°C. Immunoprecipitation took place overnight at 4°C with anti-FLAG resin. Recovered proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Proteins were immunoblotted with the indicated antibodies and visualized with ECL and exposure to film. *Top panel,* Western blot analysis of co-IP'ed receptors and G□ subunits. *Bottom panel,* Western blot analysis of expression levels of receptors and G□ subunits present in cell lysates.

first row, lanes 3 and 7), while all of the other mutant receptors bind to $G\alpha_{11}$ at levels comparable to wtPAR1 (Figure 3, second row, lanes 4-6). Furthermore, all of the mutants tested (including R205A and L216A) form complexes with $G\alpha_o$ and $G\alpha_{12}$, similar to wtPAR1 (Figure 3-3 rows 3 and 4). To our knowledge, these data are the first to show that discrete amino acid mutations in the second intracellular loop of PAR1, R205A and L216A, dictate PAR1 binding to $G\alpha_{11}$ but not to $G\alpha_o$ or $G\alpha_{12}$. Although we have not tested the importance of other cytoplasmic PAR1 residues in $G\alpha_o$ or $G\alpha_{12}$ coupling, our data suggests that the regions responsible for these receptor/ G protein interactions differ from the PAR1 i2 loop amino acids we identified here.

3.3.4. PAR1 i2 loop mutants have differential capacities to stimulate calcium signaling in astrocytes from PAR1-/- mice

For the experiments described up to this point, we have used functional PAR1-null cells (COS-7 cells) to compare the G protein coupling capacities of PAR1 i2 loop mutant receptors with wtPAR1/ G protein coupling. From these studies (Figs. 3-1 through 3-3), we have identified five amino acid residues, R205A, V209A, P212A, I213A, and L216A, that are important for the functional coupling of PAR1 to G_{11} but not to $G\alpha_o$ or $G\alpha_{12}$. Two of these residues, R205A and L216A, also disrupt the formation of a stable PAR1/ $G\alpha_{11}$ complex, as shown in our co-IP data (Figure 3-3). To further explore the physiological relevance of these findings, we shifted our focus from the COS-7 cell line into primary astrocyte cultures from PAR1-/- mice. By reconstituting either wtPAR1 or the PAR1 i2 loop mutants into these more physiologically relevant cells, we are able to study the endogenous intracellular calcium responses elicited by these receptors. We

previously have reported that activated PAR1 stimulates measurable increases in intracellular calcium levels in primary cultures of astrocytes from wt mice through a PLC-mediated (i.e., U73122-sensitive) signaling pathway (Lee et al., 2007). We also have demonstrated that astrocytes from PAR1-/- mice do not mobilize intracellular calcium in response to TFLLR agonism (Lee et al., 2007). Therefore, we used this system as a means to further characterize the G protein coupling capacities of our mutant PAR1 receptors.

We first tested whether astrocytes expressing recombinant wtPAR1 invoke measurable increases in intracellular calcium concentrations relative to cells expressing vector alone. As is shown in the vector only controls, TFLLR has no effect in astrocytes from PAR1−/− mice (n =3; Figure 3-4). ATP was used in these experiments as a positive control to ensure that the cells tested were viable and had the capacity to mobilize calcium. By contrast, in cells expressing wtPAR1, a measurable (i.e., ≥ 1.5-fold over baseline) calcium response is elicited (n=3; Figure 3-4), indicating that in the presence of reconstituted wtPAR1, TFLLR-stimulated increase in intracellular calcium is recovered. These data are consistent with several studies, including our own, that have previously demonstrated the positive effects of thrombin (i.e., mediated by PAR1 and potentially other PARs) on calcium signaling in glial cells (Suo et al., 2002;Wang et al., 2002a;Wang et al., 2002b;Sorensen et al., 2003;Junge et al., 2004;Lee et al., 2007). Interestingly, the five tested PAR1 i2 loop mutant receptors had differential capacities to elicit calcium responses when overexpressed in the PAR1-/- astrocytes (Figure 3-4 and 3-5). Similar to PAR1, the V209A, P212A, I213A, and L216A mutants stimulated increases in calcium levels when activated by TFLLR (n=3; Figure 3-5). However, the R205A mutant receptor completely lost the capacity to initiate calcium mobilization (n=3; Figure 3-4), suggesting

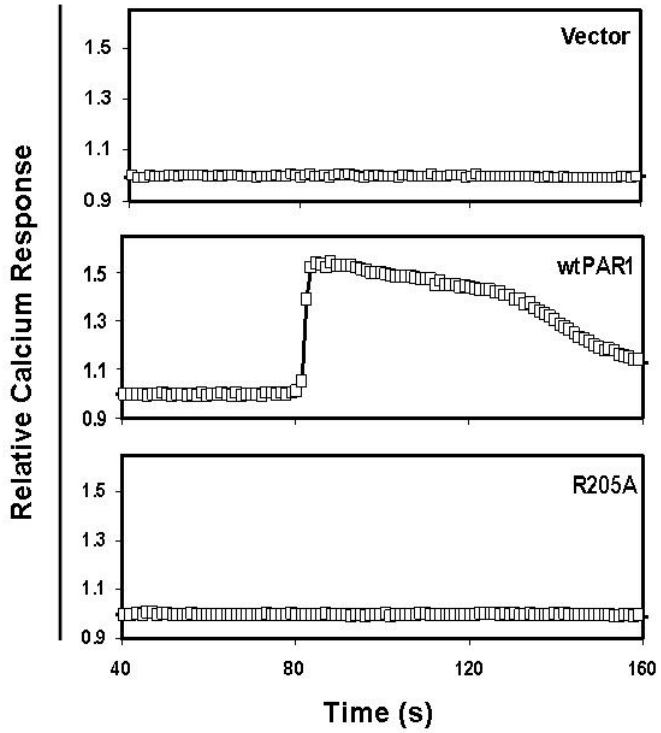

Figure 3-4. Mutant R205A disrupts PAR1-induced calcium mobilization in PAR1-/- astrocytes. Vector only, wtPAR1, or R205A cDNA was electroporated into astrocytes harvested from PAR1-/- mice for 24 h. Fura-2 was added to the cells for 30 min prior to experimentation and coverslips were transferred to a microscope stage for imaging. Imaging was performed with dual excitation at 340 nm and 380 nm wavelengths and the two resulting images were used for ratio calculations.

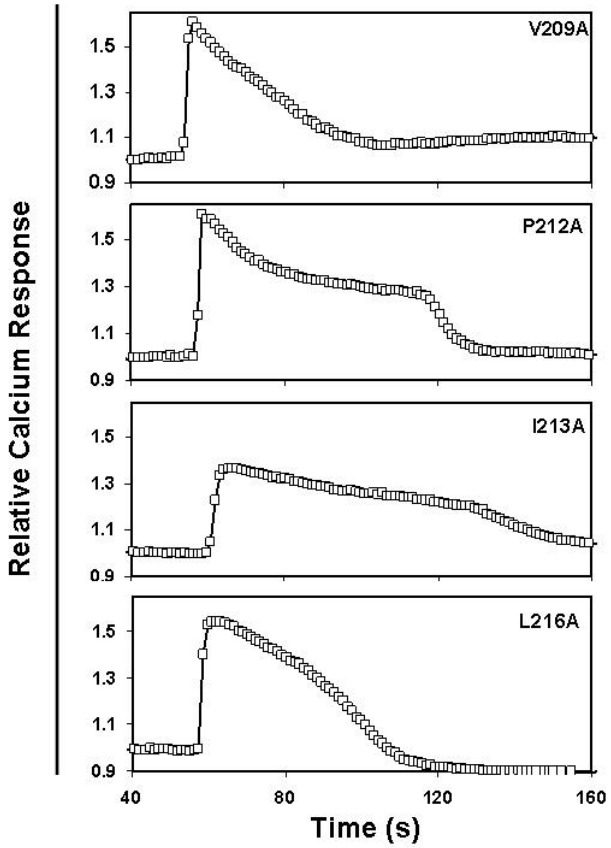

Figure 3-5. Other i2 loop mutants have no effect on PAR1-induced calcium mobilization. PAR1 it loop mutant cDNA was electroporated into astrocytes and calcium mobilization was measured as indicated in Figure 3-4.

that the arginine 205 residue located within the PAR1 i2 loop is necessary for PAR1/ $G_{q/11}$-mediated calcium responses in these cells using these methods. Of note, this same mutant receptor was one of the two of the tested mutants that also inhibit the binding of G_{11} to PAR1 in our co-IP experiments (Figure 3-3). Taken together, these findings indicate that although five point mutations in the PAR1 i2 loop reduce the receptor's capacity to couple to $G_{q/11}$-linked pathways, only one of these mutations, R205A completely abolishes both PAR1/G_{11} binding as well as calcium signaling elicited by PAR1 agonist in primary astrocytes.

3.4. Discussion

Various studies have focused on characterizing PAR1 signaling and regulation in recent years (McCoy et al., 2010). However, the underlying mechanisms dictating the coupling of PAR1 to multiple G protein families remain unresolved. The paradox of these so-called "promiscuous" receptors is that their capacity to invoke multiple G protein-linked signaling pathways could seemingly become chaotic; however they manage to initiate and maintain organized—and probably highly regulated—signal transduction.

Several published reports have described various cellular and biochemical factors that may influence selective G protein coupling to particular GPCRs. For example, G protein proximity to receptors, due to co-localization in lipid rafts and caveolae, may help put receptors and linked G proteins in favorable configurations to promote complex formation and signal activation (Ostrom and Insel, 2004). Like PAR1, S1P receptors couple to multiple G proteins (Sorensen et al., 2003). However, recent studies have

shown that unlike other S1P receptors, the $S1P_1$ receptor couples exclusively to G_i depending on its compartmentalization in caveolae (Means et al., 2008). Perhaps PAR1/G protein coupling also depends on the activating protease or peptide agonist. Ongoing research has supported this idea of ligand-induced functional selectivity, which suggests that the activating agonist plays a large role in dictating receptor/ G protein coupling. In the case of PAR1, McLaughlin and colleagues found that the receptor's affinity for coupling to either $G_{q/11}$ or $G_{12/13}$ shifted depending on which agonist was used to activate the receptor, thrombin or TFLLR (McLaughlin et al., 2005). As such, it is feasible that PAR1 has the capacity to activate multiple G proteins but interacts with and activates individual G proteins depending on its location within the cell membrane and its activating ligand.

Although the positioning and conformation of PAR1 clearly plays a role in its G protein coupling activities, our findings here and those elsewhere show that the receptor's amino acid composition and structure contribute to PAR1/ G protein signaling as well. For many conventional GPCRs that couple to only one subfamily of G proteins, the domains of the receptor responsible for binding and activating G proteins have been reported. Specifically, the i2 and i3 loops of the receptors and/or the C-terminal regions have been implicated. Somewhat surprisingly, despite studies that have identified binding sites for G proteins on individual GPCRs, these sites vary between receptors, with few conserved binding motifs identified (Hermans, 2003).

Given what is already known about conventional GPCR/ G protein coupling, our goal for the studies described here were to further explore mechanisms underlying the promiscuous G protein coupling of PAR1. Our key findings include the following: 1)

Five discrete point mutations within the PAR1 i2 loop reduce the capacity of the receptor to stimulate $G_{q/11}$/PLC-mediated inositol lipid signaling; 2) These five PAR1 i2 loop point mutations that disrupt InsP signaling have no effect on $G_{12/13}$-mediated RhoA activation or $G_{i/o}$-stimulated ERK1/2 phosphorylation; 3) Of these five point mutations that reduce PAR1/$G_{q/11}$ functional coupling, only two (R205A and L216A) prevent PAR1 and G_{11} from forming a complex; 4) Of these two mutants, only R205A disrupts stable PAR/G_{11} complex formation and also disrupts downstream calcium mobilization in astrocytes from PAR1-/- mice. Here we will discuss each of these findings.

3.4.1. Five discrete point mutations within the PAR1 i2 loop reduce the capacity of the receptor to stimulate $G_{q/11}$/PLC-mediated inositol lipid signaling

Our findings indicate that of the 21 amino acids that comprise the PAR1 i2 loop, five of them are important for $G_{q/11}$ coupling to and InsP production stimulated by PAR1. When these recombinant mutant receptors were overexpressed in COS-7 cells, they each had significantly blunted capacities to stimulate InsP production, whereas the other tested mutants retained the capacity to activate inositol lipid signaling (Figure 3-1). Our studies focused specifically on the PAR1 i2 loop since Verrall and colleagues initially reported that this domain was sufficient to invoke G_q-like coupling and inositol lipid signaling when introduced into the G_s-linked β_2-AR and the $G_{i/o}$-linked D_2R (Verrall et al., 1997). By contrast, neither the PAR1 i1 loop or i3 loop conferred this signaling capacity on non-G_q-linked receptors. Of note, the authors also found that both the N- and C-terminal regions of the PAR1 i2 loop were necessary for these chimeric receptors to initiate InsP signaling (Verrall et al., 1997). Consistent with these findings, the five point mutations

that we identified to be important for PAR1/ $G_{q/11}$ coupling in our screen span both regions of the i2 loop. Using wtPAR1 and the five PAR1 i2 loop mutants, we also confirmed that in COS-7 cells, InsP signaling is indeed mediated by $G_{q/11}$ and PLC-□ by using a selective inhibitor of this pathway, U73122 (Figure 3-2B).

3.4.2. The five PAR1 i2 loop point mutations that disrupt InsP signaling have no effect on $G_{12/13}$-mediated RhoA activation or $G_{i/o}$-stimulated ERK1/2 phosphorylation

Other than PAR1, a limited number of other GPCRs that couple to multiple G proteins are known to exist, (Hermans, 2003;Riobo and Manning, 2005) (and references therein), and some investigators have sought to define the molecular determinants of promiscuous receptor/ G protein coupling. Accumulating evidence indicates that there appear to be distinct locations on several of these receptors (e.g., the α_{2A}-adrenergic, calcitonin, cholecystokinin, endothelin, glutamate mGlu1a, and others) that disrupt activation of certain G protein pathways while preserving the signaling integrity of others (reviewed in (Hermans, 2003)). Consistent with these reports, our results indicate that the five point mutations that reduce the coupling efficacy of PAR1 and $G_{q/11}$ have no effect on $G_{12/13}$- or $G_{i/o}$-linked signaling pathways. In our studies, we assessed PAR1 activation of C3-toxin sensitive RhoA-GTP formation and PTX-sensitive ERK1/2 phosphorylation (Figure 3-2C-D). Significantly, all of the PAR1 i2 loop mutants tested retained full capacities to activate both G proteins and their linked signaling pathways, indicating i2 loop/ $G_{q/11}$ specificity. The cytoplasmic portions of PAR1 are relatively small compared to other GPCRs. As such, it might be impossible for a single region, such as the i2 loop, to simultaneously interact with three different types of G proteins due to steric hindrance.

Therefore, we conclude that the PAR1 binding sites for members of the $G_{q/11}$ subfamily likely differ from the binding sites for $G_{12/13}$ and $G_{i/o}$ subfamily members.

3.4.3. Of the five point mutations that reduce PAR1/$G_{q/11}$ functional coupling, only two prevent PAR1 and G_{11} from forming a complex

We recently demonstrated that PAR1 forms stable complexes with G protein heterotrimers (i.e., $G\alpha_{11}$ plus $G\beta\gamma$ as well as $G\alpha_o$ plus $G\beta\gamma$) that are readily recovered using immunoprecipitation techniques (McCoy et al., 2010). Here, we sought to determine whether these interactions would be disrupted by our identified PAR1 i2 loop point mutations. Consistent with our functional data, which shows that all of the PAR1 i2 loop mutants tested activate RhoA and stimulate ERK1/2 phosphorylation (Figure 3-2C-D), these same mutants also retain the capacity to interact with $G\alpha_{12}$ and $G\alpha_o$ in co-IP binding studies (Figure 3-3). Of the five PAR1 i2 loop mutants tested, all but two retained full capacities to interact with G_{11}. These results are intriguing since these receptors each contain point mutations that significantly reduced TFLLR-stimulated InsP production in COS-7 cells. It is possible that although the PAR1/ G_{11} complex remains intact, the receptor is incapable of fully activating the G protein and its linked signaling pathways. To further explore this idea, we next moved into a more physiologically relevant system and looked at signaling events that are further downstream from $G_{q/11}$-activated inositol lipid signaling.

3.4.4. A single point mutation in the PAR1 i2 loop disrupts downstream calcium mobilization in astrocytes from PAR1-/- mice

To test our PAR1 i2 loop mutants in a more physiologically relevant system, we studied their capacities to mobilize intracellular calcium in astrocytes from PAR1-/- mice. All but one of the tested PAR1 mutants stimulated increases in intracellular calcium levels comparable to those elicited by wtPAR1 (Figure 3-4). However, one receptor mutant, R205A, completely lost the capacity to signal in these cells (Figure 3-4C). This same point mutation also prevented PAR1/G_{11} complex formation, as was demonstrated in our co-IP studies shown in Figure 3-3. Somewhat surprisingly, the other receptor that contained a point mutation that prevented PAR1/G_{11} complex formation, L216A, retained a full capacity to stimulate an increase in intracellular calcium levels (Figure 3-4G). As is faintly detectible in our co-IP data (Figure 3-3), this calcium mobilization may occur as a result of a preserved, albeit weak, interaction between the L216A receptor mutant and G_{11}.

Only the most high affinity protein-protein interactions remain in a stable complex after being subjected to co-IP experimentation. Therefore, it is possible that a complex between L216A and G_{11} does form but is unable to withstand the harsh conditions (i.e., detergents and salts) used in our experimental methods. The L216A-triggered G_{11} signaling seemingly surpasses the threshold that is required to transduce InsP production into a calcium response. In doing so, its signal amplifies as it is relayed from $G_{q/11}$ to PLC, InsP, and finally to intracellular calcium stores. In this model, signaling initiated by the R205A/ G_{11} complex may not reach the necessary threshold to transduce InsP signaling into calcium mobilization, suggesting that its interaction with G_{11} and linked signaling pathways is weaker than the interaction between L216A and G_{11}.

In summary, we report here that five discrete point mutations located within the PAR1 i2 loop significantly reduce the receptor's capacity to initiate $G_{q/11}$-mediated InsP signaling. By contrast, none of these identified point mutations reduce the capacity of PAR1 to stimulate $G_{12/13}$–linked RhoA activation or $G_{i/o}$-stimulated ERK1/2 phosphorylation. Significantly, only one of these point mutations also disrupts downstream, and potentially amplified, calcium responses. Of note, this same mutation is also one of only two identified that also inhibits G_{11} binding to PAR1, an interaction that is clearly important for activation of downstream signaling. These studies highlight previously unknown molecular details underlying PAR1/G protein coupling, and begin to partially explain how one receptor can functionally interact with multiple G proteins and linked signaling pathways.

Chapter 4: RGS protein regulation of PAR1 and PAR2 is RGS-, receptor-, and G protein-dependent

4.1 Introduction

With the discovery and characterization of PARs and other unconventional GPCRs, it is now generally accepted that early models of G protein signaling (i.e., stimulated GPCRs preferentially bind and activate one specific G protein), are over-simplified. Not only can PAR1 and PAR2 activate multiple G proteins and linked signaling pathways, but extensive cross-talk among the different signaling pathways that these receptors activate also is well-documented (Dery et al., 1998;Macfarlane et al., 2001;Traynelis and Trejo, 2007). In order to preserve specificity and fidelity, it is likely that these complex receptor-initiated signals are tightly regulated at the level of the GPCR, the G protein, and the effector.

The regulatory mechanisms in place to modify PAR1 and PAR2 signaling are particularly interesting to study since the receptors are often expressed in the same cells, are irreversibly activated, and they couple to multiple G protein subfamilies. However, as described in chapter 1, these receptors tend to cause divergent physiological effects with regard to pain, inflammation, and other pathophysiological processes. The desensitization and trafficking of PAR1 and PAR2 have been well-studied (reviewed in (Traynelis and Trejo, 2007)) but to our knowledge, the involvement of other regulatory proteins, such as RGS proteins, in PAR signaling have not been explored.

RGS proteins regulate GPCR signal transduction at the level of the receptor, the G protein and the effector by selectively forming functional pairs with GPCRs (reviewed in (McCoy and Hepler, 2009)). Initially, RGS inhibition of G protein signaling was anticipated to be selective for individual pairs of RGS and Gα proteins. However, studies

later emerged that demonstrated that many RGS proteins indiscriminately inhibit G proteins from several different G protein families including $G_{i/o}$ and $G_{q/11}$ (reviewed in (Hollinger and Hepler, 2002)). Therefore, a search began for other determinants of specificity of RGS/Gα interactions that prevent chaotic cellular signaling. Studies from our lab and others have shown that GPCR interactions with RGS proteins provide such specificity and signal regulation (reviewed in (McCoy and Hepler, 2009)). Xu and colleagues first showed that RGS1, RGS2, RGS4 and RGS16 inhibit Ca^{2+} responses mediated by different $G_{q/11}$-linked receptors (Xu et al., 1999). More recently, our lab found that RGS2 directly binds to and inhibits the signaling of $G_{q/11}$-linked mAChRs, but not mAChRs that couple to $G_{i/o}$ (Bernstein et al., 2004b), which suggests that RGS proteins act in receptor- and G protein-dependent manners. Furthermore, RGS2 also has been shown to interact with and regulate the $G_{q/11}$-coupled α_{1A}-AR but not the α_{1B}-AR or α_{1D}-AR, which also link to $G_{q/11}$ (Hague et al., 2005). These findings indicate that GPCRs are the true determinants of RGS protein specificity. Taken together, these data indicate that RGS protein regulation is highly organized and involves RGS protein interactions with not only G proteins but also with receptors.

Given that RGS protein interactions with receptors may be receptor-dependent, G protein-independent, or both, the molecular details underlying RGS regulation of GPCRs must be characterized on an individual receptor basis. As such, the RGS regulation of PAR1 and PAR2 is particularly interesting to explore since these two closely related receptors have similar expression and signaling profiles yet have diverse pathophysiological effects. Therefore, they both must be individually regulated by overlapping and distinct mechanisms. In the case of PAR1, very little is known about its

RGS regulation. One study implicated the large, multifunctional RGS protein, LARG, which also is an RGS-RhoGEF, in mediating thrombin-stimulated Rho responses prostate cancer cells (Wang et al., 2004). However, roles for smaller, B/R4 RGS proteins in modulating PAR and linked G protein signaling pathways have not been described. Regarding RGS regulation of PAR2, nothing has ever been published.

In the present study, we sought to determine whether RGS proteins interact with and functionally regulate PAR1 and PAR2. Our main goal was to identify differences in such regulation, thereby providing mechanistic insight into how these two similar receptors have differential effects on cellular processes. Using *X. laevis* oocytes and COS-7 cells expressing recombinant proteins, we have found that PAR1 and PAR2 are differentially regulated by overlapping and distinct RGS proteins. In these studies, we report entirely novel interactions between PARs and RGS proteins as well as differences in the RGS regulation of these two closely related receptors.

4.2. Experimental Procedures

4.2.1. Materials

Materials were obtained from the following sources: Anti-FLAG M2 affinity gel and anti-FLAG M2 monoclonal antibody-peroxidase conjugate, bovine serum albumin (BSA), penicillin, and streptomycin from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum from Atlanta Biologicals (Atlanta, GA); trypsin, Dulbecco's modified Eagle's medium (DMEM) from Cellgro (Herndon, VA); Lipofectamine 2000 transfection

reagent from Invitrogen (Carlsbad, CA); RhoA G-LISA™ Activation Assay colorimetric format kit from Cytoskeleton, Inc. (Denver, CO); anti-HA antibody from Clontech (Palo Alto, CA); conjugated goat anti-mouse monoclonal antibody from Rockland Inc. (Gilbertsville, PA); p44/42 ERK1/2 (extracellular signal-regulated kinase 1/2) antibody, phospho-p44/42 ERK1/2 antibody from Cell Signaling Technology (Beverly, MA) anti-$G\alpha_o$ antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-$G\alpha_{q/11/14}$ antibody Z811 was kindly provided by Dr. Paul Sternweis (U. Texas Southwestern, Dallas, TX); and peroxidase-conjugated goat anti-rabbit was from Bio-Rad (Hercules, CA). The PAR-APs, TFLLR-NH_2 (TFLLR) and 2-furoyl-LIGRLO-NH_2 (LIGRLO), were synthesized by Dr. Jan Pohl at the Emory University Microchemical Facility (Atlanta, GA).

4.2.2. Methods

GST-PAR constructs—GST-PAR1-i2, GST-PAR1-i3, GST-PAR2-i2, and GST-PAR1-i3 were cloned into the pET41b vector to create a fusion protein containing an N-terminal GST epitope tag and C-terminal His epitope tag. Each PAR1 i2 or i3 loop was amplified from corresponding regions of the mouse full-length receptor. Similarly, i2 and i3 loops of PAR2 were used to create the PAR2-containing fusion proteins. PAR1 and PAR2 were amplified as *EcoRI/XhoI* fragments.

In order to use purified RGS proteins with His tags to perform these pulldown assays, fragments encoding the intracellular loops of PAR1 and PAR2 were subcloned further into the pGEX4T vector to eliminate the His tag. PAR1 and PAR2 were amplified as *EcoRI*/*XhoI* fragments and cloned in frame with an N-terminal GST tag.

PAR1 and RGS constructs: Mouse PAR1-FLAG and PAR2 are both in the pcDNA3.1 vector and were created as described above (Section 2.2). RGS1-HA, RGS2-HA, RGS4-HA, and RGS16-HA were created as previously described (Bernstein et al., 2004b).

Induction and Purification of GST fusion proteins—Glutahione-S-transferase (GST)-PAR i2 and i3 loop fusion proteins were transformed into DH5α *E. coli* and grown in cultures containing LB/carbenicillin. Induction with 500 μM isopropyl-β-D-thiogalactopyranoside was conducted for 2 h at 37°C with shaking. Cells were pelleted and then resuspended in harvest buffer (10 mM HEPES pH 8, 50 mM NaCl, 5 mM EDTA, 0.5% Triton-X100) supplemented with protease inhibitors and lysozyme. After freezing the pellets at -80°C, samples were thawed, sonicated, and then centrifuged at 4°C. Streptomycin sulfate was added to the remaining material, and the samples subsequently were centrifuged, thereby producing the bacterial lysate. Lysates were combined with Glutathione-sepharose 4B beads (Amersham-Pharmacia) for 1 h, rotating end-over-end at 4°C to allow binding. Protein-bead complexes were recovered and then washed with harvest buffer. They were stored as slurry solutions in harvest buffer at -80°C until experimentation. The concentration of the slurries was determined by Coomassie staining, and the same amount of total protein was used for in each binding reaction.

RGS pull-down assays—Pulldowns of purified proteins were performed by using using RGS-His proteins as described previously (Hague et al., 2005). Briefly, equal amounts of GST fusion proteins were added to reactions as determined by Coomassie

staining. The total reaction volume was 250 μL, which was achieved using a buffer that contained 30 mM imidazole and 80 mM NaCl to control for volume differences in the buffers among the purified RGS proteins and to prevent non-specific GST/ His tag interactions. Reactions were carried out in microcentrifuge tubes, which were rotated overnight, end-over-end at 4°C. Beads were pelleted by centrifugation and were washed with harvest buffer. Proteins bound to the beads were eluted with 2X sample buffer and were detected by immunoblot.

Cell culture and transfections—As described in section 2.2 and 3.2, COS-7 (ATCC® Number CRL-1651™) cells were propagated in DMEM with additives at 37°C with 5% CO_2. Transfections were performed according to Lipofectamine 2000® transfection reagent protocol.

Co-Immunoprecipitation of PAR/G protein/RGS complexes—Co-IP experiments were carried out in transfected COS-7 cells, as described in sections 2.2 and 3.2. The same protocol was used here except that RGS proteins were also included in these experiments. Cells were transfected with equal amounts of cDNAs in the combinations indicated (i.e., receptor + G protein, receptor + RGS protein, or receptor + G protein + RGS protein); empty vector was used in place of any component as a control) for 18-24 h.

Immunoblot Analysis—After separation by SDS-PAGE and transference to nitrocellulose membranes, immunoblots were carried out as described in sections 2.2 and 3.2. Dilutions of the antibodies used in these particular studies are listed here: anti-p44/42 ERK1/2 1:300 and anti-phospho p44/42 1:1000 in TBST with 5% BSA; anti-$G\alpha_q$ family Z811 1:2000, anti-$G\alpha_o$ 1:200, anti-HA 1:5,000 in blocking buffer. Goat anti-rabbit IgG

1:25,000 in TBST was used as a secondary antibody for all experiments. The protein bands were detected with ECL upon being exposed to film.

Two-electrode voltage clamp recordings from Xenopus laevis oocytes—Oocytes were harvested from *X. laevis* and injected as described in section 2.2. The only difference is that RGS proteins also were used in these studies, in which case 5ng of the tested RGS protein were added to either the 5ng PAR1 or PAR2 cRNA prior to injection. Recordings were performed 4-5 days after injections as detailed previously (section 2.2).

Measurement of ERK1/2 phosphorylation— ERK1/2 phosphorylation experiments were carried out in COS-7 cells as described in sections 2.2 and 3.2. The only additional measure taken was that equal amounts of different PAR/RGS cDNA pairs were separately transfected into cells prior to experimentation. Detection of the HA-tagged RGS proteins was performed by immunoblotting the same samples used in the ERK1/2 phosphorylation experiments.

Measurement of RhoA activation—RhoA-GTP levels were measured as described in sections 2.2 and 3.2, with the exception that RGS protein cDNA was also transfected into the COS-7 cells prior to experimentation. Different pairs of PAR/RGS proteins were separately transfected to use for each sample that was measured. As we noted previously, a RhoA Activation G-LISA™ kit (Cytoskeleton, Inc., Denver, CO) was used to measure activated RhoA levels.

4.3 Results

4.3.1. RGS2 and RGS4 differentially interact with PAR1 and PAR2

By employing co-immunoprecipitation techniques, we previously have shown that PAR1 and PAR2 both interact with $G\alpha_{11}$ subunits (section 2.3), which confirms that both receptors are $G_{q/11}$-linked. Our earlier findings also indicate that relative to PAR2, PAR1 couples much more strongly to $G_{i/o}$, with a strikingly robust coupling to G_o (Figure 2-3). Similar to the methods we used to study these PAR/G protein interactions, we performed a number of co-IP experiments to determine whether RGS proteins also interact with PAR1 and/or PAR2. In doing so, we first investigated whether PAR1 and/or PAR2 form a complex with RGS2 in the presence and absence of G protein α subunits (G_{11} and G_o) (Figure 4-1). We chose to first assess the role of RGS2 in PAR regulation since it is a known modulator of $G_{q/11}$ signaling and $G_{q/11}$-linked receptors. However, it is also known that RGS2 selectively regulates only "preferred" $G_{q/11}$-linked receptors and forms functional pairs with these preferred receptors to fine-tune their G protein signaling. We therefore sought to determine whether RGS2 forms preferred functional pairs with PAR1, PAR2, or both receptors.

Carboxy-terminally FLAG-tagged PAR1 or PAR2, HA-tagged RGS2, and either $G\alpha_{11}$, $G\alpha_o$, or no G protein subunits were each independently co-expressed as PAR/RGS2 ± G protein pairs in COS-7 cells. Anti-FLAG resin was used to recover the receptor/RGS2/G protein complexes, and the presence of RGS2 and/or the $G\alpha$ subunit in the recovered material was detected by immunoblotting (IP, Figure 4-1, top panels). The total protein expressed in the lysates material also is shown (input, Figure 4-1, bottom panels). We found that PAR1 and PAR2 both interact with RGS2 in similar manners. Both receptors co-IP with RGS2 in the absence of any overexpressed G protein.

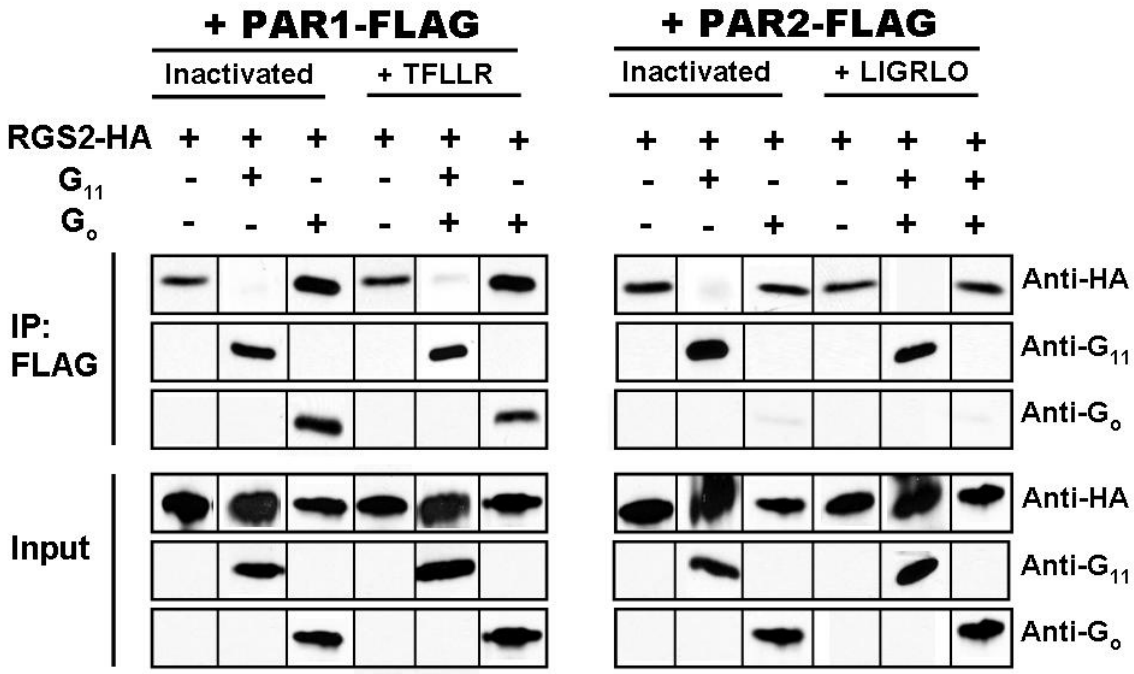

Figure 4-1. PAR1 and PAR2 form G protein-dependent complexes with RGS2. Co-IP studies were performed as described in the Materials and Methods section. For these experiments, RGS2, $G\alpha_{11}$, or both were co-transfected with PAR1 or PAR2 and pulled down in the presence and absence of agonist. *Top panel,* Immunoblot of IP'ed $G\alpha_{11}$ or $G\alpha_o$ with a corresponding G protein-specific antibodies and RGS2-HA with an anti-HA antibody. *Bottom panel,* Immunoblots of cell lysates from co-IP experiments with corresponding G protein-specific antibodies or the anti-HA antibody to detect RGS2-HA. The same goat anti-rabbit secondary antibody was used for all western blots, and proteins were visualized using ECL and exposure to film.

Surprisingly, neither PAR1 nor PAR2 co-IP with RGS2 when $G\alpha_{11}$ also is present in the reactions (Figure 4-1A). These findings suggest that RGS2 and $G\alpha_{11}$ interact with the same domains of PAR1 and PAR2 and that $G\alpha_{11}$ competitively inhibits RGS2 binding to both GPCRs. By contrast, RGS2 retains the capacity to interact with PAR1 even when $G\alpha_o$ is bound (Figure 4-1), indicating that the binding regions for $G\alpha_o$ and RGS2 differ. These findings support our conclusions from the PAR1 mutant data, which suggested that $G\alpha_{11}$ (and perhaps also RGS2) and $G\alpha_o$ interact with different cytoplasmic regions of PAR1 (sections 3.3 and 3.4). In the case of PAR2, the presence of $G\alpha_o$ did not impact RGS2 binding. These data are not surprising since our previous findings also showed that PAR2 and $G\alpha_o$ do not interact (Figure 2-3A). As such, there would be no reason for $G\alpha_o$ to preclude an interaction between PAR2 and RGS2. Of note, our data also indicate that the addition of receptor agonists to the co-IP reactions have no impact on the formation of PAR/G protein/ RGS complexes. These data also support our earlier conclusions from chapter 2, which suggest that PARs and G proteins may form inactive complexes prior to stimulation with an agonist and simply rearrange upon activation to initiate signal transduction.

To further explore PAR/RGS interactions, we also tested the capacity of RGS4 to bind to PAR1 and PAR2 in the presence and absence of $G\alpha_o$ (Figure 4-2). We found that RGS4 only binds to inactive PAR1 and PAR2 in the presence but not absence of $G\alpha_o$ overexpression. However, when stimulated with TFLLR, PAR1 gains the capacity to interact with RGS4 absence of $G\alpha_o$ overexpression, while LIGRLO-activated PAR2 does not (Figure 4-2). These data differ from our findings with RGS2, which bound to both receptors in the absence of G protein overexpression regardless of whether the receptors

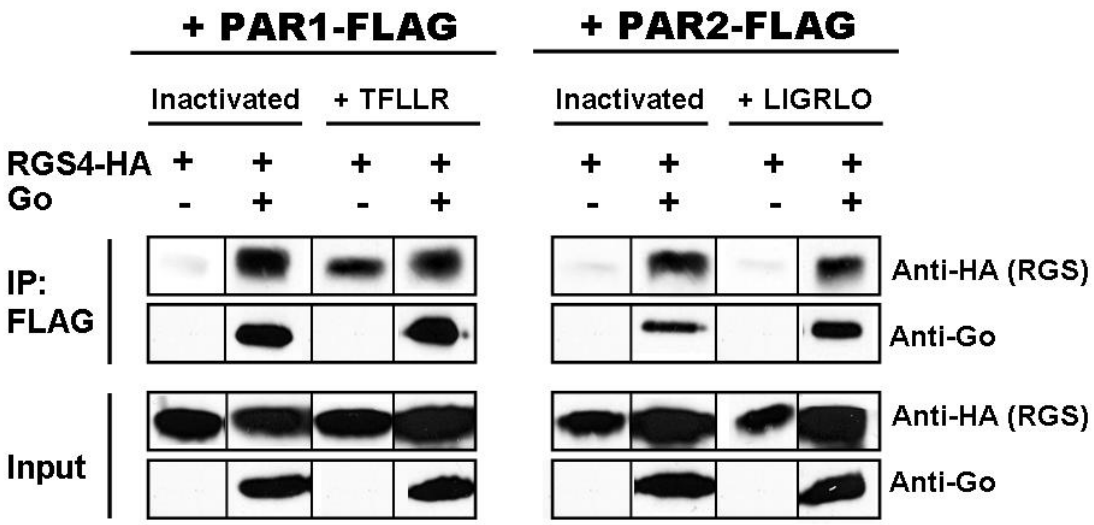

Figure 4-2. PAR1 and PAR2 interact with RGS4 in activation- and G protein-dependent manners. Similar to Figure 4-1, COS-7 cells were co-transfected with PAR/RGS4 pairs ± $G\alpha_o$, and co-IPs were performed in the presence and absence of agonist. *Top panel,* Western blot of IP'ed $G\alpha_o$ with a corresponding anti-$G\alpha_o$ antibody and of RGS2-HA with an anti-HA antibody. *Bottom panel,* Western blots of cell lysates from co-IPs with corresponding anti-$G\alpha_o$ antibody or the anti-HA antibody to detect RGS2-HA. The same goat anti-rabbit secondary antibody was used for all immunoblotting, and proteins were detected with ECL and subsequent exposure to film.

were activated or not. These findings may suggest that when inactive, PAR1 exists in a conformation that does not favor RGS4 binding. Upon TFLLR stimulation however, the receptor may change shape, thereby exposing a previously hidden binding site. Perhaps PAR1 also sits in such a favorable conformation for this RGS4 interaction when it is bound to $G\alpha_o$. However, we did not test any of these hypotheses.

In striking contrast to many of our other data (Figures 2-3, 2-5, 4-2), we also found that PAR2 gains the capacity to interact with $G\alpha_o$ in the presence of RGS4 (Figure 4-2). None of our other attempts at co-IP'ing $G\alpha_o$ with PAR2 have been successful (Figures 2-3, 2-5, 4-2). Although we did not examine these findings in any more detail, they may indicate that RGS4 serves as a scaffolding protein that is required for PAR2 to indirectly interact with and perhaps activate signaling through G_o.

4.3.2. Cytoplasmic i2 and i3 loops of PAR1 but not PAR2 are involved in interactions with RGS proteins

In addition to using co-IP techniques to study PAR/RGS interactions, we also employed a GST-pull down strategy to more precisely define the regions of the receptors that dictate PAR/RGS interactions (Figure 4-3). Using this method, our lab has previously shown that RGS2 binds to the i3 loops of the $G_{q/11}$-coupled mAChR subtypes (M1, M3 and M5) and to the $G_{q/11}$-linked α_{1A}-AR (Bernstein et al., 2004b;Hague et al., 2005). Similar reports have been published about the i3 loop and C-termini of GPCRs interacting with other members of the B/R4 family of RGS proteins, including RGS4 (reviewed in (McCoy and Hepler, 2009)).

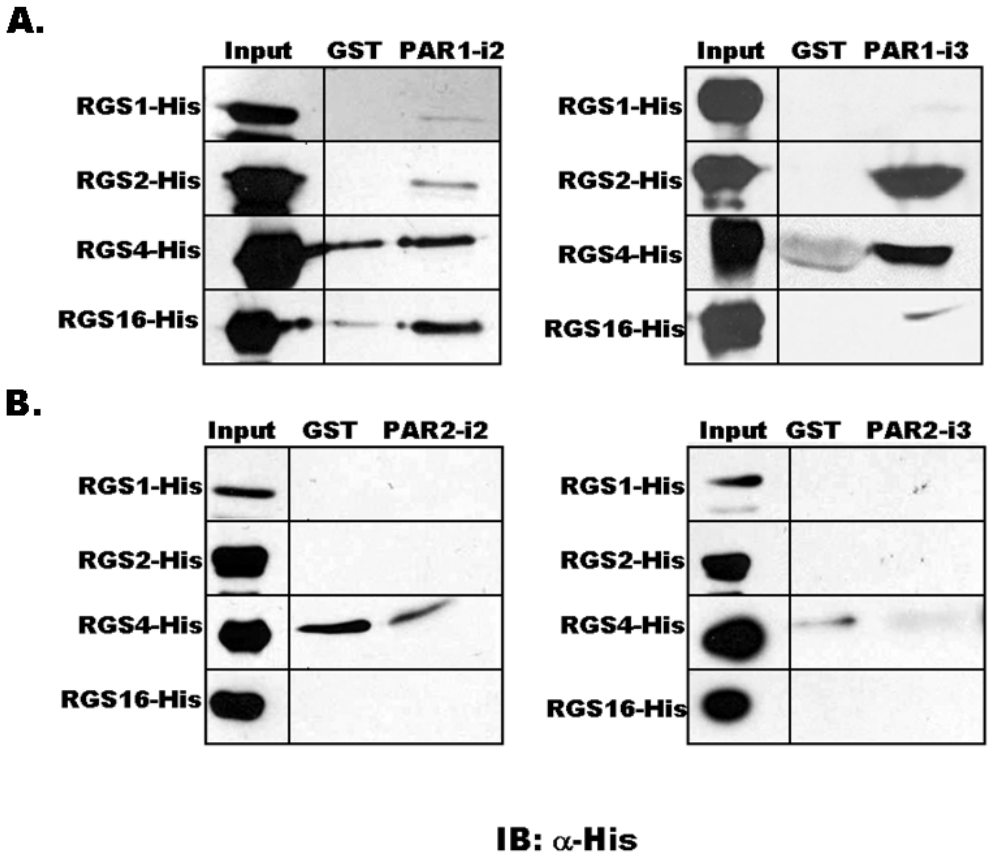

Figure 4-3. RGS proteins bind to the i2 and i3 loops of PAR1. Purified RGS1-His, RGS2-His, RGS4-His or RGS16-His were incubated with equal amounts of GST alone, GST-PAR1-i2, or GST-PAR1-i3 (A) or with GST alone, GST-PAR2-i2, or GST-PAR2-i3 (B) with the GST bound to glutathione-Sepharose beads. After centrifugation, bound RGS proteins were eluted in 2X sample buffer and subjected to SDS-PAGE. Immunoblots were performed using an anti-His antibody.

Here, we examined the capacity of several His-tagged B/R4 RGS proteins, RGS1, RGS2, RGS4, and RGS16 to associate with GST-tagged truncated PAR receptors, which contain only the isolated PAR1 or PAR2 i2 or i3 loops. As shown in Figure 4-3A, RGS4-His and RGS16-His bind to the PAR1 i2 loop, but in the case of RGS4, there is a considerable amount of background binding to GST beads as well. Whether this interaction is real or is simply the result of non-specific binding is unknown. RGS2 also may minimally bind to the PAR1 i2 loop but the association is not as strong as for the other RGS proteins (Figure 4-3A). By contrast, RGS2 does bind strongly to the PAR1 i3 loop, indicating that perhaps RGS2 contacts mainly the i3 loop but also interacts with the i2 loop of PAR1. Similarly, RGS4 also strongly binds to the PAR1 i3 loop, at levels stronger than the background binding to the GST beads. We believe this interaction to be real but remain unsure of the interaction between the PAR1 i2 loop and RGS4. By contrast, there is only very minimal RGS16 that is pulled down with the PAR1 i3 loop (Figure 4-3A), indicating that its association with the PAR1 i2 loop must be stronger than its association with the i3 loop. Of the RGS proteins tested, RGS1 does not appear to bind to either loop (Figure 4-3A), suggesting that it may not interact with PAR1 at all or it may interact with the receptor's C-terminus.

In contrast to PAR1, none of the RGS proteins tested appear to interact with the i2 or i3 loops of PAR2 (Figure 4-3B). The only material pulled down in these experiments was the background binding of RGS4-His to the GST beads, similar to Figure 4-3A. As such, it does not appear that purified RGS proteins interact with the i2 or i3 loops of PAR2. These data may indicate that RGS proteins interact either with the C-terminus of the receptor or that they require other scaffolding proteins to assist with their

interactions with PAR2. Overall, these findings suggest that RGS proteins differentially interact with PAR1 and PAR2.

4.3.3. RGS2 and RGS4 but not RGS1 block PAR-activated calcium-activated chloride currents in oocytes

To further characterize the interactions of RGS proteins with PAR1 and PAR2, we explored the functional impact of RGS proteins on PAR signaling. To do so, we again used cells that do not express PARs, including *X. laevis* oocytes. As described in section 2.3.1, *X. laevis* oocytes express calcium-activated chloride channels that can be activated by overexpressed GPCRs, including PAR1 and PAR2 (Figure 2-2B), to provide a readout of $G_{q/11}$-activated intracellular calcium mobilization (Oron et al., 1985;Dascal and Cohen, 1987;Nystedt et al., 1994;Mannaioni et al., 2008). Here we show that oocytes injected only with PAR1 or PAR2 cRNA and stimulated with either TFLLR or LIGRLO, activate calcium-activated chloride channels, which produce measurable inward currents. With a holding potential of -40 mV, separate activation of PAR1 and PAR2 evokes an inward current characteristic of the calcium-activated chloride channel, indicating that both PAR1 and PAR2 mobilize intracellular calcium in response to InsP production. When co-injected with RGS1, PAR1 and PAR2 both maintain the capacity to stimulate calcium mobilization (Figure 4-4), suggesting that this RGS protein has no effect on $G_{q/11}$-mediated PAR signaling. By contrast, the currents evoked by PAR1 and PAR2 activation are significantly reduced to almost no current when either RGS2 or RGS4 is co-injected with either receptor (Figure 4-4). These data suggest that RGS2 and RGS4 similarly regulate $G_{q/11}$-mediated PAR signaling in these cells using these methods.

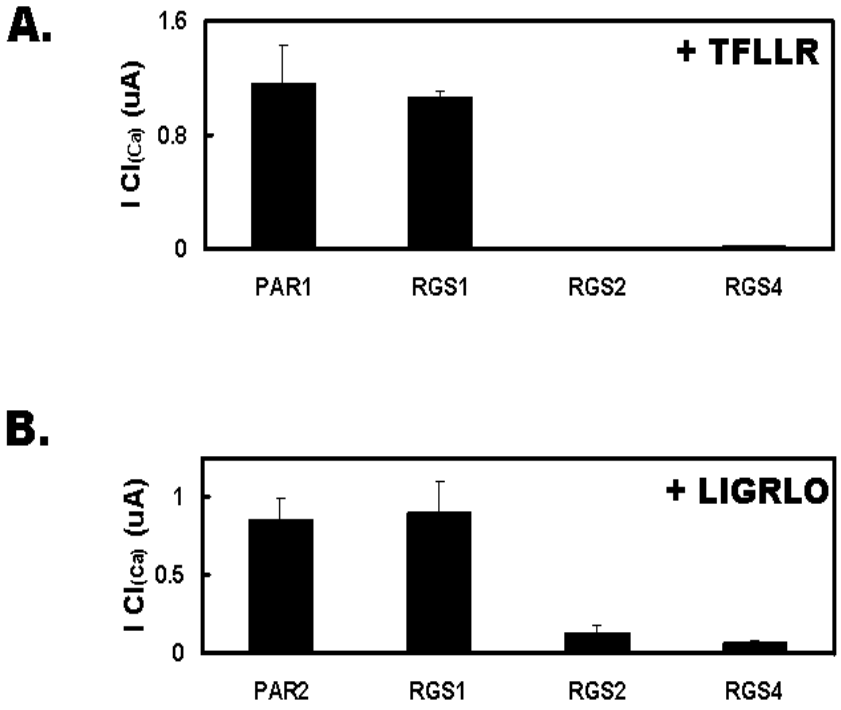

Figure 4-4. RGS2 and RGS4, but not RGS1, reduce PAR1- and PAR2-evoked calcium-activated chloride currents in oocytes. PAR1 or PAR2 cRNA alone or mixed with individual RGS protein cRNA was injected into *X. laevis* oocytes, which were sustained in 1x Barth's solution. 4-5 days after injection, $I_{Ca(Cl)}$ measurements were obtained from the oocytes in response to activation with either 30μM TFLLR or 10μM LIGRLO. A two-electrode voltage clamp was used to obtain the current changes, as described in Materials and Methods. Data are expressed as the mean change in $I_{Ca(Cl)}$ + S.E.M. (n>11 oocytes).

4.3.4. RGS2 and RGS4 differentially regulate PAR1- and PAR2-stimulated ERK1/2 phosphorylation

In addition to oocytes, we also used COS-7 cells to gain a better understanding of RGS regulation of PAR signaling. Similar to our experiments described in Chapters 2 and 3, these studies were performed by overexpressing PAR1 or PAR2 in COS-7 cells. However, here we also co-transfected HA-tagged RGS proteins into the cells to investigate their impact on PAR1- and PAR2-stimulated ERK1/2 phosphorylation (Figure 4-5). Interestingly, we found that RGS2 reduces PAR1- but not PAR2-mediated ERK1/2 phosphorylation. By contrast, we also observed that RGS4 inhibits PAR2-stimulated ERK1/2 phosphorylation but only slightly decreases PAR1-stimulated ERK1/2 phosphorylation (Figure 4-5A-B). RGS16 appears to have no influence on ERK1/2 phosphorylation simulated by either receptor.

These findings are consistent with the data presented in Chapter 2, which suggested that PAR1- and PAR2-stimulated ERK1/2 phosphorylation is mediated by $G_{q/11}$ but only PAR1-induced ERK1/2 phosphorylation also is partially mediated through $G_{i/o}$ signaling pathways. RGS2, which blocks PAR1-mediated MAPK signaling is a known regulator of $G_{q/11}$ signaling, and likely blocks the $G_{q/11}$ component of PAR1-stimulated ERK1/2 phosphorylation. Similarly RGS4, which reduces PAR1- and PAR2-mediated ERK1/2 phosphorylation, also is known to modulate signaling through $G_{q/11}$ but also has the capacity to block $G_{i/o}$–linked pathways. Consistent with our previous data, we believe that RGS4 blocks the $G_{q/11}$-mediated PAR2-stimulated ERK1/2 phosphorylation and also has the capacity to block $G_{i/o}$-mediated PAR1-stimulated ERK1/2 phosphorylation.

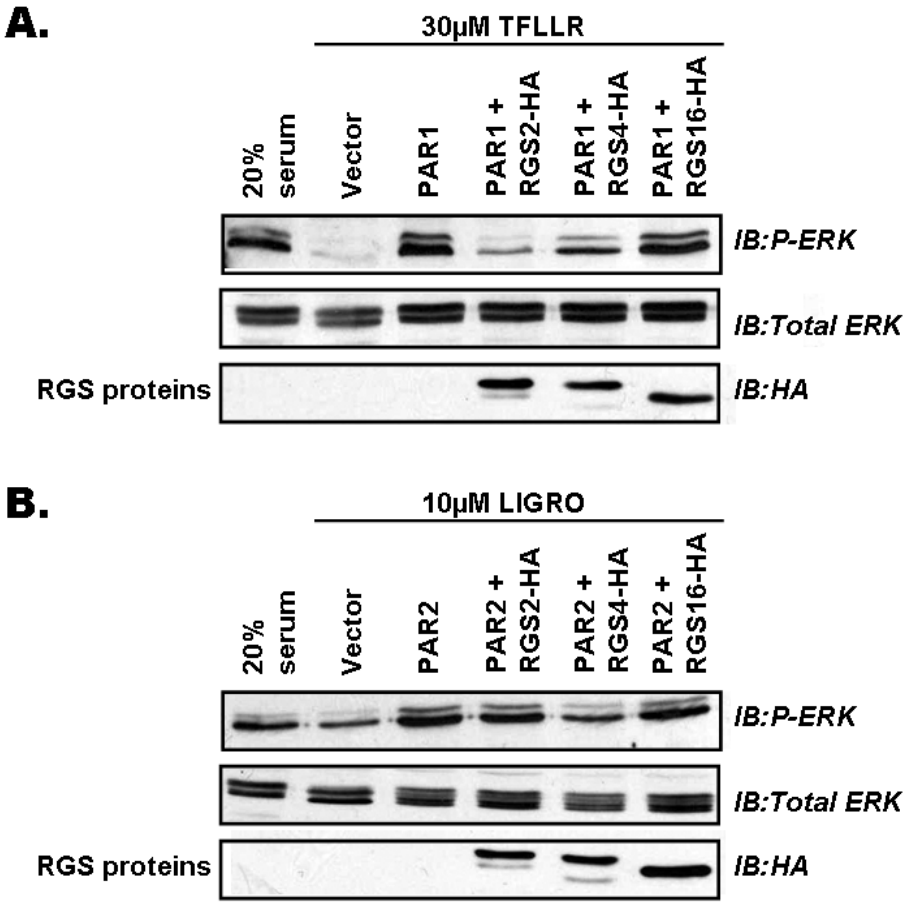

Figure 4-5. RGS 2 and RGS4 differentially block PAR1-and PAR2- stimulated ERK1/2 phosphorylation. Vector alone, PAR1 alone, PAR2 alone or pairs of PAR1 or PAR2 and the indicated RGS proteins were separately transfected into COS-7 cells. Cells were either stimulated with 20% serum, 30μM TFLLR or 10μM LIGRLO, as indicated, for 5 min. Immunoblots were performed with either phospho-ERK1/2, total ERK1/2, or an anti-HA antibody, followed by a goat-anti rabbit secondary antibody and detected by ECL.

4.3.5. RGS4 and RGS16 regulate PAR2- but not PAR1-stimulated RhoA activation

COS-7 cells were also used to explore the RGS regulation of a third G protein-linked pathway activated by PARs, RhoA signaling. Previously, we have shown that PAR-stimulated RhoA activation is mediated by $G_{12/13}$ in COS-7 cells (McCoy et al., 2010), and here we sought to determine whether any of the B/R4 RGS proteins tested in our studies had the capacity to modulate this signaling pathway (Figure 4-6). Of note, $G_{12/13}$ interactions with small RGS proteins have not been studied, and to our knowledge B/R4 family members have not been shown to regulate $G_{12/13}$-linked pathways. By contrast, larger RGS-RhoGEF proteins have been reported to themselves be regulated by $G_{12/13}$ (Tanabe et al., 2004). Despite this lack of insight into RGS regulation of $G_{12/13}$, we attempted to determine whether RGS proteins regulate $G_{12/13}$-mediated signaling by PAR1 and/or PAR2.

To perform these studies, we again used the chemiluminescence-based G-LISA™ Rho assay system that has been described in Chapters 2 and 3. We found that the levels of activated RhoA-GTP evoked by PAR1 activation are not decreased in the presence of any of the tested RGS proteins (Figure 4-6A). However, PAR2-activated increases in Rho-GTP formation are markedly decreased to control levels in the presence of RGS4 and RGS16. Taken together, these findings indicate that PAR2- but not PAR1-stimulated RhoA activation is regulated by RGS proteins. To our knowledge, this is the first evidence of RGS proteins inhibiting $G_{12/13}$-activating signaling pathways.

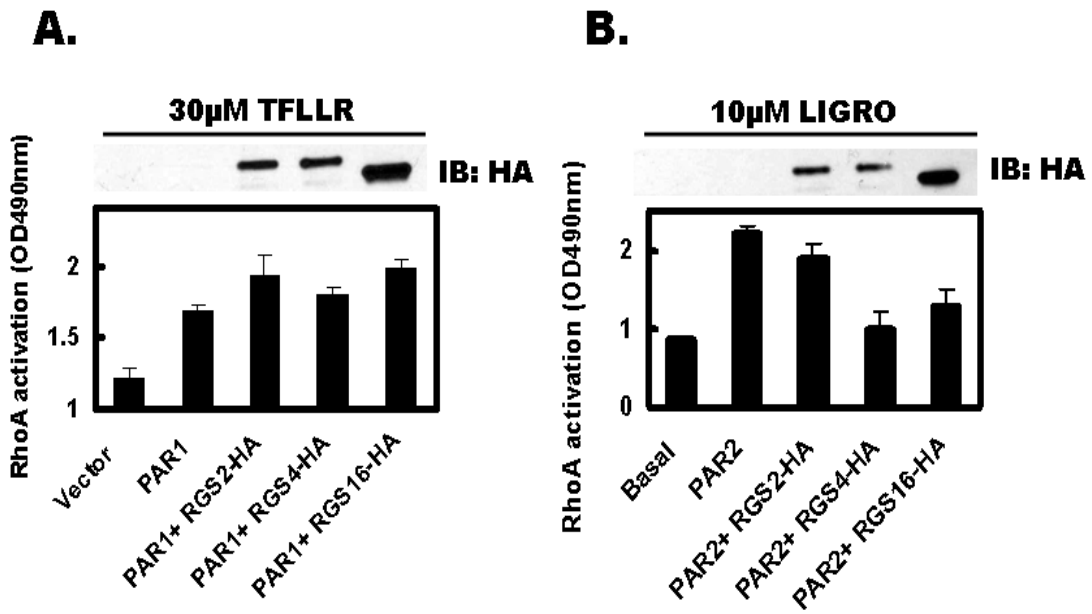

Figure 4-6. PAR1 and PAR2-mediated RhoA activation is differentially regulated by RGS proteins. As described in the materials and methods section, RhoA activation was measured using a RhoA G-LISA™ Assay kit. PAR1, PAR2, or PAR/RGS pairs were separately transfected into COS-7 cells for 5 h before an overnight period of serum-starvation. The next day, cells were stimulated with 30μM TFLLR or 10μM LIGRLO, as indicated, for 2 min prior to cell lysis. The manufacturer's protocol was followed throughout the experiment, and the absorbance of each well was read with a spectrophotometer wavelength of 490nm.

4.4 Discussion

In recent years, RGS proteins have become accepted core components of GPCR signaling complexes. The full range of roles of RGS proteins in these GPCR signaling complexes are not yet fully elucidated, but remain an important topic of investigation. Here we compared the mechanisms of RGS regulation of PAR1 and PAR2 signaling using purified proteins, *X. laevis* oocytes, and COS-7 cells. Our key findings indicate the following: 1) PAR1 and PAR2 interact with overlapping and distinct sets of RGS proteins in receptor- and G protein-dependent manners; 2) RGS proteins regulate PAR1 but not PAR2 by interacting with its i2 and i3 loops; 3) PAR1 and PAR2 signaling is differentially regulated by RGS proteins at the level of the receptor and the level of the G protein. In this section, each of these findings is discussed.

4.4.1. PAR1 and PAR2 interact with overlapping and distinct sets of RGS proteins in receptor- and G protein-dependent manners

We found that PAR1 and PAR2 both form complexes with RGS2 (in the absence of $G\alpha_{11}$) and that PAR1 also forms a multi-protein complex with $G\alpha_o$ and RGS2. In addition, we also have shown that RGS4 only interacts with inactive PAR1 and PAR2 in the presence of $G\alpha_o$. However, upon activation, PAR1 but not PAR2, gains the capacity to bind to RGS4, regardless of whether $G\alpha_o$ is overexpressed in the cells. Overall, our most important finding is that these observations support our overarching hypothesis that RGS proteins differentially regulate PAR1 and PAR2. These findings are entirely novel since B/R4 RGS proteins have never been reported to interact with either PAR1 or PAR2.

However, these observations are not altogether surprising, given what is known about PAR signaling and RGS regulation of GPCRs.

Emerging models of GPCR signaling indicate that receptors serve as signaling platforms that assemble multiple signaling components, including G proteins and RGS proteins to maintain organized signal transduction within cells. We first chose to investigate the role of RGS2 in PAR signing since it exhibits a strong specificity for $G\alpha_{q/11}$ (Heximer et al., 1997), but has the capacity to discriminate which receptors it regulates, thereby only modulating signaling of "preferred" receptors (reviewed in (Neitzel and Hepler, 2006;McCoy and Hepler, 2009)). As such, we thought it might be interesting to determine whether PAR1 and/or PAR2 were preferred receptors. As indicated in Figure 4.3.1I, RGS2 interacts with both receptors. Although this interaction is not receptor-dependent (RGS2 interacts with *both* PAR1 and PAR2), it is G protein-dependent (the presence of $G\alpha_{11}$ but not $G\alpha_o$ inhibits RGS2 binding to PAR1 and PAR2). Significantly, these data are the first to show that RGS regulation of PAR1 and PAR2 is G protein-dependent.

We have not fully studied the mechanism of this competitive inhibition of RGS2 binding to PAR1 or PAR2, but we believe that $G\alpha_{11}$ and RGS2 compete for binding sites within the PAR1 i2 loop (most likely at Arg205, as demonstrated in chapter 3), and that relative to RGS2, $G\alpha_{11}$ must have a higher affinity for this binding site since it maintains the capacity to bind to PAR1, while RGS2 does not. By contrast, the presence of $G\alpha_o$ in these reactions does not influence the formation of PAR/RGS2 complexes. Given our conclusions about RGS2 and $G\alpha_{11}$ sharing a binding site on PAR1, we also conclude that

RGS2 does not share a binding site with $G\alpha_o$, since both proteins have the capacity to simultaneously interact with PAR1 (Figure 4-1).

Our co-IP data with PARs, RGS4, and $G\alpha_o$ is intriguing. We found that when transfected only with PAR1 or PAR2 and left unstimulated, RGS4 does not interact with either receptor (Figure 4-2). However, upon activation, PAR1, but not PAR2, gains the capacity to co-IP with RGS4, suggesting that the receptor undergoes a conformational change when simulated with an agonist, thereby potentially unmasking a previously hidden binding site for RGS4. Because such an interaction between RGS4 and stimulated PAR2 does not occur, it is likely that PAR2 either does not contain a binding site for RGS4 or it does not change conformation in a manner that favors RGS4 binding to it. Interestingly, in the presence of $G\alpha_o$ both receptors gain the capacity to form a complex with RGS4. These findings may suggest that RGS4 serves as a scaffolding protein that is required for PAR2 to indirectly interact with and perhaps activate signaling through G_o. In recent years, a growing appreciation for the role scaffolding proteins play in GPCR signaling has emerged. In the RGS field, it has been reported that several GPCR/RGS interactions (i.e., α_{1B}-AR with RGS2 (Liu et al., 2006) and RGS4 (Wang et al., 2005); μ-opioid with RGS9-2 (Charlton et al., 2008), D_2R with RGS19 (Jeanneteau et al., 2004), and others) are indirect and require the presence of intermediate proteins (i.e., spinophilin (Liu et al., 2006) (Wang et al., 2005), β-arrestin2 (Charlton et al., 2008), GIPC (Jeanneteau et al., 2004), respectively) in order to form multi-protein signaling complexes. Of note, this is also the first instance of $G\alpha_o$ interacting with PAR2 in our studies. As mentioned above, these findings contradict some of our other data, which indicate that PAR2 and $G\alpha_o$ do not interact in COS-7 cells using these methods. As such,

it is possible that the $G\alpha_o$ interaction with PAR2 requires such an intermediate protein, like RGS4, in order to bind. Because our co-IP data with PARs and RGS4 identified differences in RGS4 binding to PAR1 and PAR2, we now know that RGS regulation of PAR1 and PAR2 is not only G protein-dependent but it is receptor-dependent as well.

In conclusion, our findings from co-IP experiments support a mechanism whereby PAR1 and PAR2 interact with distinct and overlapping sets of RGS proteins. Both receptors have the capacity to interact with RGS2 and RGS4 but these interactions change depending on the presence or absence of specific G proteins (G_{11} or G_o) and on whether or not the receptor is activated (i.e., in the case of PAR1 binding to RGS4 only when stimulated with TFLLR). Other than our data that shows PAR1 activation enhancing its capacity to interact with RGS4, our observations indicate that the majority of PAR/ RGS interactions are neither promoted nor disrupted upon receptor activation. These findings are somewhat surprising since it would be reasonable to hypothesize that RGS proteins, which serve as GAPs to limit the duration of Gα signaling events, may not be recruited to a receptor/G protein complex until it is activated. However, our observations support a model whereby certain PAR/G protein/RGS protein complexes are pre-bound prior to receptor activation and signal transduction. Being situated in such proximity to the receptor and G protein, the RGS protein is therefore in a position where it has the capacity to modulate linked signaling events that occur upon PAR activation.

4.4.2. RGS proteins regulate PAR1 but not PAR2 by interacting with its i2 and i3 loops

In our attempts to define the cytoplasmic regions of PAR1 and PAR2 that dictate their interactions with RGS proteins, we found that RGS2 and RGS4 mainly bind to the

i3 loop of PAR1 and minimally binding to its i2 loop. RGS16, on the other hand, binds mainly to the PAR1 i2 loop, with minimal binding to its i3 loop (Figure 4-3A). The implications of these findings raise some interesting questions as they seemingly contradict some of our earlier conclusions about PAR/ G protein interactions. First, we hypothesized that RGS2 and G_{11} bind to the same regions of PAR1 (i.e., to its i2 loop, as shown for G_{11} in Chapter 3) since these two PAR1 binding proteins compete with each other to co-IP with the receptor (Figure 4-1). However, our results here implicate the PAR1 i3 loop in RGS2 binding (Figure 4-3B). There are two potential explanations for this discrepancy. One explanation is that RGS2 binding to the i3 loop is prevented when G_{11} is bound to the i2 loop due to steric hinderance. As discussed previously (Chapter 2), the intracellular loops of PAR1 are small, and it is feasible to believe that only one or few binding partners can interact with the cytoplasmic portions of the loops at any one time. An alternative explanation is that RGS2 preferentially binds the PAR1 i3 loop when both binding partners are purified proteins. In intact cells however, RGS2 may shift its affinity and more strongly couple to the PAR1 i2 loop. This explanation may be feasible given that minimal amounts of RGS2 are pulled down with the PAR1 i2 loop, as shown in Figure 4-3A.

A second contradiction is that our co-IP data suggests that RGS4 does not bind to PAR1 except in the presence of agonist or $G\alpha_o$ (Figure 4-2). However, our pure protein pull-down data indicates that RGS4 does in fact interact with the PAR1 i3 loop (Figure 4-3). Our potential explanation for these confounding findings highlights a caveat to using truncated receptor intracellular loops to study binding interactions. Whether the GST-tagged PAR1 i2 and i3 loops exist in their native conformations (i.e., how they

structurally sit as full receptors in COS-7 cells) is unknown. Accordingly, it is plausible to conclude that the purified i3 loop exists in a conformation that favors RGS4 binding, similar to the conformation that allows activated PAR1 to interact with RGS4 in intact cells (Figure 4-2). If this were the case, it would explain why RGS4 binds to PAR1 in our pull-down but not co-IP experiments.

Our findings with PAR2, which suggest that its i2 and i3 loops are not involved in RGS protein binding, are not unexpected. Our data indicate that RGS2 and RGS4 may interact with the receptor's C-terminus instead of its i2 or i3 loops since we recovered no RGS protein in pull-downs with GST-tagged PAR2 i2 or i3 loops. These findings are consistent with previously published reports, which have shown that RGS2 directly binds to the C-terminus of cholecystokinin-2 (CCK2) (Langer et al., 2009), and that RGS4 binds to the C-terminus of the μ-opioid and δ-opioid receptors (Georgoussi et al., 2006;Itoh et al., 2006). Therefore, although we did not test the C-terminal region of PAR2 for its capacity to bind to RGS proteins, it is plausible that this domain contains binding sites where RGS proteins interact.

Taken together, our data with PAR1 and PAR2 have identified novel differences between the RGS regulation of these two closely related receptors. The regulation of PAR1 by the RGS proteins tested appears to require the i2 and i3 loops of the receptor. By contrast, the C-terminus of PAR2 is potentially involved in PAR/RGS interactions since no RGS proteins bind to the PAR2 i2 or i3 loops. These conclusions highlight a previously unknown mechanism of RGS regulation of PAR1 and PAR2 whereby RGS2 and RGS4 interact with different cytoplasmic regions of PAR1 and PAR2.

4.4.3. PAR1 and PAR2 signaling is differentially regulated by RGS proteins at the level of the receptor and the level of the G protein-linked signaling pathway

Our previously reported findings showed that PAR1 and PAR2 both couple to $G_{q/11}$, and $G_{12/13}$, and that PAR1 also couples to $G_{q/11}$ (Chapters 2 and 3). When we tested the capacity of B/R4 RGS protein family members to regulate these G proteins and their linked signaling pathways, we found that RGS regulation of PAR1 and PAR2 is receptor- and G protein-dependent. First we reported that RGS2 and RGS4 but not RGS1 inhibit PAR1- and PAR2-evoked calcium-activated chloride currents in oocytes. These data did not provide insight into differential regulation of the two receptors by RGS proteins. However, these findings did show that different RGS proteins have differential impacts on PAR signaling. Next we found that RGS2 but not RGS4 or RGS16 blocks PAR1-stimulated ERK1/2 phosphorylation but that RGS4 but not RGS2 or RGS16 reduces PAR2-activated ERK1/2 phosphorylation. These data are significant since they show a differential regulation of PAR1 and PAR2, which are two very closely related receptors. As such, our ERK1/2 phosphorylation data indicate that RGS regulation is not only G protein-dependent (i.e., RGS4 does not block PAR1-stimulated ERK1/2 phosphorylation but does inhibit PAR1-mediated calcium mobilization in oocytes) but is also receptor-dependent since PAR1- and PAR2-induced MAPK signaling is modulated by RGS2 and RGS4, respectively. Receptor and G protein differences also were noted when we explored the effect of RGS proteins on PAR-mediated RhoA activation. In COS-7 cells, we showed that PAR1-stimulated increases in RhoA activation is not affected by the presence of any RGS proteins tested. By contrast, RGS4 and RGS16 appear to decrease PAR2-activated RhoA-GTP formation. Similar to our ERK1/2 data, our RhoA signaling

data indicate that RGS protein regulation of $G_{12/13}$-linked pathways is receptor- and G protein-dependent.

In summary, our findings reported here are the first to show that PAR1 and PAR2 are differentially regulated by overlapping and distinct sets of RGS proteins. Although they are mostly preliminary, these data provide a potentially important mechanism that may explain how PAR1 and PAR2, which are often expressed together and activate overlapping G protein-linked pathways, are fine-tuned by distinct regulatory mechanisms and have differential effects on cellular functioning and physiology.

CHAPTER 5: Discussion[4]

[4] A version of a portion of this chapter has been published elsewhere. McCoy KL and Hepler JR (2009) Regulators of G protein signaling proteins as central components of G protein-coupled receptor signaling complexes. *Progress in Molecular Biology and Translational Science.* 86:49-74

5.1. PAR1 and PAR2 couple to overlapping and distinct sets of G proteins

5.1.1. PAR1 but not PAR2 interacts with $G_{i/o}$ family members

Many reports have linked PAR activation to second messenger signaling, presumably through their association with G proteins (reviewed in (Coughlin, 2000;Macfarlane et al., 2001;Coughlin, 2005;Bunnett, 2006;Traynelis and Trejo, 2007)). However, to our knowledge, only one paper has ever investigated direct interactions between PARs and G proteins. Inn 1996, Ogino and colleagues found that G_{i2} and $G_{q/11}$ both co-IP with PAR1 in human neuroblastoma cells (Ogino et al., 1996). Besides G_{i2} and $G_{q/11}$, the only other G protein tested in these experiments was G_s, which did not associate with PAR1 (Ogino et al., 1996). Given the small scale of this screen, I chose to perform a larger screen, where I tested members of every G protein family with which PARs have been associated (Figure 2-3). Moreover, I compared the G protein interactions with both PAR1 and PAR2, which are closely related receptors that have both been reported to activate three different G protein families. In doing so, we identified the first differences in PAR1 and PAR2 G protein coupling that laid the foundation for the rest of our studies. My findings demonstrated that PAR1 and PAR2 interact equally well with $G\alpha_{q/11}$ and $G\alpha_{12/13}$ family members. However, only PAR1, but not PAR2, interacts strongly with $G_{i/o}$ family members. A caveat to these binding studies is that they require overexpression of receptors and G proteins in cells that do not natively express PAR1 or PAR2. Therefore, we cannot be sure that our observations truly reflect similar interactions between endogenous receptors and G proteins cells that natively express PARs. To address these problems in the future, it may be possible to perform similar co-IP experiments in Neu7

cells, which endogenously express PAR1 and PAR2. Antibodies to PAR1 and PAR2 are now commercially available, and we have been able to detect native $G\alpha_{q/11}$, $G\alpha_{i1}$, $G\alpha_{o}$, and $G\alpha_{12}$ in Neu7 cell lysates (data not shown). As such, recovering complexes of native proteins in Neu7 cells may provide insight as to whether these protein-protein interactions occur endogenously. Despite the limitations of our co-IP experiments, it is important to note that these data are the first direct evidence of PAR1 and PAR2 coupling to overlapping and distinct sets of G proteins.

5.1.2. PAR1 but not PAR2 interacts with $G_{i/o}$-linked signaling pathways

Consistent with our co-IP data, PAR1 and PAR2, which both interact with $G_{q/11}$ and $G_{12/13}$ in binding studies, elicit similar signaling responses through $G_{q/11}$– and $G_{12/13}$–linked signaling pathways. Both receptors trigger PLC-mediated InsP signaling and RhoA activation in COS-7 cells and Neu7 cells (Figures 2-5 and 2-6). Importantly, these data confirm that PAR2 activates Rho through a $G_{12/13}$-linked pathway. Other published reports have alluded to PAR2/$G_{12/13}$ coupling (Vouret-Craviari et al., 2003;Yagi et al., 2006), but it has never been directly confirmed with $G_{12/13}$ inhibitors. Furthermore, the novel difference identified between PAR1 and PAR2 interactions with $G_{i/o}$ family members also translated into differences between the receptors' downstream signaling capacities. We demonstrated that PAR1-induced ERK1/2 phosphorylation and PAR1-mediated inhibition of cAMP production in COS-7 and Neu7 cells is at least in part PTX-sensitive. Similarly PAR1-stimulated Neu7 cell migration also is PTX-dependent. By contrast, PAR2-stimulated ERK1/2 phosphorylation and Neu7 cell migration is not PTX

sensitive (Figures 2-6 and 2-9), and PAR2 activation has no effect on isoproterenol-stimulated cAMP production in COS-7 or Neu7 cells (Figures 2-6 and 2-9).

Our findings are consistent with other published reports that have described a robust coupling of PAR1 to $G_{i/o}$-linked signaling pathways. In platelets, PAR1 stimulates $G_{i/o}$ signaling to initiate PI3K signaling. In turn, PI3K modulates activation of platelet integrin αIIbβ3, which sustains PAR1-induced rises in intracellular calcium and contributes to platelet activation (Voss et al., 2007). In addition, the same group also has implicated G_o in PAR1-directed intracellular calcium signaling and cytoskeletal rearrangements in endothelial cells (Vanhauwe et al., 2002). Although we did not measure the specific contributions of individual $G_{i/o}$ family members in our studies, it would not be surprising to find that PAR1-stimulated ERK1/2 phosphorylation and reductions in cAMP production are at least partially attributable to G_o, particularly in our astrocyte cell line. G_o is especially abundant in the brain, making up approximately 1% of its total protein composition (Sternweis and Robishaw, 1984). As such, G_o is likely involved in a number of PAR1-mediated signaling process in the brain, though this remains to be demonstrated.

The selectivity of G_o for PAR1 and not for PAR2 in our studies is intriguing, particularly because a recent study has demonstrated a role for PAR2/$G\alpha_i$ coupling in cell migration (Su et al., 2009). In breast cancer cells, which express high levels of PAR2, trypsin and the PAR2 agonist, LIGRLO, have been shown to induce chemokinesis and cell migration through a $G\alpha_i$-activated, JNK-mediated pathway (Su et al., 2009), which clearly demonstrate that PAR2 has the capacity to couple to G_i. These findings directly oppose what we have found, which is that $G_{i/o}$ is not involved in PAR2-induced MAPK-

mediated cell migration in Neu7 cells. G_o, however, was not implicated in the breast cancer cell migration, and as such may serve as a cell-type specific modulator of PAR1 but not PAR2 signaling. In the brain cells that we tested, G_o might be more highly expressed than other members of the $G_{i/o}$ subfamily of G proteins, and as such, may skew receptor/$G_{i/o}$ coupling toward increased receptor/G_o coupling and less receptor/G_i coupling. By contrast, perhaps G_i is more abundant than G_o in the breast cancer cells tested by Su and colleagues (Su et al., 2009), and lends itself to more receptor/G_i interactions, in which PAR2 may participate. These ideas are merely speculation but do provide evidence that PAR regulation is likely to be cell type-specific, which may explain our findings with robust $G_{i/o}$ coupling to PAR1 and not PAR2 in Neu7 cells.

To further explore the signaling events that lead to PAR2-stimulated ERK1/2 phosphorylation and cell migration in Neu7 cells, inhibitors of other signaling pathways known to induce ERK1/2 signaling also were tested in our MAPK signaling experiments. We found that PAR1 and PAR2 both at least partially rely on PLC to stimulate ERK1/2 signaling (Figure. 2-9C-D). However, in Neu7 cells, $G_{i/o}$-linked signaling pathways only contribute to ERK1/2 phosphorylation mediated by PAR1—not by PAR2 (Figure. 2-9A-B). Consistent with these data, PAR2-stimulated Neu7 cell migration is not affected by PTX but is mediated through a MAPK signaling pathway (Figure 2-10). Taken together, our overall findings are illustrated in the model shown in Figure 5-1, whereby PAR1 and PAR2 activate MAPK signaling through $G_{q/11}$ pathways but only PAR1-mediated $G_{i/o}$ activation also contributes to MAPK signaling in Neu7 cells.

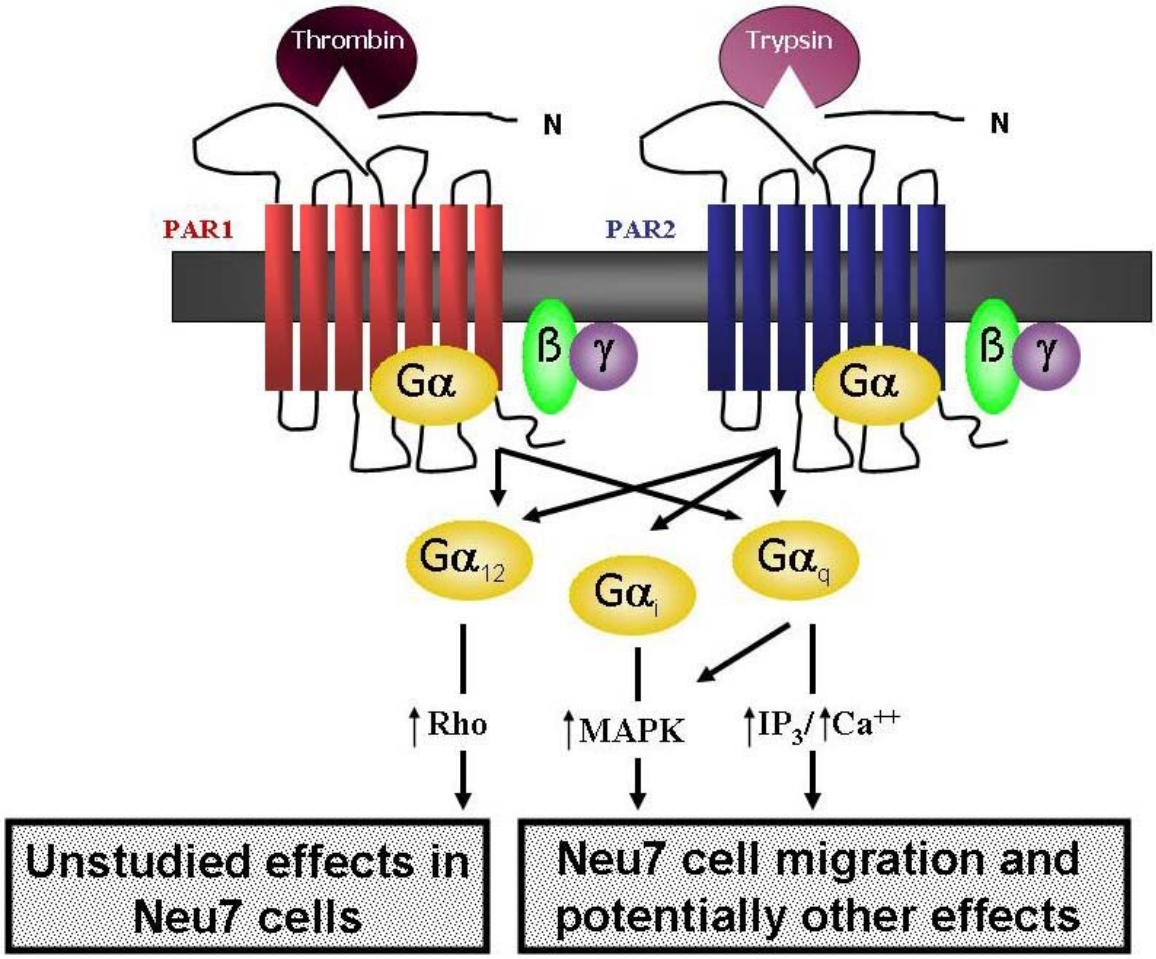

Figure 5-1. PAR1 and PAR2 couple to overlapping and distinct sets of G proteins to regulate Neu7 cell migration.

5.2. Arg205 amino acid residue in the PAR1 i2 loop dictates receptor binding to G_{11} but not to G_o or G_{12}

Our findings that PAR1 and PAR2 couple to distinct and overlapping G proteins prompted us to ask the next obvious questions: How do PARs couple to multiple G protein subfamilies? Do G proteins bind to the same receptor at different locations (i.e., can they all bind to a single receptor at the same time)? Or do G proteins have overlapping binding sites on PAR1?

To begin to answer some of these questions, we created sequential PAR1 mutants, each containing a discrete point mutation within a cytoplasmic region of the receptor (Figure 5-2). Similar to many GPCRs, the PAR1 i2 and i3 loops have been implicated in interactions between PARs and G proteins (Verrall et al., 1997;Swift et al., 2006). Verrall and colleagues identified the PAR1 i2 loop as a region that, when transferred to either the G_s- or G_i-linked chimeric receptors, is sufficient to confer coupling to InsP and intracellular calcium (i.e., $G_{q/11}$-mediated) signaling pathways (Verrall et al., 1997). Therefore, we screened 21 receptor mutants that each contained an individual point mutation in the PAR1 i2 loop. We found that five of the tested mutants significantly reduced InsP signaling but not ERK1/2 phosphorylation or RhoA activation, in COS-7 cells. Of these mutants, only one point mutation, located at the Arg205 residue, disrupted G_{11} binding to PAR1 and also evoked no intracellular calcium response when introduced into primary astrocytes from PAR1 mice (Figure 3-4). However, this residue does not appear to be important for PAR1 coupling to $G_{i/o}$ or $G_{12/13}$ as signaling mediated by these G proteins was not disrupted. These findings, which indicate that $G_{i/o}$ and $G_{12/13}$ have

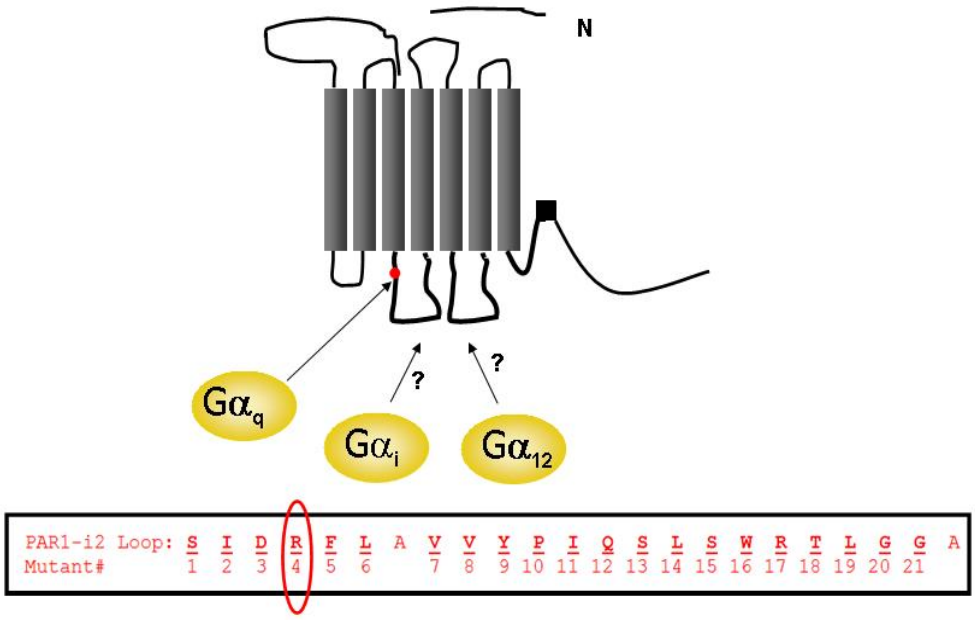

Figure 5-2. The fourth amino acid residue (Arg205) of the PAR1 i2 loop is important for PAR1 coupling to $G_{q/11}$ but not to $G_{i/o}$ or $G_{12/13}$.

different PAR1 binding sites than $G_{q/11}$ are illustrated in Figure 5-2. To our knowledge, our results are the first to shown that PAR1 can be uncoupled from one G protein whiles its coupling to other G proteins is preserved.

The results obtained in our studies with mutant PARs are consistent with reports from other mutagenesis studies that have investigated G protein coupling to promiscuous GPCRs. For example, removing the i1 loop of the CCK receptor only affects the receptor's capacity to interact with G_s- and not $G_{q/11}$–linked pathways (Wu et al., 1997). Similar studies also have identified binding regions to GPCRs (i.e., α_{2A}-AR, parathyroid receptors, and others) that dictate coupling to one G protein but not others (reviewed in (Hermans, 2003)). Similarly, our findings demonstrate that amino acids that are important for $G_{q/11}$ coupling to PAR1 do not affect its interactions with $G_{12/13}$ or $G_{i/o}$. Further exploring PAR1 domains that confer coupling to $G_{12/13}$ and $G_{i/o}$ may provide additional insight into promiscuous G protein coupling and the same mechanisms could potentially be applied to PAR2 and PAR4. Investigating coupling and signaling mechanisms of receptors that are closely related to PAR1 may be a quick and easy step in characterizing G protein coupling to two other receptors, each of which is involved in diverse physiological events.

5.3. RGS regulation of PAR1 and PAR2 is receptor- and G protein- dependent

RGS proteins regulate GPCR signal transduction at the level of the receptor, the G protein and the effector by selectively forming functional pairs with GPCRs (reviewed in

(McCoy and Hepler, 2009)). Because they regulate integral membrane proteins, RGS proteins rely on cellular mechanisms to translocate to and attach at the plasma membrane. G proteins likely contribute to recruiting RGS proteins to the membrane, but accumulating evidence suggests that receptors also are able to promote membrane translocation of RGS protein partners independent of their linked G protein (Druey et al., 1998;Dulin et al., 1999;Masuho et al., 2004;Heximer and Blumer, 2007). Consistent with these reports, we found that signaling by PAR1 and PAR2 is differentially regulated by RGS proteins in receptor- and G protein-dependent manners.

5.3.1. PAR1 and PAR2 interact with overlapping and distinct sets of RGS proteins

Similar to our PAR/ G protein interaction studies, we used co-IP techniques and pure protein pull-down experiments to identify RGS proteins that interact with PAR1 and PAR2. Our pure protein data indicates that RGS2, RGS4, and RGS16 interact with either the PAR1 i2 or i3 loop but not with the i2 or i3 loops of PAR2. However, in our co-IP experiments, we found that both receptors form complexes with RGS2 and RGS4. However, these interactions only occurred under certain conditions. For example, RGS2 binds to PAR1 and PAR2 only in the absence of overexpressed $G\alpha_{11}$ but PAR1 retains the capacity to interact with RGS2 in the presence of $G\alpha_o$. Since RGS protein interactions with PARs differ depend on whether $G\alpha_{11}$ or $G\alpha_o$ are present in the reactions, our initial results indicate that RGS protein regulation of PARs is G protein-dependent. In addition, RGS4 only interacts with inactive PAR1 and PAR2 in the presence of $G\alpha_o$. However, upon activation, PAR1 but not PAR2, gains the capacity to bind to RGS4, in the absence of $G\alpha_o$ overexpression in the cells. These findings suggest that RGS regulation of PARs

also is receptor-dependent since PAR1 and PAR2 have different capacities to interact with RGS4 in the presence of agonist. As described in Chapter 4, our PAR/RGS findings are entirely novel since small RGS proteins have never been reported to interact with PARs.

5.3.2. PAR1 and PAR2 signaling is differentially regulated by RGS proteins at the level of the receptor and the level of the G protein-linked signaling pathway

Using the same effector activation experiments as we used in our PAR/G protein studies (e.g., calcium mobilization, ERK1/2 phosphorylation, and RhoA experiments), we confirmed what we reported in our PAR/RGS interaction studies—that RGS regulation of PAR1 and PAR2 is receptor- and G protein-dependent. RGS2 regulates PAR1-activated $G_{q/11}$– and $G_{i/o}$–linked signaling but not signaling mediated by $G_{12/13}$. However, RGS4 only modulates PAR1 signaling through $G_{q/11}$, as illustrated in Figure 5-3A. Patterns of RGS regulation of PAR2 are different. Although RGS2 and RGS4 inhibit $G_{q/11}$–linked PAR2 signaling, they do not affect $G_{i/o}$– or $G_{12/13}$—linked PAR2 signaling, as depicted in Figure 5-3B. Importantly, RGS16 appears to have little-to-no effect on PAR signaling, despite its small, potentially non-specific pure protein interaction with PAR1 (Figure 4-3). Taken together, our data demonstrate that RGS proteins functional regulate PARs, and these findings are the first to show that RGS proteins differentially regulate PAR1 and PAR2 in receptor- and G protein-dependent manners.

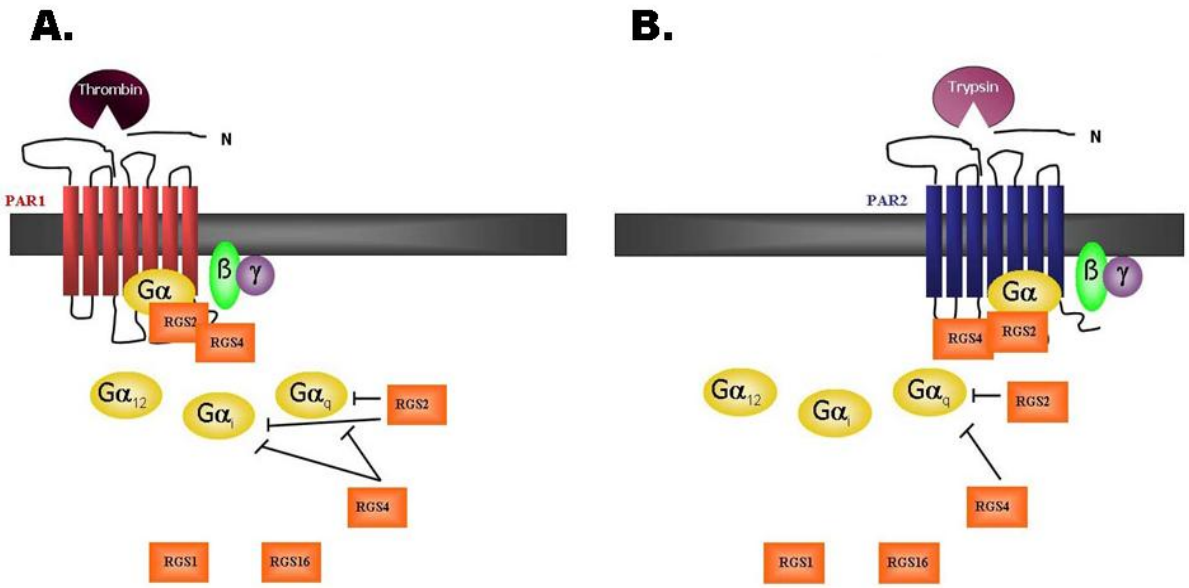

Figure 5-3. RGS proteins differentially regulate PAR1 and PAR2.

5.4. Overall Conclusions and Implications

From the data presented in this dissertation, we conclude that PAR1 and PAR2 have overlapping and distinct G protein signaling and regulation that mediate the selective physiological effects of each receptor. Gaining a better understanding of these unconventional receptors introduces new concepts into the PAR field, which also may be applicable to GPCR pharmacology as a whole. Namely, this information adds to the accumulating evidence that establishes working models of GPCRs and their linked G proteins and downstream signaling pathways are more complex than originally thought. Recently solved crystal structures of rhodopsin (Palczewski et al., 2000) and β2-AR (Rasmussen et al., 2007) indicate that these seven-transmembrane-spanning proteins have the intracellular surface area and capacity to form multi-protein complexes. As such, GPCRs may serve as nucleation centers for various proteins to come together and perform a shared, though receptor-specific, signaling task (Ferguson, 2001;Moore et al., 2007).

In newly emerging models of GPCR signaling, G proteins and regulatory proteins exist in close proximity with receptors, perhaps even pre-associated with inactive receptor. Established models of G protein signaling (Hepler and Gilman, 1992) propose that, upon agonist stimulation, receptors and G proteins undergo a conformational change that dissociate G protein subunits to reveal binding sites on the receptor and G proteins to which regulatory and signaling proteins could attach. However, recent evidence, including our own, suggests that some GPCRs and G protein subunits remain complexed following agonist stimulation, and merely rearrange *in situ* to present newly revealed

binding interfaces for effectors and transduce signals to nearby signaling partners (Bunemann et al., 2003;Hein and Bunemann, 2008;Hoffmann et al., 2008;Zurn et al., 2009). Our observations with PAR/ G protein interactions are consistent with this theory. We showed that PAR1 and PAR2 both associate with Gα subunits in the absence of agonist stimulation (Figure 2-3). Unexpectedly, we also demonstrated that upon agonist stimulation and in the presence of GTPγS, heterotrimeric G proteins remain assembled with both PAR1 and PAR2 (Figure 2-5). These findings support a model whereby PARs and G proteins form an inactive complex that upon stimulation rearranges—but does not dissociate—to activate intracellular signaling. In this model, signaling and regulatory proteins must be pre-positioned within close proximity of the GPCR prior to receptor stimulation and move to the GPCR/G protein complex for signal transduction to occur.

Besides heterotrimeric G proteins, proteins involved in regulating PARs and other GPCRs also interact with receptors as part of the multi-protein signaling complex. Arrestins and GRKs, which are best know for their involvement in the termination of signaling, may serve as scaffolding proteins by binding other signaling molecules when linked to receptors (DeFea et al., 2000;Tohgo et al., 2003;Macey et al., 2006;Scott et al., 2006;Shenoy et al., 2006;Pfleger et al., 2007;Zheng et al., 2008). For example, considerable evidence, including data from PAR2 studies (DeFea et al., 2000;Stalheim et al., 2005), shows that arrestins can recruit various components of the MAPK signaling pathways to initiate ERK signaling outside of the nucleus (DeWire et al., 2007). More recently, GPCRs were linked to PIP2 production via an arrestin-mediated interaction with 4-phosphate 5-kinase (PIP5K) which converts PIP to PIP2 (Nelson et al., 2008). Other scaffolding proteins that interact with GPCRs include spinophilin and GIPC, which link

various other signaling molecules (i.e., certain RGS proteins) to GPCRs and G proteins as well. As such, RGS proteins likely are part of and play a role in PAR (and other GPCR) signaling complexes. My data, which demonstrates that PARs form inactive and active complexes with RGS proteins in the presence and absence of G proteins (Figures 4-1 and 4-2), provides evidence that RGS proteins may be involved in these signaling complexes. I found that PAR1 and PAR2 are differentially regulated by RGS proteins, which suggests that each receptor may dictate which RGS proteins interacts with and regulates it. Thus, PARs, like some other GPCRs, may serve as platforms for multiple scaffolding proteins that engage a variety of signaling proteins and pathways that, in combination, initiate a unique profile of shared and distinct signaling outputs specific to that receptor.

Overall, the functional coupling and pairing of RGS proteins, G proteins and GPCRs may have broad implications for future therapeutic interventions. GPCRs regulate nearly all aspects of cell and organ physiology, and exhibit discrete tissue distribution patterns making them ideal as front-line therapeutic targets. For example, even within the brain, PAR expression is region- layer- and cell-type specific (Striggow et al., 2001). Like PARs, RGS proteins also exhibit discrete cellular and tissue distribution patterns and have been shown to play important roles in receptor functions critical for the cardiovascular, immune, and nervous systems (Hollinger and Hepler, 2002;Chidiac and Roy, 2003;Bansal et al., 2007). In the CNS, RGS proteins play key roles in relating to receptors involved with drug abuse, addiction, and drug tolerance (Neubig, 2002;Garzon et al., 2005;Xie and Palmer, 2005;Hooks et al., 2008). Therefore, small molecule inhibitors of PAR/G protein/RGS interactions and RGS regulation of GPCR could help to reduce "dirty" drug cross reactivity and extend the specificity of existing drugs that act on

GPCRs, or perhaps offer new therapies to boost GPCR function where it is diminished (Neubig, 2002). Thus, understanding of the underlying mechanisms of how PARs, G proteins, and RGS proteins interact are important goals for future research.

5.5. Future Directions

The work presented in this dissertation has laid the foundation for future studies that may potentially help us better understand PAR/ G protein coupling and RGS regulation of PARs. In this section, I present some ideas for future studies that may partially address some unanswered questions that have been prompted by my work. Key outstanding questions raised by my dissertation include the following:

1. What are the functional and physiological consequences of differential PAR1 and PAR2 signaling in native cells (e.g., neurons and astrocytes)?
2. How does G protein coupling to PAR4 compare and contrast with our findings with PAR1 and PAR2?
3. Where do $G_{i/o}$ and $G_{12/13}$ bind to PAR1?
4. Can point mutations in PAR2 and/or PAR4 also disrupt coupling to one G protein while preserving the functioning of others?
5. Where do RGS proteins bind to PARs?
6. Do RGS proteins and G proteins compete for PAR binding sites?

First I showed that PAR1, but not PAR2, couples to $G_{i/o}$ and linked signaling pathways in COS-7 and Neu7 cells. Following up on these findings with additional PAR1 mutant studies could help determine which PAR1 residues are important for PAR1/$G_{i/o}$ (and PAR1/ $G_{12/13}$) coupling. Furthermore, creating point mutants in the cytoplasmic portions of PAR2 may identify similar and/or different amino acids that dictate its interactions with various G proteins. Doing so would potentially yield additional insight as to why PAR1 and PAR2 differentially couple to $G_{i/o}$ in our experiments.

Researching whether this PAR1/$G_{i/o}$ preferred coupling is preserved in other cells warrants further attention. Similarly, determining whether preferred coupling between PAR2 and $G_{i/o}$ occurs in other cells also is worth investigating. In addition to the breast cancer cell migration studies described in section 5-1 (Su et al., 2009), another indication that PAR2 may interact with $G_{i/o}$ under certain circumstances came from our PAR/RGS co-immunoprecipitation data. Albeit unexpected, we found that in the presence of RGS4, PAR2 gains the capacity to co-IP with $G\alpha_o$, a phenomenon that we did not observe in any other situation (i.e., with PAR2/RGS2 co-IPs, in the presence of $G\alpha_{11}$ or in the presence or absence of agonist). As such, binding partners, such as RGS4, or unidentified scaffolding proteins may promote PAR2 coupling to $G_{i/o}$ family members in certain cells. In turn, reinforcing interactions between PAR2 and $G_{i/o}$ may allow PAR2 to elicit functional $G_{i/o}$–mediated signaling. Therefore, it would be interesting to determine whether PAR2-stimulated MAPK activation and Neu7 cell migration gains a PTX-sensitive component in the presence of scaffolding proteins, like RGS4. Moreover, to activate platelets, PAR1, but not PAR4, couples to G_o and linked signaling pathways (Voss et al., 2007). Investigating whether PAR4, like PAR2, gains the capacity to interact

with G_o and activate platelets in the presence of other modulatory proteins may uncover a conserved mechanism by which PAR/ G protein coupling is selectively regulated.

Another avenue that warrants further investigation is RGS regulation of PARs. In general, our studies remain incomplete, yet lay the foundation for an entirely novel story. To our knowledge, RGS regulation of receptors that couple to multiple G proteins has never been explored and may potentially highlight previously unidentified mechanisms of GPCR regulation. In our studies, we identified only very few RGS binding partners for PAR1 and PAR2. Others may exist and such interactions easily can be identified with a large screen using our co-IP techniques. Furthermore, our studies were limited to testing RGS protein interactions with only a few different combinations of PARs and G proteins. However, several more permutations of PAR/ G protein/ RGS pairs can be tested.

After identifying candidate PAR/RGS interactions, it also may be useful to employ the PAR1 i2 loop mutants that we created to identify PAR1 binding sites for RGS proteins. In doing so, it may be possible to locate overlapping and distinct binding sites for RGS proteins and G proteins, thereby determining whether G proteins and RGS proteins may compete for binding sites on PARs. Together, these studies would shed light on how GPCRs form complexes with multiple signaling partners and regulatory proteins at one time. As mentioned above, exploring such interactions may be useful for future therapeutic interventions that target these interactions, once their implications for physiology and disease are more completely understood.

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