

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Functional characterization of protease-activated receptor -1 palmitoylation in receptor signaling and trafficking /

Permalink

<https://escholarship.org/uc/item/3r35x8xj>

Author

Cordova, Isabel Canto

Publication Date

2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Functional characterization of protease-activated receptor-1 palmitoylation in receptor
signaling and trafficking

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Isabel Canto Cordova

Committee in charge:

Professor JoAnn Trejo, Chair
Professor Joan Heller Brown
Professor Marilyn G. Farquhar
Professor Tracy M. Handel
Professor Tony Hunter

2014

Copyright

Isabel Canto Cordova, 2014

All rights reserved

The Dissertation of Isabel Canto Cordova is approved, and it is acceptable in quality
and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014

DEDICATION

I would like to dedicate this dissertation to my husband and my family. Without them, none of this would have been possible. Their love, values, and encouragement were huge factors for my success. I am truly blessed to have such wonderful people in my life. I hope I have made you proud, thank you for all of your support.

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Table of Contents	v
List of Abbreviations	ix
List of Figures	xii
Acknowledgements.....	xv
Vita.....	xviii
Abstract of the Dissertation.....	xx
Chapter 1: An introduction to palmitoylation of GPCRs and PAR1 signaling	1
1.1 Introduction	1
1.1.2 Thrombin generation and cellular responses.....	1
1.1.3 PAR1 expression and function.....	3
1.1.4 PAR1 signaling and regulation.....	6
1.1.5 PAR1 trafficking	8
1.1.6 Mechanism of protein palmitoylation.....	10
1.1.7 Functional implications of protein palmitoylation.....	12
1.1.8 Palmitoylation and GPCRs	14
1.2 Acknowledgements.....	20
1.3 Figures	21
1.4 References	27

Chapter 2: Characterization of PAR1 palmitoylation and other post-translational modifications	44
2.1 Abstract.....	44
2.2 Introduction	45
2.3 Materials and Methods	48
2.4 Results	53
2.4.1 PAR1 C-terminal tail palmitoylation sites are highly conserved across multiple species	53
2.4.2 PAR1 is modified with palmitoylation	54
2.4.3 Palmitoylation-deficient PAR1 glycosylation and surface expression....	55
2.4.4 Palmitoylation is required for agonist-induced PAR1 phosphorylation and ubiquitination.....	56
2.4.5 Palmitoylation-deficient PAR1 in endothelial cells	57
2.5 Conclusions	59
2.6 Acknowledgements.....	62
2.7 Figures	63
2.8 References	69
Chapter 3: PAR1 trafficking is regulated by palmitoylation	74
3.1 Abstract.....	74
3.2 Introduction	75
3.3 Materials and Methods	78
3.4 Results	83
3.4.1 Palmitoylation is not required for PAR1 caveolae localization	83

3.4.2 Palmitoylation-deficient PAR1 exhibits enhanced constitutive internalization.....	84
3.4.3 PAR1 CC/AA constitutive internalization is AP-2 dependent.....	85
3.4.4 Palmitoylation is important for proper utilization of tyrosine based sorting motifs	86
3.4.5 Palmitoylation deficient PAR1 is basally degraded more rapidly than wildtype PAR1.....	88
3.5 Conclusions	91
3.6 Acknowledgements.....	93
3.7 Figures.....	95
3.8 References	107
Chapter 4: The role of palmitoylation in PAR1 signaling	113
4.1 Abstract.....	113
4.2 Introduction	114
4.3 Materials and Methods	116
4.4 Results	121
4.4.1 Gq signaling in PAR1 CC/AA expressing HeLa cells.....	121
4.4.2 PAR1 CC/AA elicits ERK1/2 activation in response to thrombin	123
4.4.3 Palmitoylation is required for thrombin induced barrier permeability in endothelial cells	124
4.4.4 Thrombin-induced RhoA activation is reduced in PAR1 CC/AA expressing endothelial cells	125

4.4.5 Thrombin-induced p38 MAPK activation is reduced in PAR1 CC/AA expressing cells	126
4.4.6 Thrombin-induced RhoA activation is not mediated by p38 MAPK	128
4.4.7 Receptor palmitoylation is critical for $G\alpha_i$ association with PAR1	129
4.5 Conclusions	130
4.6 Acknowledgements.....	134
4.7 Figures	135
4.8 References	145
Chapter 5: Conclusions	151
5.1 PAR1 palmitoylation and other post-translational modifications	152
5.2 Palmitoylation regulates accessibility of tyrosine-based sorting motifs and PAR1 trafficking	154
5.3 Palmitoylation of PAR1 specifies distinct G-protein signaling pathways	158
5.4 Concluding Remarks.....	161
5.5 Acknowledgements.....	164
5.6 Figures	165
5.7 References	166

LIST OF ABBREVIATIONS

A	alanine
ABTS	step 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid
ATP	adenosine triphosphate
ANOVA	analysis of variance
APC	activated protein C
AP-2	adaptor protein complex-2
AP-3	adaptor protein complex-3
APT1	Acyl-protein thioesterase-1
BRET	bioluminescence resonance energy transfer
β 2-AR	β 2- Adrenergic receptor
BSA	bovine serum albumin
C	cysteine
cAMP	3'-5' cyclic adenosine monophosphate
Cav1	caveolin-1
CHX	cycloheximide
DHHC	aspartate-histidine-histidine-cysteine motif
DMEM	Dulbecco's Modified Eagle Medium
DAG	diacyl glycerol
EEA1	early endosomal antigen-1
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase

EPCR	endothelial protein C receptor
FLAG	DYKDDDDK epitope tag
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate
HRP	horseradish peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP	inositol phosphate
K	lysine
LAMP1	lysosomal-associated membrane protein 1
MAPK	mitogen-activated protein kinase
PAR1	protease-activated receptor-1
PAT	palmitoyl acyl transferase
PI	phosphoinositide
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PNGase F	peptide <i>N</i> -glycosidase F
PKC	protein kinase C
PLC	phospholipase C
PPT	protein-palmitoylthioesterase-1
R	arginine
Rac1	Ras-related C3 botulinum toxin substrate 1

RBD	Rho Binding Domain
RhoA	Ras homologue gene family, member A
Rluc	<i>Renilla reniformis</i> luciferase
α -Th	α -thrombin
S	serine
shRNA	short hairpin RNAi
TfR	transferrin receptor
TM	transmembrane
TP	thromboxane A2 receptor
UT	untransfected
WT	wild type
YFP	yellow fluorescent protein

LIST OF FIGURES

Figure 1.1: Thrombin mediated cellular responses in the vasculature.	21
Figure 1.2: PAR1 G-protein signaling pathways.	22
Figure 1.3: PAR1 trafficking pathways.	23
Figure 1.4: Modifications and motifs important for PAR1 signal regulation.	24
Figure 1.5: A model for the mechanism of protein palmitoylation.	25
Figure 1.6: Sequence alignment of the C-terminal tails of select GPCRs.	26
Figure 2.1: PAR1 C-tail species sequence alignment.	63
Figure 2.2: PAR1 is posttranslationally modified by palmitoylation.	64
Figure 2.3: Palmitoylation is not required for cell surface expression or receptor glycosylation.	65
Figure 2.4: Palmitoylation is important for agonist-induced phosphorylation of PAR1.	66
Figure 2.5: Palmitoylation is required for agonist-induced receptor ubiquitination.	67
Figure 2.6: Characterization of palmitoylation-deficient PAR1 in endothelial cells.	68
Figure 3.1: Palmitoylation is not required for PAR1 recruitment to caveolin-rich microdomains.	95
Figure 3.2: Palmitoylation-deficient PAR1 displays enhanced constitutive internalization.	96
Figure 3.3: Palmitoylation deficient PAR1 is sorted to early endosomes.	97
Figure 3.4: AP-2 mediates enhanced constitutive internalization of palmitoylation- deficient PAR1.	98

Figure 3.5: Palmitoylation sites are near tyrosine-based sorting motifs in the PAR1 C-tail.	99
Fig 3.6: Palmitoylation of PAR1 is important for proper utilization of C-tail tyrosine-based sorting motifs.....	100
Figure 3.7: Mutagenesis of the proximal tyrosine-based sorting motif prevents PAR1 CC/AA enhanced constitutive internalization.	101
Figure 3.8: AP-2 mediates constitutive internalization of PAR1 CC/AA+AKKAA and CC/AA+ASIA mutants.....	102
Figure 3.9: The palmitoylation-deficient PAR1 mutant exhibits enhanced degradation.	103
Figure 3.10: Mutagenesis of the proximal tyrosine-based sorting motif prevents PAR1 CC/AA sorting to LAMP1 positive endosomes.....	104
Figure 3.11: Intracellular palmitoylation-deficient PAR1 exhibits enhanced degradation.	105
Figure 3.12: Enhanced degradation of the palmitoylation-deficient PAR1 mutant is AP-3 dependent.	106
Figure 4.1: Signaling by the palmitoylation-deficient PAR1 mutant.	135
Figure 4.2: Palmitoylation-deficient PAR1 elicits ERK1/2 phosphorylation in response to thrombin.....	136
Figure 4.3: Palmitoylation is required for thrombin-induced endothelial barrier permeability.	137
Figure 4.4: Palmitoylation is required for thrombin mediated RhoA activation.	138

Figure 4.5: Palmitoylation is required for thrombin-induced p38 MAPK activation in endothelial cells.	139
Figure 4.6: PAR1 CC/AA endothelial stable cell lines are not defective for ligand-induced p38 signaling.	140
Figure 4.7: Palmitoylation is required for thrombin-induced p38 MAPK activation in HeLa cells.	141
Figure 4.8: Thrombin-induced RhoA activation is not mediated by p38 MAPK.	142
Figure 4.9: Palmitoylation of PAR1 is required for interaction with $G\alpha_i$	143
Figure 4.10: A model for the role of PAR1 palmitoylation in activated receptor signaling pathways.	144
Figure 5.1: Palmitoylated cysteine residues and localization of nearby sorting motifs.	165

ACKNOWLEDGEMENTS

I would like to acknowledge my thesis advisor, Dr. JoAnn Trejo, for her guidance and support throughout my graduate school experience. She has instilled in me the importance of being meticulous in all aspects of scientific research, from planning experiments to careful interpretation of data. She has also taught me the importance of becoming a well-rounded scientist and provided me with many opportunities to present my research, mentor students, improve my writing skills, and explore different career opportunities.

I would like to acknowledge current and former Trejo Lab members for their valuable insights and thoughtful discussions. I feel fortunate to have been a part of such a talented group of scientists. Their suggestions and feedback have been very helpful in progressing my projects forward. I would like to thank the undergraduate students that I have mentored throughout the years, and the contributions they have made to some of my projects. I would also like to thank my thesis committee members, Drs. Joan Heller Brown, Marilyn Farquhar, Tracy Handel, and Tony Hunter for their insightful suggestions, advice, and development of my thesis project. I would also like to thank the Pharmacology Department and the Biomedical Sciences graduate program for their support and guidance throughout this process. I would also like to acknowledge my funding sources: The Tobacco Related-Disease Research Program (TRDRP) Dissertation Award (21DT-0096) and the Pharmacology Training Grant (T32 GM007752).

I would like to thank all my friends who have supported me throughout this process. To all my friends outside of science, I appreciate your sincere efforts to try and understand my research. To my friends in graduate school, I will always appreciate your support in those moments of commiseration, and cherish those moments of celebration when a paper got published, a grant was accepted, or an experiment worked. In particular, I would like to thank Stephanie Huelga, Joshua Lerman, and Spencer Wei for their constant support and for reminding me to have a life outside of the lab. This journey has been full of highs and lows, and I'm grateful to have a wonderful group of friends that understood what I was going through, and kept me motivated to continue working towards this goal.

Last but not least, I would like to thank my family. My husband, David Cordova, has been supportive every step of the way, from my application to grad school, to moving to San Diego, to patiently waiting for me to finish up experiments on the weekend, and encouraging me accomplish what I had set out to do. His unending support has kept me motivated, especially in times when I felt I had no motivation left. I would like to thank my parents, Jorge and Maria Canto, for always encouraging me to pursue my dreams, and instilling in me the values of hard work and dedication. I would like to thank my Nana, who unfortunately passed away while I was in grad school. Every time I would say goodbye after a visit, she would always give me a hug and say "never give up, keep going"- a simple gesture that meant so much at times. I would like to thank my siblings for always making me laugh and reminding me to have fun. My family has always reminded me about the importance of maintaining a

balance between hard work and relaxation. This grad school journey would not have been the same without them.

The majority of chapter 3, and parts of chapters 2 and 4, are published as a manuscript entitled “Palmitoylation of protease-activated receptor-1 regulates adaptor protein complex-2 and -3 interaction with tyrosine-based motifs and endocytic sorting” in the *Journal of Biological Chemistry*. The dissertation author is the primary author who wrote the manuscript, directed experiments, and analyzed the data.

The majority of content in chapter 4, and parts of chapters 2 and 3, are being prepared for publication: Canto Cordova I, Saucedo C., and Trejo J. Palmitoylation regulates PAR1 G-protein signaling pathways and thrombin-induced endothelial barrier permeability. *Journal TBD*. The dissertation author is the primary author who directed experiments and analyzed the data.

VITA

Education

- 2014 Doctor of Philosophy, Biomedical Sciences
University of California, San Diego
- 2007 Bachelor of Science, Biological Sciences
University of California, Irvine

Publications

Canto I and Trejo J. Palmitoylation of protease-activated receptor-1 regulates adaptor protein complex-2 and -3 interaction with tyrosine-based motifs and endocytic sorting. (2013) *Journal of Biological Chemistry*. May 31; 288 (22):15900-12

Canto I, Soh UJ, Trejo J. Allosteric Modulation of Protease-activated Receptor Signaling. (2012) *Mini-Reviews in Medicinal Chemistry*. Aug 2012; 12(9):804-11

Chen B, Dores M, Grimsey N, **Canto I**, Barker BL and Trejo J. Adaptor Protein Complex-2 (AP-2) and Epsin-1 Mediate Protease-activated Receptor-1 Internalization via Phosphorylation- and Ubiquitination-dependent Sorting Signals. (2011) *Journal of Biological Chemistry*. Nov 25;286(47):40760-70

Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, **Canto I**, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K. Somatic coding mutations in human induced pluripotent stem cells. (2011) *Nature*. Mar 3; 471(7336):63-7.

Kayed R, **Canto I**, Breydo L, Rasool S, Lukacs T, Wu J, Albay R, Pensalfini A, Yeung S, Head E, Marsh L and Glabe CG. Conformation Dependent Monoclonal Antibodies Distinguish Different Replicating Strains or Conformers of Prefibrillar A β Oligomers. (2010) *Molecular Neurodegeneration*. Dec 13;5:57

Shah S, Nolan R, Davis E, Stokin G, Niesman I, **Canto I**, Glabe, C, Goldstein L. Pathways for General and Amyloid-Induced Neuronal Transport Defects. (2009) *Neurobiology of Disease*. 36(1):11-25.

Canto I. The effect of tranylcypromine stereoisomers on the rewarding and reinforcing properties of nicotine. (2007) *The Journal of Undergraduate Research in the Biological Sciences*. 37: 55-66

Abstracts

Canto I, Grimsey N, and Trejo J. Palmitoylation is required for activated PAR1 ubiquitination and p38 MAPK signaling. Experimental Biology Meeting, 2014

Coronel L, Grimsey N, **Canto I**, Stephens B, Phan C, Handel T, and Trejo J. Rab11A and Rab11B distinctly regulate protease-activated receptor-1 recycling and constitutive degradation. Experimental Biology Meeting, 2014

Canto I and Trejo J. Palmitoylation of PAR1 regulates the accessibility of C-tail YXX ϕ motifs to AP-2 and internalization. Experimental Biology Meeting, 2012

Chen B, Dores MR, Grimsey N, **Canto I**, Barker BL and Trejo J. Adaptor Protein Complex-2 and epsin-1 mediate Protease-activated Receptor-1 internalization via phosphorylation- and ubiquitination-dependent sorting signals. Experimental Biology Meeting, 2012

Canto I and Trejo J. PAR1 palmitoylation: regulation of receptor ubiquitination and intracellular trafficking. Experimental Biology Meeting, 2011

***FASEB MARC travel award recipient**

Canto I and Leslie FM. The Role of Monoamine Oxidase in Tobacco Addiction. Society for the Advancement of Chicanos and Native Americans in Science Meeting, 2006

Canto I and Martinez-Torres A. Functional characterization of 1kb fragment upstream of the GABA $_c$ receptor rho2 subunit gene. Annual Biomedical Research Conference for Minority Students, 2005

ABSTRACT OF THE DISSERTATION

Functional characterization of protease-activated receptor-1 palmitoylation in receptor signaling and trafficking

by

Isabel Canto Cordova

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2014

Professor JoAnn Trejo, Chair

G protein-coupled receptors (GPCRs) are the largest family of signaling receptors that respond to diverse stimuli and regulate many physiological responses. GPCRs elicit their cellular responses by coupling to distinct subtypes of heterotrimeric G-proteins composed of $G\alpha$ and $G\beta\gamma$ subunits. Activated GPCRs undergo conformational changes that allow the receptor to exchange GDP for GTP on the $G\alpha$ subunit, which induces dissociation from the $\beta\gamma$ subunits and subsequent downstream signaling. Protease-activated receptor-1 (PAR1) is a member of a family of GPCRs

that are proteolytically activated. PAR1 is a receptor for the coagulant protease thrombin, and is capable of coupling to multiple G-protein subtypes to elicit various cellular responses; however, the mechanisms that regulate this selectivity are not well understood. Palmitoylation is a post-translational modification that many GPCRs possess, and is defined as the addition of palmitate, a 16 carbon fatty acid, to a cysteine residue via a thioester linkage. Many GPCRs are palmitoylated on their C-terminal tails, but the role of this modification differs based on the GPCR being examined. In this dissertation, I examined the role of palmitoylation in PAR1 signaling and trafficking. I defined the sites of PAR1 palmitoylation to occur on conserved C-tail cysteine residues C³⁸⁷ and C³⁸⁸. I discovered that palmitoylation is important for other PAR1 post-translational modifications, specifically phosphorylation and ubiquitination. I also show that palmitoylation of PAR1 regulates the accessibility of a nearby tyrosine-based sorting motif to the adaptor-protein complex-2 (AP-2) and -3 (AP-3), which controls receptor internalization and degradation. Additionally, palmitoylation appears to be important for the regulation of selective PAR1-induced signaling pathways such as G $\alpha_{12/13}$ -induced RhoA activation, G α_i coupling, and thrombin-stimulated p38 MAPK signaling pathways. However, thrombin-induced G α_q -mediated phosphoinositide hydrolysis and ERK1/2 activation are unperturbed in the absence of PAR1 palmitoylation. Taken together, the studies summarized in this dissertation highlight the relevance of palmitoylation for PAR1 function, and suggest that palmitoylation governs a C-tail conformation that is important for accessibility of other proteins such as ligases, kinases, adaptor proteins, and G-proteins, ultimately regulating PAR1 signaling and trafficking.

Chapter 1: An introduction to palmitoylation of GPCRs and PAR1 signaling

1.1 Introduction

The seven transmembrane superfamily of G protein-coupled receptors (GPCRs) are highly targeted for drug development, as they are the most abundant group of signaling receptors in the mammalian genome and implicated in multiple disease states (1-3). A better understanding of GPCR signal regulation is critical for the development of therapeutics for the prevention and treatment of various diseases. Post-translational modifications are one mechanism that modulate GPCR signal transduction. It has been demonstrated that GPCRs are modified with ubiquitination (4), phosphorylation (5), and glycosylation (6). Palmitoylation is one such modification in which palmitate, a 16 carbon fatty acid, is added to a cysteine residue through a thioester linkage. It has been shown that many GPCRs are palmitoylated on cysteine residues in the C-terminal tail (7). Palmitoylation has been shown to be important for some soluble proteins to associate with membranes (8,9). However, considering that GPCRs are seven transmembrane proteins, palmitoylation may serve a function other than plasma membrane association. The focus of this thesis dissertation is to examine the function of palmitoylation in the signaling and trafficking of protease-activated receptor-1 (PAR1), a GPCR for the coagulant protease thrombin.

1.1.2 Thrombin generation and cellular responses

Thrombin is generated at sites of vascular injury and in thrombotic disease. As the main effector protease of the coagulation cascade, thrombin regulates

hemostasis and thrombosis, and has also been implicated in inflammation, blood vessel formation, and cancer progression (10,11). Thrombin is generated through a series of zymogen conversions, which are dependent upon the presence of tissue factor, an integral membrane protein that is expressed by cells that are normally segregated from blood (Fig. 1.1). Thrombin cleaves the N-terminus of soluble fibrinogen to form fibrin monomers, which then undergo end-to-end polymerization to form protofibrils, and ultimately lateral association to form a fibrin network (12). As a mechanism to terminate coagulation, thrombin binds to thrombomodulin, an integral membrane protein expressed on the surface of endothelial cells. The thrombin-thrombomodulin complex enhances thrombin-mediated cleavage of protein C to generate activated protein C (APC) (13,14). APC, along with its cofactor protein S, inactivates the upstream coagulation factors Va or VIIIa, to prevent further thrombin generation (15).

Thrombin also acts on multiple cell types in the vasculature, such as platelets and endothelial cells (Fig. 1.1). Thrombin-mediated platelet activation causes cell shape change, aggregation, upregulation of P-selectin and integrins, and granule secretion which results in the release of ADP, serotonin, thromboxane A₂, chemokines and growth factors (16). Platelet aggregation is mediated by the binding of surface integrin $\alpha_{IIb}\beta_3$ to fibrinogen and von Willebrand factor (17). Thrombin also activates endothelial cells by upregulating cytokines such as interleukin (IL-8) (18) and monocyte chemotactic protein 1 (MCP-1) (19). Thrombin activation of endothelial cells also upregulates expression of the adhesion molecule P-selectin at the cell surface (20), which is critical for leukocyte and platelet rolling (21). Thrombin also

induces endothelial cell rounding and gap formation, resulting in increased endothelial monolayer barrier permeability (22) and vasodilation (23).

1.1.3 PAR1 expression and function

Thrombin mediates cellular responses mainly through activation of the G protein-coupled protease-activated receptor-1 (PAR1). PAR1 belongs to a unique family of GPCRs that are proteolytically cleaved for receptor activation. The family of PARs is comprised of four members including: PAR1, PAR2, PAR3 and PAR4. PAR1 was discovered as the first human thrombin receptor in 1991 through an expression cloning screen using *Xenopus laevis* oocytes. Briefly, mRNA isolated from megakaryocyte cell lines was microinjected into oocytes, and measured for $^{45}\text{Ca}^{2+}$ release and inward current in response to thrombin. It was shown that these oocytes responded to thrombin at nanomolar concentrations, similarly to the pharmacology observed in platelet aggregation (24). The isolated mRNA was then size fractionated by sucrose centrifugation, and assayed in the oocyte system for thrombin responsiveness. It was determined that the 4kb fraction conferred the most thrombin receptor activity, and thus was used to generate a cDNA library. The library was plated in 50 pools, at 20,000 clones per pool, then capped cRNA from each pool was screened by the oocyte system for thrombin activity. A single 3.4 kb cDNA clone was identified from the screen, and sequenced to identify the thrombin receptor (24). The thrombin cleavage site was identified to occur at arginine (R)⁴¹-serine (S)⁴². Furthermore, a synthetic peptide that mimics the newly cleaved N-terminus of PAR1 was shown to be sufficient to stimulate Ca^{2+} mobility in *Xenopus laevis* oocytes (24). Knockout of the PAR1 gene in mice resulted in 50% lethality, wherein most embryos

died at embryonic day 9-10, suggesting a role for PAR1 in vascular development (25). In fact, endothelial cell specific expression of PAR1 in a transgenic mouse model was sufficient to rescue lethality in PAR1 $-/-$ mice (26). Additionally, platelets from the PAR1 $-/-$ mice retained thrombin responsiveness, whereas fibroblasts did not. Furthermore, these PAR1 $-/-$ platelets did not respond to the human or mouse PAR1 agonist peptides (25). The results from this study prompted the discovery of the other thrombin receptors PAR3 and PAR4 (27,28). PAR2 is the only member of the PAR family that is not activated by thrombin, but instead is activated by trypsin-like serine proteases (29). Murine platelets express PAR3 and PAR4, whereas human platelets express PAR1 and PAR4, thus there are species-specific differences in PAR expression depending on the cell type.

The mechanism of PAR1 activation by the coagulant protease thrombin has been most extensively studied (24,30). Thrombin binds to the PAR1 N-terminal LDPR⁴¹-S⁴²FLLRN sequence and cleaves the R⁴¹-S⁴² peptide bond exposing a new N-terminal ligand sequence (Fig. 1.2). Thrombin also has a second site of interaction termed exosite I, which is targeted by the leech anticoagulant peptide hirudin (31). PAR1 contains an N-terminal acidic region distal to the cleavage site termed the “hirudin”-like domain, based on sequence homology to the leech anticoagulant hirudin (Fig 1.2). This domain interacts with exosite I of thrombin and significantly enhances thrombin's affinity for the receptor. Amongst PARs, only PAR1 and PAR3 contain the hirudin-like domain. PAR4 is also cleaved by thrombin, but it lacks the hirudin-like domain thus making it a low affinity receptor for thrombin.

PAR1 is expressed in a variety of cell types including platelets, endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, and astrocytes. In endothelial cells, PAR1 is critical for barrier regulation, expression of adhesion molecules, and release of growth factors and cytokines (10) (Fig 1.1). In smooth muscle cells and fibroblasts, PAR1 is important for cell proliferation and migration, which could be important in proper wound healing (32,33). PAR1 is also expressed in T-lymphocytes, where activation of PAR1 induces cytokine production (34). PAR1 activation in sensory neurons causes release of substance P and edema (35). PAR1 is also expressed in astrocytes and glioblastoma cell lines (36). PAR1 activation in glial cells can trigger astrogliosis in the event of brain injury (37). PAR1 is also highly expressed in aggressive melanoma, colon cancer, prostate cancer, and invasive breast cancers (11). Knockdown of PAR1 in breast carcinoma has been shown to decrease invasiveness (38). However, the mechanisms of PAR1 mediated tumor progression can vary and have been attributed to defects in PAR1 trafficking (38), transactivation of other receptors such as EGFR/ErbB2 (11,39) or proteases available within the tumor microenvironment that can activate PAR1 (40).

In addition to its broad expression, PAR1 is also capable of being cleaved by multiple proteases to elicit cellular responses. PAR1 is cleaved by factor Xa (41), plasmin (42), and kallikreins (43). Matrix metalloprotease 1 (MMP-1) has been shown to be activated on platelets in response to collagen exposure, and cleaves PAR1 at a site that is distinct from that of thrombin (44) (Fig 1.4). Interestingly, plasmin can cleave PAR1 at the thrombin cleavage site arginine (R)⁴¹ at high concentrations. Alternatively, plasmin can also cleave at sites R⁷⁰, lysine (K)⁷⁶, or K⁸² to disarm the

receptor and desensitize PAR1 (42). PAR1 can also be cleaved by the anticoagulant protease activated protein C (APC). APC is generated on the endothelial cell surface when endogenous protein C (PC) bound to the endothelial protein C receptor (EPCR) is cleaved by the thrombin-thrombomodulin complex (45). APC degrades upstream coagulant factors Va and VIIa to diminish thrombin generation. However, when APC is bound to endothelial protein C receptor (EPCR), it directly activates PAR1 and elicits anti-inflammatory and endothelial barrier-protective responses, opposite of what is observed with thrombin-activation of PAR1 (45-47). APC has been shown to cleave at the R⁴⁶ rather than the R⁴¹ site utilized by thrombin (48). Our lab has shown that localization of PAR1 to caveolae is required for APC, but not thrombin activation of PAR1 signaling and endothelial barrier protection (46,47,49). Although PAR1 exhibits protease dependent biased agonism, it is not known how PAR1 couples to multiple G-protein subtypes in response to thrombin stimulation.

1.1.4 PAR1 signaling and regulation

The heterotrimeric G proteins are comprised of α - and $\beta\gamma$ - subunits, which are each encoded by distinct genes and are grouped into four different families based on sequence homology and include the $G_{\alpha_q/11}$, G_{α_s} , $G_{\alpha_i/o}$ and $G_{\alpha_{12/13}}$ subtypes. Once activated, GPCRs act as guanine nucleotide exchange factors and promote GTP exchange for GDP on the α -subunit, which causes dissociation from the $\beta\gamma$ subunit and is the rate-limiting step for activation of subsequent signaling responses. PAR1 activates multiple heterotrimeric G-protein subtypes including $G_{\alpha_{12/13}}$, G_{α_q} , $G_{\alpha_i/o}$, and $G\beta\gamma$, allowing for diverse cellular responses (10,11) (Fig. 1.2). $G_{\alpha_{12/13}}$ can bind to Rho-guanine nucleotide exchange factors (GEFs) to activate RhoA (50), which mediates

cytoskeletal responses that are involved in platelet shape changes (51) as well as cell migration and endothelial permeability (52). G_{α_q} activates phospholipase C- β (PLC- β) mediated phosphoinositide (PI) hydrolysis, calcium mobilization, and protein kinase C activation (53,54). G_{α_q} signaling mediates platelet aggregation and endothelial cell transcriptional responses through the activation of calcium-regulated kinases and phosphatases, RasGEFs, and mitogen-activated protein (MAP) kinase cascades (55,56). PAR1 activation of $G_{\alpha_{i/o}}$ inhibits adenylyl cyclase (54). $G\beta\gamma$ subunits have been shown to activate phosphoinositide-3-kinase, which generates phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), important attachment sites at the inner leaflet of the plasma membrane for a host of signaling effectors; $G\beta\gamma$ can also activate other lipid-modifying enzymes, protein kinases and channels (57). It is not clear how PAR1 selectively couples to specific G-protein subtypes within the same cell, and there is limited knowledge about the regulation of PAR1 signal termination. Post-translational modifications of PAR1 could provide critical regulatory functions for these processes, and thus are examined in this dissertation.

PAR1 signal termination mechanisms are critical for the fidelity of signaling. Similar to most classic GPCRs, activated PAR1 is rapidly desensitized by phosphorylation and β -arrestin binding (58,59). PAR1 phosphorylation is mediated by G-protein receptor kinases (GRKs), as overexpression of GRKs 3 and 5 enhance PAR1 phosphorylation (60,61). β -arrestin-1 is an important mediator of PAR1 desensitization, as PAR1 activation shows increased PI hydrolysis in β -arrestin-1, but not β -arrestin-2, knockout mouse embryonic fibroblasts (MEFs) (58). β -arrestin recruitment to PAR1 is also independent of phosphorylation. A phosphorylation-

deficient mutant of PAR1 displays enhanced PI hydrolysis in response to thrombin, a phenotype that is rescued with overexpression of β -arrestin-1 (59). In addition to desensitization, trafficking of PAR1 is critical for regulation of thrombin signaling (11,38). However, unlike most GPCRs, PAR1 displays constitutive and agonist-induced internalization that occur independent of β -arrestins (58).

1.1.5 PAR1 trafficking

Trafficking of PAR1 is crucial for regulating signaling responses. PAR1 displays two modes of trafficking that are critical for regulating the amount of receptor expressed at the cell surface (Fig.1.3). Unactivated PAR1 is constitutively internalized and recycled, which generates a pool of uncleaved PAR1 that replenishes the cell surface and is important for rapid resensitization after thrombin exposure (62). PAR1 constitutive internalization requires the clathrin adaptor protein complex-2 (AP-2) (63), which is comprised of α , β 2, μ 2 and σ adaptin subunits. AP-2 regulates internalization of transmembrane proteins via μ 2-adaptin subunit interaction with tyrosine-based YXX ϕ motifs, where Y is tyrosine, X denotes any amino acid and ϕ is a bulky hydrophobic residue (64,65). PAR1 contains two tyrosine-based motifs within its C-tail: one located proximal to the seventh transmembrane domain and the other at the distal end of the C-tail. The μ 2-subunit of AP-2 directly binds to a tyrosine-based motif localized within the PAR1 C-tail and promotes constitutive internalization (63) (Fig. 1.3). PAR1 contains multiple lysine residues in its C-tail which can be post-translationally modified with ubiquitin (Figure 1.4). Basal ubiquitination at PAR1 C-tail lysine residues negatively regulates constitutive internalization, as a ubiquitin deficient mutant of PAR1 is rapidly internalized in the

absence of agonist (66). The distal tyrosine-based motif localized within the PAR1 C-tail has two lysine residues that are likely modified with ubiquitin, and thus impede AP-2 interaction (66) (Fig. 1.3).

Internalization and lysosomal sorting of activated PAR1 is important for preventing persistent signaling. Activated PAR1 internalization is clathrin and dynamin dependent (67), but independent of β -arrestins (58). Receptor phosphorylation is critical for PAR1 internalization (59), but ubiquitination is not (66). AP-2 also mediates activated PAR1 internalization through a distinct mechanism that requires phosphorylation at the distal C-terminus and not the distal tyrosine based sorting motif (68). Basal ubiquitination of activated PAR1 is necessary for internalization mediated by epsin-1, a clathrin adaptor protein that binds to polyubiquitin via its ubiquitin-interacting motifs (68).

Following internalization, PAR1 is sorted to endosomes and then to the intraluminal vesicles of multivesicular bodies (MVBs) for degradation. PAR1 degradation requires the proximal tyrosine-based sorting motif (YSIL), which is located near the putative palmitoylation sites in the PAR1 C-tail. Mutagenesis of the proximal motif does not affect PAR1 internalization, but prevents PAR1 degradation (69). Adaptor protein complex-3 (AP-3) interacts with a C-tail proximal tyrosine motif YSIL at the sorting endosome to facilitate degradation of activated PAR1 (70). Sorting of activated PAR1 to MVBs occurs through an ESCRT-III-dependent pathway independent of ubiquitination (71). This trafficking is mediated by a YPX(3)L motif in the PAR1 2nd intracellular loop that interacts with ALIX, a charged MVB protein 4-

ESCRT-III interacting protein. Additionally, AP-3 mediated PAR1 sorting to MVBs is critical for ALIX binding to PAR1 (71).

Although many components that regulate PAR1 trafficking have already been defined, it is not known if palmitoylation is a post-translational modification that occurs on PAR1, and if this modification regulates some of PAR1 trafficking events. PAR1 is post-translationally modified with glycosylation, phosphorylation, and ubiquitination (Fig. 1.4). Glycosylation of the second extracellular loop was demonstrated to be important for G-protein signaling and early internalization (72). Phosphorylation is not only critical activated receptor desensitization and internalization (59), but it is also important for activated PAR1 ubiquitination to occur (68). Receptor ubiquitination is important for retention of unactivated PAR1 at the cell surface, but not for activated internalization or degradation (66). Ubiquitination specifies engagement with the adaptor protein epsin-1, which contributes to efficient receptor internalization following activation (68) (Fig 1.3). It is not known if PAR1 palmitoylation has an effect on any of these post-translational modifications, and thus is one of the main topics of this dissertation.

1.1.6 Mechanism of protein palmitoylation

S-Palmitoylation involves covalent attachment of a 16-carbon fatty acid chain to a cysteine residue through a labile thioester linkage. N-palmitoylation can also occur when a 16-carbon fatty acid chain is attached to a glycine or cysteine residue through an amide linkage. Palmitoylation serves to increase the hydrophobicity of its target proteins and facilitates membrane association. This modification is a dynamic process, and the palmitoyl group is added enzymatically through palmitoyl acyl

transferases (PATs) and removed by palmitoyl protein thioesterases (PPTs). Other lipid modifications such as myristoylation, and prenylation are irreversible, thus making palmitoylation unique in its potential to transiently modulate protein signaling. Despite this structured enzymatic process, a clear consensus sequence for the palmitoylation modification is currently unavailable.

The first PATs were identified in yeast (73,74) and were found to be transmembrane proteins that have a conserved aspartate-histidine-histidine-cysteine (DHHC) motif (75) (Fig. 1.5). This family of enzymes appears to regulate the majority of protein palmitoylation, as knockout of 6 out of 7 yeast PATs eliminated protein palmitoylation (74). Recently, 23 DHHC proteins have been identified in the human genome and shown to localize to distinct intracellular compartments including the Golgi, the Endoplasmic Reticulum (ER), the plasma membrane, and endosomes (74,76-78). Some studies have already confirmed that some of these DHHC proteins have a physiological role in protein palmitoylation (79,80). The family of DHHC containing PATs is further diversified through alternative splicing of PAT mRNA (81). It has been suggested that alternative splicing can affect the presence of post-translational modifications, leading to changes in subcellular localization, substrate recognition, and protein interactions (81). It has been demonstrated that mutagenesis of the DHHC motif in the PATs is sufficient to prevent auto-palmitoylation and palmitoylation of substrates (73). Although some studies have also shown the capacity of some proteins to bypass enzymatic addition of palmitate *in vitro*, this autoacylation could be influenced through a myristoylation modification or association with other proteins (82).

An enzymatic group of palmitoyl-protein thioesterases (PPTs) catalyze the removal of palmitate from proteins and are also modified with palmitoylation (83). Only a few PPTs have been identified thus far. Acyl-protein thioesterase-1 (APT1) has been shown to depalmitoylate $G\alpha_i$ and Ras *in vitro* (84). APT1 was also shown to act on $G\alpha_{13}$ (85). Furthermore, Palmostatin-B, an inhibitor that targets APT1, has shown to be effective in disrupting the Ras palmitoylation cycle (9). APT2 is a homologue of APT1 that is expressed in mammals. Interestingly, APT1 has been shown to catalyze its own deplamitoylation as well as APT2, ultimately affecting membrane localization (83). Palmitoylation removal usually occurs as an essential step in the degradation process (86). The thioesterase protein-palmitoylthioesterase-1 (PPT1) is localized to the lysosome and functions in fatty acid hydrolysis during protein degradation (87). This enzyme was shown to be defective in the disorder infantile neuronal ceroid lipofuscinosis (88). The PPT1 homologue PPT2 was identified to also have lysosomal localization, but to act on substrates that are distinct from PPT1 (89). Although only four PPTs have been identified, it is likely that many more exist given the diversity of the palmitoylproteome. It is possible that cross- and auto-regulation by thioesterases is one mechanism by which these enzymes increase their diversity of substrates. However, depalmitoylation activity has been shown to be widely distributed within the cell (77), further suggesting that there are other PPTs that have yet to be identified.

1.1.7 Functional implications of protein palmitoylation

For some proteins, such as the Ras proto-oncogenes H-Ras and N-Ras, cycles of palmitoylation and depalmitoylation are important for stable association with

the plasma membrane and the Golgi (90). In a similar manner, it has been demonstrated that palmitoylation cycles of $G\alpha_i$ and Fyn mediate association with the Golgi and the plasma membrane (8,77). In these cases, the proteins already contain irreversible lipid modifications, and the addition of palmitoylation serves to aid in the hydrophobicity and association of the protein with the membrane. Some neuronal proteins such as GAP43 and PSD 95 solely utilize multiple palmitoylated cysteines for their membrane association, and also undergo cycles of palmitoylation and depalmitoylation (91).

As for transmembrane proteins, palmitoylation could serve a purpose other than plasma membrane association. Palmitoylation could regulate the conformation of a protein, and thus promote protein-protein interactions, or alternatively shield binding domains from being recognized. Palmitoylation could affect the accessibility of the endocytic machinery and adaptor proteins to a particular transmembrane protein. For the transferrin receptor (TfR), it was demonstrated that palmitoylation was important for plasma membrane retention, and a palmitoylation-deficient mutant was rapidly internalized (92). In a similar manner, a palmitoylation deficient anthrax toxin receptor also underwent enhanced endocytosis, a phenotype that was a result of increased ubiquitination by the ubiquitin ligase Cbl (93). Palmitoylation has been shown to be involved in the recycling of some proteins, such as the mucin-like transmembrane protein (MUC1) (94). Additionally, palmitoylation has been shown to play a protective role by preventing some proteins such as the mannose-6-phosphate receptor (MPR) and TEM8 from premature lysosomal degradation (93,95). As for

MPR, it has been determined that palmitoylation is important for association with the retromer complex for proper trafficking from the endosome to the TGN (96).

It has been proposed that the addition of palmitate will increase the affinity of some transmembrane proteins to associate with cholesterol-enriched raft like domains. Indeed it has been shown that palmitoylation is critical for some proteins, such as the linker of activated T cells (LAT) and the integrin $\alpha 6\beta 4$ (97,98), to associate with lipid rafts. However, not all raft-associated proteins require palmitoylation for their association. It has been demonstrated that caveolin-1 does not require palmitoylation to associate with caveolin microdomains (99). In some cases, palmitoylation is a negative regulator of association with lipid rafts. The transferrin receptor is not localized to lipid rafts despite being basally palmitoylated (92). In a similar manner, the anthrax toxin receptor TEM8 is also not associated with lipid rafts basally, but a palmitoylation-deficient mutant is (93).

1.1.8 Palmitoylation and GPCRs

A number of GPCRs have been reported to be palmitoylated and this modification has been implicated in proper processing from immature to mature receptor in the biosynthetic pathway, as well as influencing G-protein coupling and internalization (100). The first example of GPCR palmitoylation was demonstrated with the rhodopsin receptor (101). The cysteine residues located 14 residues downstream of the 7th transmembrane domain were identified as the palmitoylation sites (102). Furthermore, the rhodopsin crystal structure demonstrated that the palmitoylation sites stabilized a 4th intracellular loop that is α -helical in structure (103). Many of the lipidation patterns occur on the carboxyl terminus of GPCRs, especially

cytoplasmic juxtamembrane cysteine residues (104). A sequence alignment analysis of select GPCR C-tails indicates that many of these receptors possess cysteine residues approximately 15 residues from the 7th transmembrane domain (Fig 1.6). Although many GPCRs are palmitoylated, the function of palmitoylation varies depending on the GPCR being examined.

Palmitoylation has been shown to modulate signaling for some GPCRs. Palmitoylation-deficient rhodopsin displays a less stable C-terminus, a slower activation of the G-protein transducin *in vitro* (105), and more rapid phosphorylation and desensitization following light stimulation *in vivo* (106). Palmitoylation-deficient β_2 -adrenergic receptor resulted in reduced stimulation of adenylyl cyclase (107), suggesting reduced coupling to G_{α_s} . Reduced coupling to G_{α_i} and G_{α_q} were observed with a palmitoylation-deficient Endothelin B receptor (108). In a similar manner, GPCR palmitoylation was important for G_{α_i} coupling for the 5-hydroxytryptamine-1A (5-HT_{1A}) receptor (109). Although, the chemokine CCR5 receptor retained coupling to G_{α_i} , the duration of the response was reduced with the palmitoylation-deficient mutant. However, this is not the case for the α_{2A} -adrenergic receptor, as a palmitoylation mutant had no effect on G-protein coupling, or the ability to bind agonists and antagonists (110), even though it was demonstrated that palmitoylation was dynamically regulated for this receptor (111). Other examples of palmitoylation-deficient GPCRs exhibiting similar G-protein coupling to their wildtype counterparts include the vasopressin receptor V_{1A} (112), the dopamine D₁ (113), the Bradykinin B₂ (114), and luteinizing hormone LH receptors (115). For some of these GPCRs, the palmitoylation was required for trafficking, rather than signaling, of the receptors.

Palmitoylation has been shown to be important for G-protein coupling specificity for some GPCRs. For the Endothelin receptor A (ET_A), palmitoylation is important for coupling to G α_q , since palmitoylation-defective mutants display impaired agonist induced phospholipase C- β activation, but are unaffected in agonist induced adenylyl cyclase stimulation (116). Likewise, constitutive G α_s activity of the 5-HT_{7a} receptor was increased in the absence of the proximal palmitoylation site, whereas constitutive G α_{12} activity was unaffected (117). In a similar manner, palmitoylation of the V₂ receptor is important for signaling to the MAPK pathway, but not for the adenylate cyclase signaling, suggesting that palmitoylation is important for interaction with β -arrestin, but not G α_s (118). Interestingly, a palmitoylation deficient Endothelin B (ET_B) receptor displayed reduced signaling to G α_i and G α_q . However, G α_q signaling was retained in an ET_B mutant containing a truncation after the palmitoylation site, suggesting that the conformation of the C-tail induced by the palmitoylation site is important for G α_i coupling (108).

There are some examples where palmitoylation was important for GPCR trafficking. A palmitoylation defective CCR5 receptor, although capable of coupling to G α_i , has decreased cell surface expression that appears to be due to a decreased half-life and increased sorting to the lysosome (119). In a similar manner, the adenosine A₁ receptor was more susceptible to degradation in the absence of palmitoylation, despite having comparable signaling and internalization compared to wildtype A₁ receptors (120). The human luteinizing hormone (LH) receptor also exhibited rapid internalization and decreased recycling in the absence of palmitoylation (115,121), due to increased phosphorylation and β -arrestin recruitment

(115). In a similar manner, a palmitoylation-deficient 5-HT_{4a} receptor mutant showed enhanced phosphorylation and β -arrestin recruitment that resulted in more efficient endocytosis (122). Alternatively, the vasopressin V₂ receptor displayed a lack of β -arrestin recruitment in the absence of palmitoylation, which resulted in reduced endocytosis as well as decreased MAPK activation (118).

There have been a few examples where palmitoylation can function to stabilize GPCR association to lipid rafts. Palmitoylation of the 5-hydroxytryptamine-1A receptor is important for retention in lipid rafts (123). Palmitoylation was also important for CCR5 targeting to lipid rafts (124). Palmitoylation of the D₁ receptor appears to specify a slower internalization through caveolae rather than clathrin-coated pits, as a palmitoylation deficient mutant displayed increased clathrin-mediated endocytosis (83). Although many GPCRs localize to lipid rafts (125), it is not clear if this localization is dependent upon palmitoylation. The localization of the μ -opiate receptor (MOR) to lipid rafts requires stable interaction with G α_i and regulates agonist selective signaling (126), but itself does not appear to be palmitoylated, indicating that palmitoylation is not absolutely required for lipid raft localization of some GPCRs.

An intriguing function of GPCR palmitoylation is the formation and stability of a 4th intracellular loop that is α -helical in structure. For the rhodopsin receptor, it was demonstrated that this 8th helix terminated immediately following palmitoylated cysteine residues 322 and 323 (103). The crystal structure for the β 2-adrenergic receptor also revealed an 8th helix that was anchored by the palmitoylated cysteine residue 341 (127). PAR1 modeling studies that are based on the x-ray structure of

rhodopsin indicate that palmitoylated residues C³⁸⁷ and C³⁸⁸ serve an important function in allowing the 8th helix to interact with the NPXXY motif of the 7th transmembrane, an important component for receptor G-protein coupling (128). However, the crystal structure recently published for PAR1 did not resolve an 8th helix due to weak electron density on residues following cysteine 378 (129). It is uncertain if the lack of helix 8 is indicative of PAR1 in its native state, or whether it is a factor of the crystallization conditions utilized in the study.

Although it is speculated that palmitoylation is important for stability of the 8th helix, it is possible that it is not a requirement for the 8th helix to form. There are examples in the literature where an 8th helix is resolved in GPCR crystal structures that lack palmitoylation sites. For example, the 8th helix was seen in the A_{2A} adenosine receptor crystal structure, but is stabilized with helix 1 rather than palmitoylated cysteines, as this receptor lacks a palmitoylation site similar to that seen with other GPCRs (130). The 8th helix was also ordered in the β 1-adrenergic receptor despite the receptor having a mutation at the site of palmitoylation to aid in crystallization (131). Furthermore, the crystal structure for the histamine H1 receptor also revealed an 8th helix, but also lacks a palmitoylation site at the end of the helix (132). Despite this discrepancy, there have been many cases where point mutations within the 8th helix have resulted in signaling defects for GPCRs (133-137). The 8th helix has opened up a new potential to develop novel allosteric modulators to alter G-protein signaling. In recent work, Dowal *et al.* identified an inhibitory molecule termed J5F that appears to act at the intracytosolic face of the receptor that requires the PAR1 putative 8th helix to inhibit G α_q signaling stimulated by SFLLRN in platelets (138). Remarkably, however, the same compound J5F failed to affect SFLLRN-

mediated signaling to $G\alpha_{12}$ when examined in MDCK epithelial cells. This compound was capable of acting on other GPCRs that were predicted to form an 8th helix.

Although PAR1 has not been shown to be palmitoylated, sequence alignments indicate that it has two juxtamembrane cysteine residues that are highly conserved among rhodopsin-like GPCRs and are likely to be targets of palmitoylation. Interestingly mouse PAR4, the main thrombin receptor on mouse platelets, contains a cysteine residue that could serve as a palmitoylation site based on its location in the C-tail (Fig. 1.6). There are no cysteine residues present in human PAR3 or PAR4 C-tails making it unlikely that these receptors are modified by palmitoylation. PAR2 contains a cysteine residue that has been demonstrated to be modified with palmitoylation (139). Mutagenesis studies have been done on the putative PAR1 palmitoylation sites, but palmitoylation was not directly assessed. Mutagenesis of the C³⁸⁷ and C³⁸⁸ to serine residues did not appear to affect PLC- β activation nor activation via thrombin cleavage, but did reduce the receptor's ability to respond to the synthetic peptide SFLLRN ligand (128). Whether such changes in PAR1 are actually due to palmitoylation was not determined in this study and is further complicated by the finding that serine can be used as an alternative palmitoylation site as shown previously for the human transferrin receptor (140).

In this dissertation, the function of palmitoylation was established for PAR1 signaling and trafficking. I have demonstrated that PAR1 is modified by palmitoylation (141) and that this modification is critical for receptor phosphorylation and ubiquitination. Additionally, palmitoylation is critical for regulating adaptor protein recognition of tyrosine-based sorting motifs in the PAR1 C-tail (141) and proper

trafficking of the receptor through the endocytic system. Finally, I demonstrate that palmitoylation of PAR1 is not required for G_{α_q} -mediated phosphoinositide hydrolysis or ERK1/2 signaling pathways. However, $G_{\alpha_{12/13}}$ -mediated RhoA activation, G_{α_i} coupling, and thrombin-stimulated p38 MAPK signaling pathways are impaired in the absence of PAR1 palmitoylation. Furthermore, a palmitoylation-deficient PAR1 mutant fails to display thrombin-induced barrier permeability in endothelial cells. Taken together, the data presented in this dissertation suggest that palmitoylation serves to stabilize the C-tail of PAR1 so that it interacts properly with adaptor proteins and signaling molecules, ultimately affecting PAR1 signaling and trafficking.

1.2 Acknowledgements

Chapter 1, in part, is published as a review article entitled “Allosteric Modulation of Protease-activated Receptor Signaling” in *Mini-Reviews in Medicinal Chemistry* (142). The dissertation author is the primary author of the manuscript.

1.3 Figures

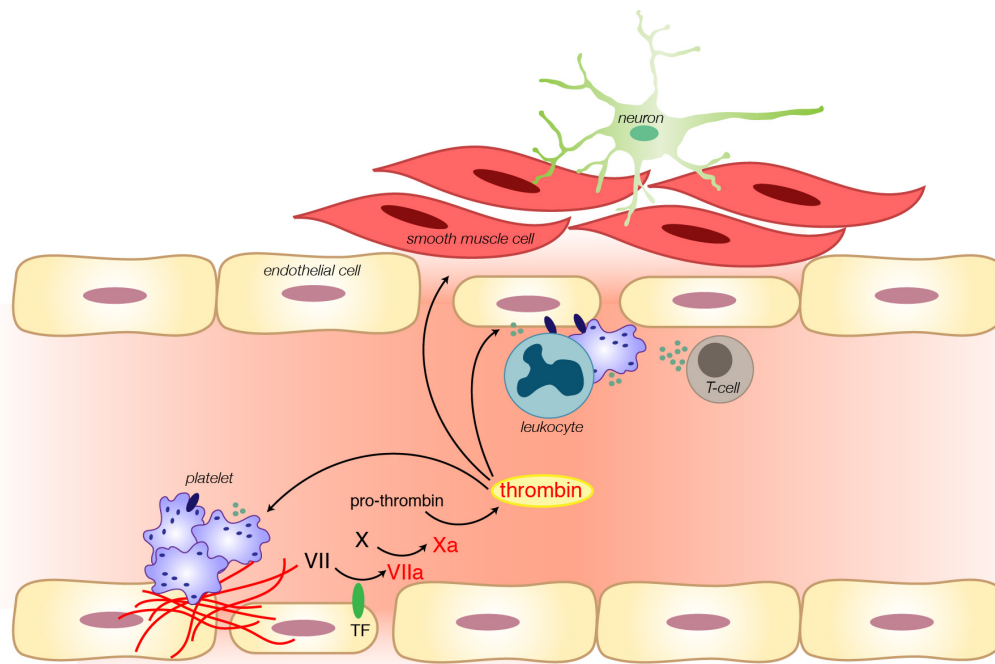


Figure 1.1: Thrombin mediated cellular responses in the vasculature. The coagulation cascade is initiated when tissue factor (TF) comes into contact with zymogens circulating in the blood. Thrombin is the main effector protease of the coagulation cascade, and converts fibrinogen to an insoluble fibrin clot, upon which platelets aggregate. Thrombin activates various cell types in the vasculature. In platelets, thrombin causes aggregation, granule release, and presentation of P-selectin to the cell surface. In endothelial cells, thrombin induces barrier permeability, cytokine release, and upregulation of P-selectin, which functions in recruitment of platelets and leukocytes to the site of injury. Thrombin can also activate T-cells, resulting in cytokine release. Thrombin's actions on smooth muscle cells and fibroblasts cause cell proliferation and motility, an important function in wound healing. In neuronal cells, thrombin can induce release of substance P, causing pain perception.

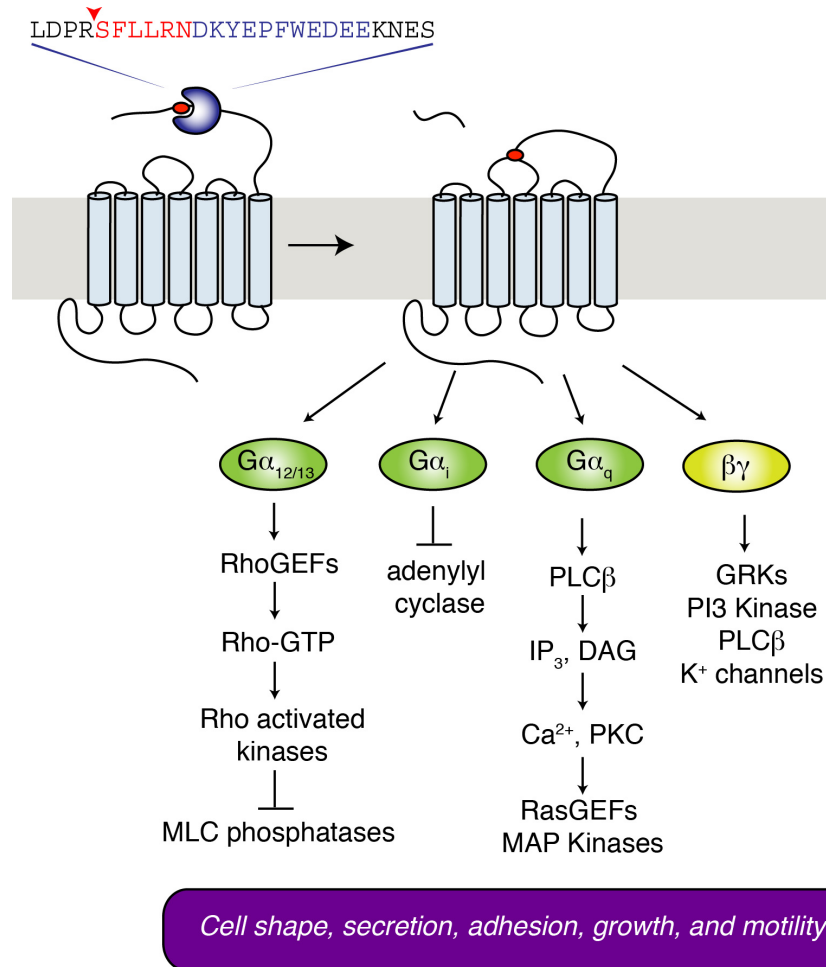


Figure 1.2: PAR1 G-protein signaling pathways. Thrombin interacts with PAR1 at a hirudin-like domain (blue) and cleaves the N-terminus to reveal a new sequence (red) that acts as a tethered ligand. The new N-terminus binds to the second extracellular loop to initiate G-protein signaling. PAR1 activates multiple heterotrimeric G-protein subtypes including $G_{\alpha_{12/13}}$, G_{α_q} , $G_{\alpha_i/o}$, and $G_{\beta\gamma}$, allowing for diverse cellular responses. $G_{\alpha_{12/13}}$ can bind to Rho-guanine nucleotide exchange factors (GEFs) to activate RhoA, which mediates cytoskeletal responses that are involved in platelet shape changes as well as cell migration and endothelial permeability. PAR1 activation of $G_{\alpha_i/o}$ inhibits adenylyl cyclase. G_{α_q} activates phospholipase C (PLC) mediated phosphoinositide (PI) hydrolysis, calcium mobilization, and protein kinase C activation. G_{α_q} signaling mediates platelet aggregation and endothelial cell transcriptional responses through the activation of calcium-regulated kinases and phosphatases, RasGEFs, and mitogen-activated protein (MAP) kinase cascades. $G_{\beta\gamma}$ subunits activate phosphoinositide-3-kinase which generates PIP3, phosphatidylinositol-(3,4,5)-trisphosphate, and also activate other lipid-modifying enzymes, protein kinases and channels.

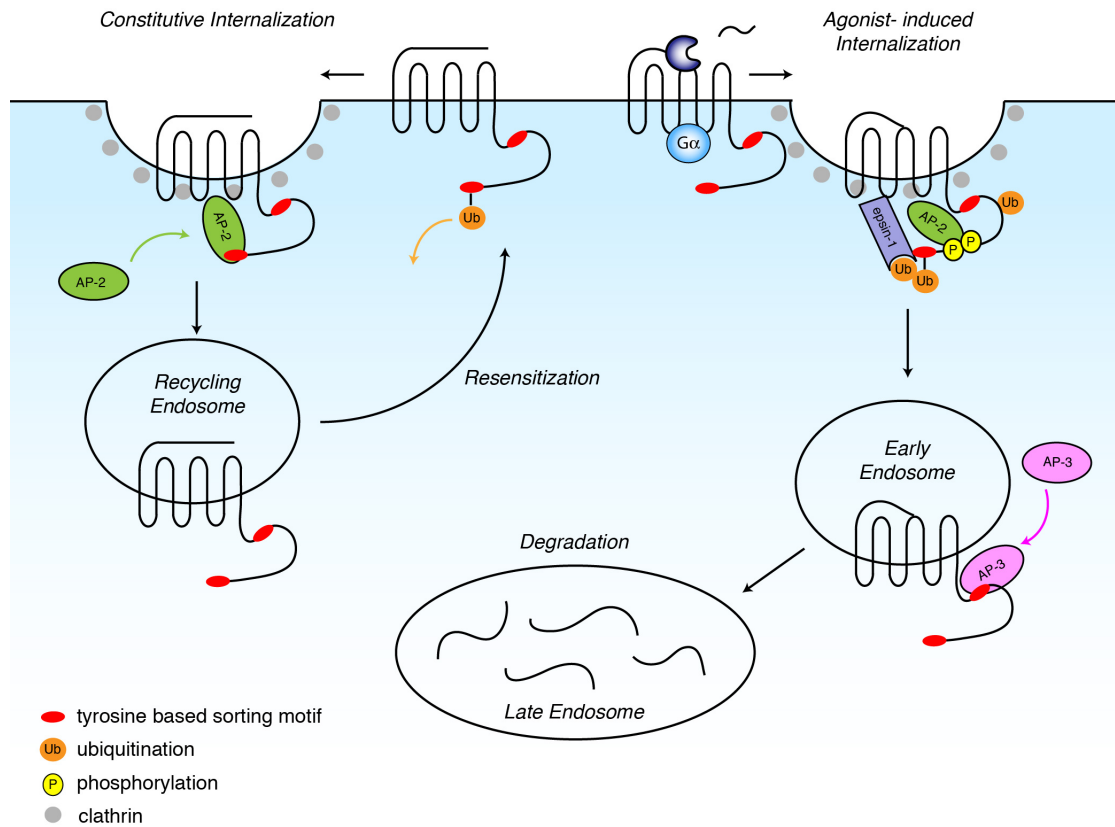


Figure 1.3: PAR1 trafficking pathways. PAR1 displays two modes of internalization. Constitutive internalization is mediated by the clathrin adaptor protein-2 (AP-2) binding to a distal tyrosine-based sorting motif. Constitutive internalization serves to populate an intracellular pool of naïve receptors that are trafficked to the cell surface following thrombin stimulation for rapid resensitization. Constitutive internalization is negatively regulated by ubiquitination at the tyrosine-based sorting motif, which sterically hinders AP-2 binding. Following thrombin cleavage, and G-protein signaling, PAR1 is rapidly phosphorylated and internalized by AP-2 and epsin1 adaptor proteins. Epsin1-dependent PAR1 internalization is dependent on ubiquitination of the receptor. AP-2-dependent internalization is dependent on distal C-tail phosphorylation sites rather than the tyrosine-based sorting motif utilized in the constitutive internalization pathway. Following internalization, PAR1 is sorted to early endosomes. The adaptor protein-3 (AP-3) interacts with the proximal tyrosine-based sorting motif to sort cleaved receptors to late endosomes for degradation.

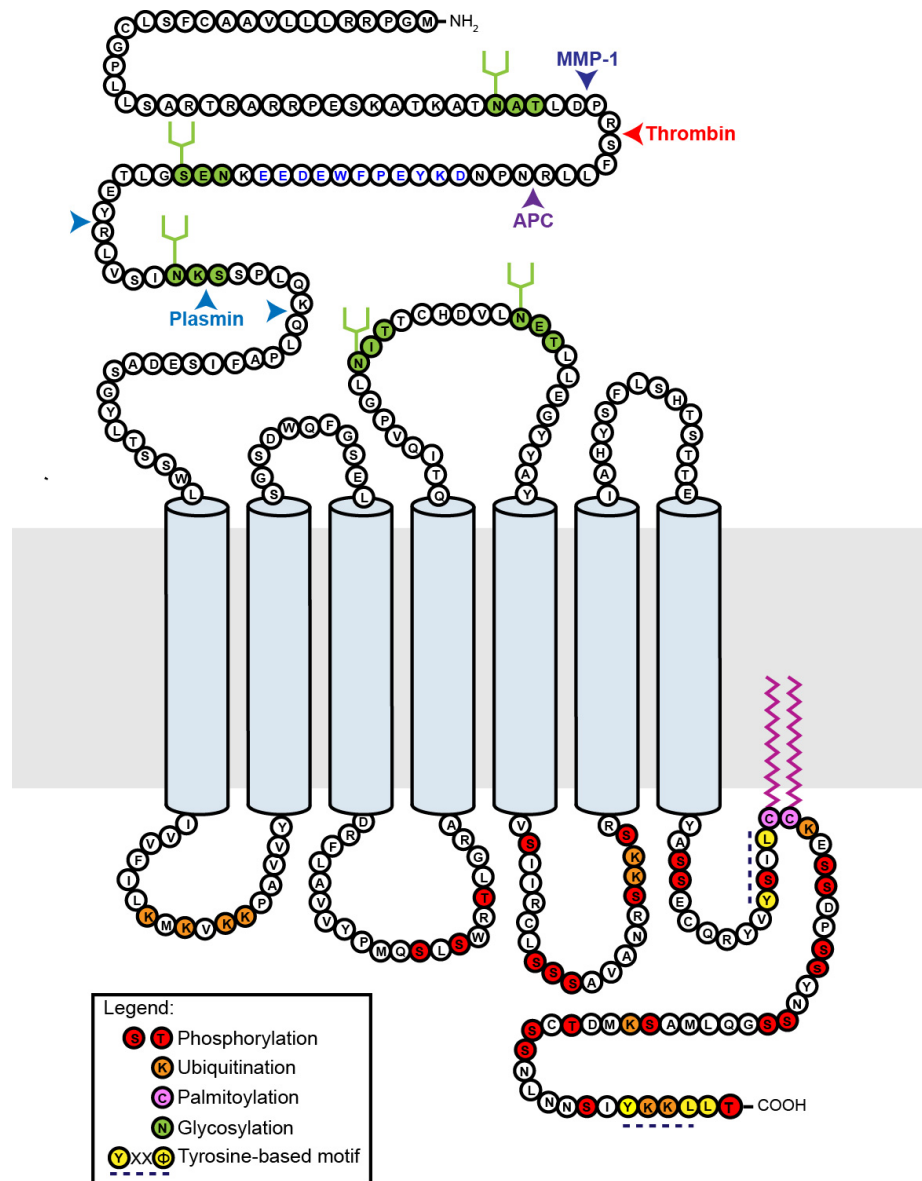


Figure 1.4: Modifications and motifs important for PAR1 signal regulation.

PAR1 is post-translationally modified with phosphorylation (red) and ubiquitination (orange) primarily in the intracellular loops and C-terminal tail. PAR1 is also modified with glycosylation (green) on the extracellular surfaces of the receptor. The putative palmitoylation sites for PAR1 are highlighted in pink. PAR1 also contains two linear sorting sequences in its C-tail that are important for trafficking, and are highlighted in yellow. Thrombin interacts with PAR1 through a hirudin binding domain, which is noted with blue residues. PAR1 is cleaved by multiple proteases, and the cleavage sites are highlighted for thrombin (red), matrix metalloprotease-1 (MMP-1) (dark blue), activated protein C (APC) (purple), and plasmin (light blue).

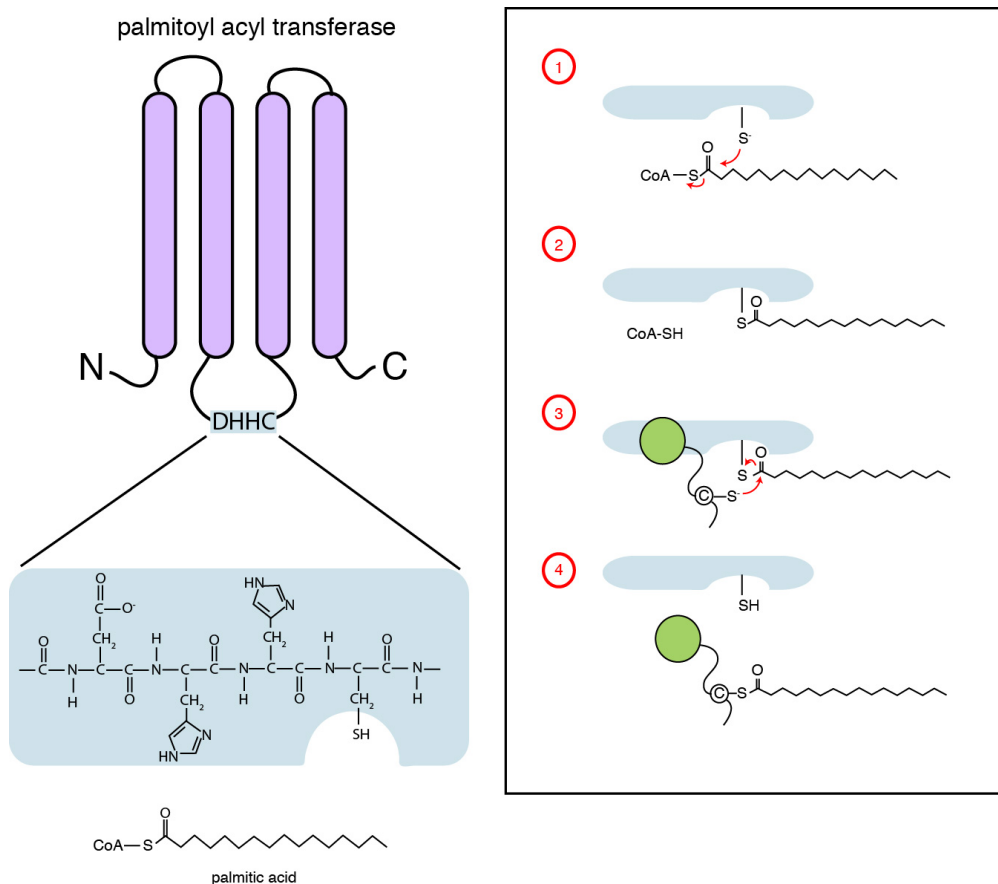


Figure 1.5: A model for the mechanism of protein palmitoylation. Palmitate is added to cysteine residues by a family of transmembrane enzymes called palmitoyl acyl transferases (PATs). PATs contain a conserved aspartate (D), histidine (H), histidine (H), cysteine (C) (DHHHC) cysteine-rich domain. The DHHHC motif has been demonstrated to be critical for enzymatic activity and for the formation of a thioester intermediate. The DHHHC motif is highlighted in blue and represented as the blue shape in the boxed diagram, which demonstrates the mechanism of protein palmitoylation.

GPCR	C-terminal tail
PAR1	..DPLIIYYASSECQRYVYSIL C KESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKLLT
PAR2	..DFVYYFVSHDFRDHAKNALL C RSVRTVKQMQLSTSKKHSRKSSSYSSSTTVKTSY
PAR3	..DPFLYFLMSKTRNHSTAYLTK
PAR4	..DPFIYYVSAEFRDKVRAGLFQSPGDTVASKASAGGSRGMGTHSSLLQ
PAR4 (mouse)	..DPFIYYVSHFEKVRAML C RQPEASSSSQASREAGSRGTAICSSLT
Adenosine A ₁	..NPIVYAFRIQKFRVTFKLIWNDHFF C QPAPIDEDLPEERPD
Adenosine A _{2A}	..NPFIYAYRIREFRQTFRKIIRSHVLQQQEPFKAAGTSARVLAHGSDEQVSLRLNGHPGPGVWANGSAPHERRPNGYALGLVS..
Adenosine A ₃ (rat)	..NPIVYACKIKKFKETIFYVILRA C RL C QTSDSLSDSLNEQTTE
α _{2A} -adrenergic:	..NPVIYTI FNHDFRRAFKKIL C RGDRKRIV
β ₁ -adrenergic	..NPFIYCRSPDFRKAQRL C ARRAARRRHATHGDRPRAS C CLARPGPPSPGAASDDDDDDVVGATPPARLLEPWAGCNGGAA..
β ₂ -adrenergic	..NPFIYCRSPDFRIAFQEL C LRSSSLKAYGNGYSSNGTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPDNDISQGRN..
Bradykinin B	..NPLVYVIVGKRFRKKSWEVYQGV C QKGG C RSEPIQMENSMTGLRTSISVERQIHKLDWA
Chemokine CCR:	..NPFIYAFVGEKFRNYLLVFFQKHIAKRF C CCSIFQQEAPERASSVYTRSTGEQEISVGL
δ opioid	..NPVLYAFLDENFKRCFRQL C RKPCGRDPSSFSRAREATARERTACTPSDGPGGGAA
Dopamine D ₁	..NPFIYAFNADFRKAFSTLL C CYRLCPATNNAIETVSINNNGAAMFSSHHEPRGSGISKECNLVYLIPHAVGSSEDLKKEEAAGIA..
Endothelin A	..NPFIYAFVSKFKKNCQ C CCCCYQSKSLMTSVPMNGTSIQWKNHDQNNHNTDRSSHKDSMN
Endothelin B	..NPFIYALVSKRFRKNCFKSCL C WCQSFEEKQSLEEKQSCLFKANDHGYDNFRSSNKYS
Histamine H ₂ (canine)	..NPFIYATLNRDFTAYQQLFRCPASHNAQETSLRSNSSQLARNQSRPMRQEEKPLKLQVWSGTEVTAAPRGATDR
Luteinizing Hormone	..NPFIYAFITKTTFQRDFLLSKFG C CKRRAELYRRKDFSAYTSNCKNGFTGSNKPSQSTLKLSTLHCQGTALLDKTRYTEC
Lysophosphatidic acid LPA ₂	..NAAVYSCRDAEMRRTFRRL C ACLRQSTRESVHYTSSAQGGASTRIMLPENHPLMDSTL
μ opioid (rat)	..NPVLYAFLDENFKR C REF C IPTSSTIEQQNSTVRQNTREHPSTANTVDRTNHQLNLEAETAPLP
Muscarinic M ₂	..NPACYALCNATFKTKFKHLL C CHYKNIGATR
Muscarinic M ₃ (rat)	..NPVCYALCNKTFRTTTFKTL C CCDKRRKRKQYQQRQSVIFHKRVPEQAL
Neuropeptide Y ₁ (rat)	..NPFIYGFNLKNFQRDLQFFNE C DFRSRDDDIETIAMSTMHTDVSKTSLKQASPVAFKKISMNDNEKI
Oxytocin (rat)	..NPWIYMLFTGHLFHELVQRFF C CSARYLKGSRPGETSVSKKNSSTFVLSRRSSSQRCSCQPSA
Prostacyclin	..NPILDPWFVILFRKAVFQRLKLW C CLGPAHGDSQTPLSQLASGRDPRAPSAFVGKEGSCVPLSAWGEQVEPLPTQSS..
Rhodopsin	..NPVIYIMNKFQFRNCLMT C CGKNPLGDDASATVSKTETSQVAPA
Sphingosine S1P ₁	..NPFIYTLTKEMRRAFIRIMS C CKCPSGDSAGKFKRPIIAGMEFSRSKSDNSSHPPQKDEGNPETIMSSGNVNSS
Sphingosine S1P ₂	..NPVIYTWRSRDLRREVLRLP C WRPGVGVQGRRGTPGHLLPLRSSSLERGMHMTSPTFLEGNTVV
Sphingosine S1P ₃	..NPVIYTLASKEMRRAFFRLV C NCLVRGRGASPIQALDPSRSKSSSSNNSHSPKVKEDLPHTAPSSCIMDKNAALQNGIFCN
Sphingosine S1P ₄	..NPFIYSFRSREVCRAVLSFL C CGCLRLGRMGPGDCLARAVEAHSGASTDSSLRPRDSFRGSRSLSPFRMREPLSSISSVRSI
Sphingosine S1P ₅	..NPFIYTLNRDLRHALLRLV C GRHSCGRDPGSGQSSASAAEASGGLRCLPPLDGSFSGSERSSPQRDGLDTSSTGSGSPGAPT..
Somatostatin SSTR5 (rat)	..NPLLYGFLSDNFRQSFVKVLCRRGYGMEADADIEPRPKSGRPQATLPTRSCANGLMQTSRI
Serotonin 5-HT _{1A} (mouse)	..NPVIYAYFNKDFQNAFKKII C CKFCR
Serotonin 5-HT _{4A} (mouse)	..NPFIYAFNLKSFERRAFIL C DDERYKRPPILGQTV C STTTINGSTHVLRYTVLHSGHHQLEKPLIHNDPES..
Serotonin 5-HT _{7A} (mouse)	..NPFIYAFNRDLRTTYRSL C QYRNINRKLASAGMHEALKLAERPERSEFVL C CH C GKKGHDT
Thromboxane A2 TPβ:	..DPWVYILFRRAVLRRLQPRLSRPRSLTLWPSLEYSGTISA C NLRLPGSSDSRASASRAAGITGVSH C AR C MLFDPEFDLLA..
Vasopressin V _{1a}	..NPWIYMFSGHLLQDCVQSF C QNMKEFNKEDTDSMSRRQTFYSNNRSPNTSTGMWKDSPKSKSIKFIPIVST
Vasopressin V ₂	..NPWIYASFSSSVSSELRLSL C ARGRTPPSLGQDESCDTASSSLAKDTS

Legend	
C	confirmed palmitoylation site with mutagenesis
C	confirmed palmitoylation, but no mutagenesis studies to confirm site
C	confirmed to lack palmitoylation
C	putative palmitoylation site mutated, no palmitoylation data
C	putative site

Figure 1.6: Sequence alignment of the C-terminal tails of select GPCRs.

Sequence alignment begins from the conserved [D/N]PXXY motif of the 7th transmembrane domain. Palmitoylated cysteine residues are highlighted in blue: a bolded C indicates the palmitoylation site was confirmed with mutagenesis, whereas a non-bolded C indicates that the GPCR was confirmed to be palmitoylated but no mutagenesis studies were done to confirm the site. Cysteine residues that are highlighted in red were confirmed to lack palmitoylation. Cysteine residues highlighted in orange are putative palmitoylation sites that were mutated, but palmitoylation was not directly assessed. Cysteine residues highlighted in pink are putative palmitoylation sites based on sequence alignment.

1.4 References

1. Heng, B. C., Aubel, D., and Fussenegger, M. (2013) An overview of the diverse roles of G-protein coupled receptors (GPCRs) in the pathophysiology of various human diseases. *Biotechnology advances* **31**, 1676-1694
2. Lappano, R., and Maggiolini, M. (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. *Nature reviews. Drug discovery* **10**, 47-60
3. Christopoulos, A. (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nature reviews. Drug discovery* **1**, 198-210
4. Marchese, A., and Trejo, J. (2013) Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling. *Cellular signalling* **25**, 707-716
5. Tobin, A. B., Butcher, A. J., and Kong, K. C. (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends in pharmacological sciences* **29**, 413-420
6. Wheatley, M., and Hawtin, S. R. (1999) Glycosylation of G-protein-coupled receptors for hormones central to normal reproductive functioning: its occurrence and role. *Human reproduction update* **5**, 356-364
7. Qanbar, R., and Bouvier, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol Ther* **97**, 1-33
8. Rocks, O., Peyker, A., Kahms, M., Verveer, P. J., Koerner, C., Lumbierres, M., Kuhlmann, J., Waldmann, H., Wittinghofer, A., and Bastiaens, P. I. (2005) An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science* **307**, 1746-1752
9. Dekker, F. J., Rocks, O., Vartak, N., Menninger, S., Hedberg, C., Balamurugan, R., Wetzel, S., Renner, S., Gerauer, M., Scholermann, B., Rusch, M., Kramer, J. W., Rauh, D., Coates, G. W., Brunsveld, L., Bastiaens, P. I., and Waldmann, H. (2010) Small-molecule inhibition of APT1 affects Ras localization and signaling. *Nature chemical biology* **6**, 449-456

10. Coughlin, S. R. (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. *Journal of thrombosis and haemostasis : JTH* **3**, 1800-1814
11. Arora, P., Ricks, T. K., and Trejo, J. (2007) Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. *J Cell Sci* **120**, 921-928
12. Hermans, J., and McDonagh, J. (1982) Fibrin: structure and interactions. *Seminars in thrombosis and hemostasis* **8**, 11-24
13. Esmon, C. T., and Owen, W. G. (1981) Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2249-2252
14. Esmon, C. T. (1995) Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **9**, 946-955
15. Esmon, C. T., Fukudome, K., Mather, T., Bode, W., Regan, L. M., Stearns-Kurosawa, D. J., and Kurosawa, S. (1999) Inflammation, sepsis, and coagulation. *Haematologica* **84**, 254-259
16. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407**, 258-264
17. Projahn, D., and Koenen, R. R. (2012) Platelets: key players in vascular inflammation. *Journal of leukocyte biology* **92**, 1167-1175
18. Kaplanski, G., Fabrigoule, M., Boulay, V., Dinarello, C. A., Bongrand, P., Kaplanski, S., and Farnarier, C. (1997) Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. *Journal of immunology* **158**, 5435-5441
19. Grandaliano, G., Valente, A. J., and Abboud, H. E. (1994) A novel biologic activity of thrombin: stimulation of monocyte chemotactic protein production. *The Journal of experimental medicine* **179**, 1737-1741

20. Subramaniam, M., Frenette, P. S., Saffaripour, S., Johnson, R. C., Hynes, R. O., and Wagner, D. D. (1996) Defects in hemostasis in P-selectin-deficient mice. *Blood* **87**, 1238-1242
21. Frenette, P. S., Johnson, R. C., Hynes, R. O., and Wagner, D. D. (1995) Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7450-7454
22. Lum, H., and Malik, A. B. (1994) Regulation of vascular endothelial barrier function. *The American journal of physiology* **267**, L223-241
23. Hamilton, J. R., Moffatt, J. D., Frauman, A. G., and Cocks, T. M. (2001) Protease-activated receptor (PAR) 1 but not PAR2 or PAR4 mediates endothelium-dependent relaxation to thrombin and trypsin in human pulmonary arteries. *Journal of cardiovascular pharmacology* **38**, 108-119
24. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068
25. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V., Jr., and Coughlin, S. R. (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381**, 516-519
26. Griffin, C. T., Srinivasan, Y., Zheng, Y. W., Huang, W., and Coughlin, S. R. (2001) A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science* **293**, 1666-1670
27. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386**, 502-506
28. Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jr., Tam, C., and Coughlin, S. R. (1998) A dual thrombin receptor system for platelet activation. *Nature* **394**, 690-694

29. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) Molecular cloning of a potential proteinase activated receptor. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9208-9212
30. Vu, T. K., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (1991) Domains specifying thrombin-receptor interaction. *Nature* **353**, 674-677
31. Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J. W., 2nd. (1990) The structure of a complex of recombinant hirudin and human alpha-thrombin. *Science* **249**, 277-280
32. McNamara, C. A., Sarembock, I. J., Gimple, L. W., Fenton, J. W., 2nd, Coughlin, S. R., and Owens, G. K. (1993) Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *The Journal of clinical investigation* **91**, 94-98
33. Chen, L. B., and Buchanan, J. M. (1975) Plasminogen-independent fibrinolysis by proteases produced by transformed chick embryo fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 1132-1136
34. Mari, B., Imbert, V., Belhacene, N., Far, D. F., Peyron, J. F., Pouyssegur, J., Van Obberghen-Schilling, E., Rossi, B., and Auberger, P. (1994) Thrombin and thrombin receptor agonist peptide induce early events of T cell activation and synergize with TCR cross-linking for CD69 expression and interleukin 2 production. *The Journal of biological chemistry* **269**, 8517-8523
35. de Garavilla, L., Vergnolle, N., Young, S. H., Ennes, H., Steinhoff, M., Ossovskaya, V. S., D'Andrea, M. R., Mayer, E. A., Wallace, J. L., Hollenberg, M. D., Andrade-Gordon, P., and Bunnett, N. W. (2001) Agonists of proteinase-activated receptor 1 induce plasma extravasation by a neurogenic mechanism. *British journal of pharmacology* **133**, 975-987
36. Junge, C. E., Lee, C. J., Hubbard, K. B., Zhang, Z., Olson, J. J., Hepler, J. R., Brat, D. J., and Traynelis, S. F. (2004) Protease-activated receptor-1 in human brain: localization and functional expression in astrocytes. *Experimental neurology* **188**, 94-103
37. Nicole, O., Goldshmidt, A., Hamill, C. E., Sorensen, S. D., Sastre, A., Lyuboslavsky, P., Hepler, J. R., McKeon, R. J., and Traynelis, S. F. (2005)

Activation of protease-activated receptor-1 triggers astrogliosis after brain injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**, 4319-4329

38. Booden, M. A., Eckert, L. B., Der, C. J., and Trejo, J. (2004) Persistent signaling by dysregulated thrombin receptor trafficking promotes breast carcinoma cell invasion. *Mol Cell Biol* **24**, 1990-1999
39. Bergmann, S., Junker, K., Henklein, P., Hollenberg, M. D., Settmacher, U., and Kaufmann, R. (2006) PAR-type thrombin receptors in renal carcinoma cells: PAR1-mediated EGFR activation promotes cell migration. *Oncology reports* **15**, 889-893
40. Boire, A., Covic, L., Agarwal, A., Jacques, S., Sherifi, S., and Kuliopulos, A. (2005) PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* **120**, 303-313
41. Schuepbach, R. A., Velez, K., and Riewald, M. (2011) Activated protein C up-regulates procoagulant tissue factor activity on endothelial cells by shedding the TFPI Kunitz 1 domain. *Blood* **117**, 6338-6346
42. Kuliopulos, A., Covic, L., Seeley, S. K., Sheridan, P. J., Helin, J., and Costello, C. E. (1999) Plasmin desensitization of the PAR1 thrombin receptor: kinetics, sites of truncation, and implications for thrombolytic therapy. *Biochemistry* **38**, 4572-4585
43. Oikonomopoulou, K., Hansen, K. K., Saifeddine, M., Vergnolle, N., Tea, I., Blaber, M., Blaber, S. I., Scarisbrick, I., Diamandis, E. P., and Hollenberg, M. D. (2006) Kallikrein-mediated cell signalling: targeting proteinase-activated receptors (PARs). *Biological chemistry* **387**, 817-824
44. Trivedi, V., Boire, A., Tchernychev, B., Kaneider, N. C., Leger, A. J., O'Callaghan, K., Covic, L., and Kuliopulos, A. (2009) Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell* **137**, 332-343
45. Stearns-Kurosawa, D. J., Kurosawa, S., Mollica, J. S., Ferrell, G. L., and Esmon, C. T. (1996) The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proceedings of the*

National Academy of Sciences of the United States of America **93**, 10212-10216

46. Russo, A., Soh, U. J., Paing, M. M., Arora, P., and Trejo, J. (2009) Caveolae are required for protease-selective signaling by protease-activated receptor-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6393-6397
47. Bae, J. S., Yang, L., Manithody, C., and Rezaie, A. R. (2007) The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood* **110**, 3909-3916
48. Schuepbach, R. A., Madon, J., Ender, M., Galli, P., and Riewald, M. (2012) Protease-activated receptor-1 cleaved at R46 mediates cytoprotective effects. *Journal of thrombosis and haemostasis : JTH* **10**, 1675-1684
49. Bae, J. S., Yang, L., and Rezaie, A. R. (2008) Lipid raft localization regulates the cleavage specificity of protease activated receptor 1 in endothelial cells. *Journal of thrombosis and haemostasis : JTH* **6**, 954-961
50. Majumdar, M., Seasholtz, T. M., Buckmaster, C., Toksoz, D., and Brown, J. H. (1999) A rho exchange factor mediates thrombin and Galpha(12)-induced cytoskeletal responses. *The Journal of biological chemistry* **274**, 26815-26821
51. Klages, B., Brandt, U., Simon, M. I., Schultz, G., and Offermanns, S. (1999) Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *The Journal of cell biology* **144**, 745-754
52. Vouret-Craviari, V., Boquet, P., Pouyssegur, J., and Van Obberghen-Schilling, E. (1998) Regulation of the actin cytoskeleton by thrombin in human endothelial cells: role of Rho proteins in endothelial barrier function. *Molecular biology of the cell* **9**, 2639-2653
53. Vaidyula, V. R., and Rao, A. K. (2003) Role of Galphaq and phospholipase C-beta2 in human platelets activation by thrombin receptors PAR1 and PAR4: studies in human platelets deficient in Galphaq and phospholipase C-beta2. *British journal of haematology* **121**, 491-496

54. Hung, D. T., Wong, Y. H., Vu, T. K., and Coughlin, S. R. (1992) The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *The Journal of biological chemistry* **267**, 20831-20834
55. Lopez, I., Mak, E. C., Ding, J., Hamm, H. E., and Lomasney, J. W. (2001) A novel bifunctional phospholipase c that is regulated by G α 12 and stimulates the Ras/mitogen-activated protein kinase pathway. *The Journal of biological chemistry* **276**, 2758-2765
56. Offermanns, S., Toombs, C. F., Hu, Y. H., and Simon, M. I. (1997) Defective platelet activation in G α (q)-deficient mice. *Nature* **389**, 183-186
57. Leervers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Current opinion in cell biology* **11**, 219-225
58. Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) β -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *The Journal of biological chemistry* **277**, 1292-1300
59. Chen, C. H., Paing, M. M., and Trejo, J. (2004) Termination of protease-activated receptor-1 signaling by β -arrestins is independent of receptor phosphorylation. *The Journal of biological chemistry* **279**, 10020-10031
60. Ishii, K., Chen, J., Ishii, M., Koch, W. J., Freedman, N. J., Lefkowitz, R. J., and Coughlin, S. R. (1994) Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. *The Journal of biological chemistry* **269**, 1125-1130
61. Tiruppathi, C., Yan, W., Sandoval, R., Naqvi, T., Pronin, A. N., Benovic, J. L., and Malik, A. B. (2000) G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7440-7445
62. Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (1994) Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. *The Journal of biological chemistry* **269**, 27719-27726

63. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Mol Cell Biol* **26**, 3231-3242
64. Bonifacino, J. S., and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* **72**, 395-447
65. Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* **269**, 1872-1875
66. Wolfe, B. L., Marchese, A., and Trejo, J. (2007) Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. *The Journal of cell biology* **177**, 905-916
67. Trejo, J., Altschuler, Y., Fu, H. W., Mostov, K. E., and Coughlin, S. R. (2000) Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *The Journal of biological chemistry* **275**, 31255-31265
68. Chen, B., Does, M. R., Grimsey, N., Canto, I., Barker, B. L., and Trejo, J. (2011) Adaptor Protein Complex-2 (AP-2) and Epsin-1 Mediate Protease-activated Receptor-1 Internalization via Phosphorylation- and Ubiquitination-dependent Sorting Signals. *The Journal of biological chemistry* **286**, 40760-40770
69. Paing, M. M., Temple, B. R., and Trejo, J. (2004) A tyrosine-based sorting signal regulates intracellular trafficking of protease-activated receptor-1: multiple regulatory mechanisms for agonist-induced G protein-coupled receptor internalization. *The Journal of biological chemistry* **279**, 21938-21947
70. Does, M. R., Paing, M. M., Lin, H., Montagne, W. A., Marchese, A., and Trejo, J. (2012) AP-3 regulates PAR1 ubiquitin-independent MVB/lysosomal sorting via an ALIX-mediated pathway. *Molecular biology of the cell*
71. Does, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., Meerloo, T., and Trejo, J. (2012) ALIX binds a YPX(3)L motif of the GPCR

PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *The Journal of cell biology* **197**, 407-419

72. Soto, A. G., and Trejo, J. (2010) N-linked glycosylation of protease-activated receptor-1 second extracellular loop: a critical determinant for ligand-induced receptor activation and internalization. *The Journal of biological chemistry* **285**, 18781-18793
73. Lobo, S., Greentree, W. K., Linder, M. E., and Deschenes, R. J. (2002) Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. *The Journal of biological chemistry* **277**, 41268-41273
74. Roth, A. F., Wan, J., Bailey, A. O., Sun, B., Kuchar, J. A., Green, W. N., Phinney, B. S., Yates, J. R., 3rd, and Davis, N. G. (2006) Global analysis of protein palmitoylation in yeast. *Cell* **125**, 1003-1013
75. Tsutsumi, R., Fukata, Y., and Fukata, M. (2008) Discovery of protein-palmitoylating enzymes. *Pflugers Arch* **456**, 1199-1206
76. Ohno, Y., Kihara, A., Sano, T., and Igarashi, Y. (2006) Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochimica et biophysica acta* **1761**, 474-483
77. Rocks, O., Gerauer, M., Vartak, N., Koch, S., Huang, Z. P., Pechlivanis, M., Kuhlmann, J., Brunsveld, L., Chandra, A., Ellinger, B., Waldmann, H., and Bastiaens, P. I. (2010) The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins. *Cell* **141**, 458-471
78. Linder, M. E., and Deschenes, R. J. (2007) Palmitoylation: policing protein stability and traffic. *Nature reviews. Molecular cell biology* **8**, 74-84
79. Ducker, C. E., Stettler, E. M., French, K. J., Upson, J. J., and Smith, C. D. (2004) Huntingtin interacting protein 14 is an oncogenic human protein: palmitoyl acyltransferase. *Oncogene* **23**, 9230-9237
80. Noritake, J., Fukata, Y., Iwanaga, T., Hosomi, N., Tsutsumi, R., Matsuda, N., Tani, H., Iwanari, H., Mochizuki, Y., Kodama, T., Matsuura, Y., Bredt, D. S., Hamakubo, T., and Fukata, M. (2009) Mobile DHHC palmitoylating enzyme

mediates activity-sensitive synaptic targeting of PSD-95. *The Journal of cell biology* **186**, 147-160

81. Planey, S. L. (2013) Discovery of Selective and Potent Inhibitors of Palmitoylation. in *Drug Discovery* (El-Shemy, H. ed.), InTech. pp 251-288
82. Duncan, J. A., and Gilman, A. G. (1996) Autoacylation of G protein alpha subunits. *The Journal of biological chemistry* **271**, 23594-23600
83. Kong, M. M., Verma, V., O'Dowd, B. F., and George, S. R. (2011) The role of palmitoylation in directing dopamine D1 receptor internalization through selective endocytic routes. *Biochem Biophys Res Commun* **405**, 445-449
84. Duncan, J. A., and Gilman, A. G. (1998) A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). *The Journal of biological chemistry* **273**, 15830-15837
85. Siegel, G., Obernosterer, G., Fiore, R., Oehmen, M., Bicker, S., Christensen, M., Khudayberdiev, S., Leuschner, P. F., Busch, C. J., Kane, C., Hubel, K., Dekker, F., Hedberg, C., Rengarajan, B., Drepper, C., Waldmann, H., Kauppinen, S., Greenberg, M. E., Draguhn, A., Rehmsmeier, M., Martinez, J., and Schratt, G. M. (2009) A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. *Nature cell biology* **11**, 705-716
86. Lu, J. Y., and Hofmann, S. L. (2006) Thematic review series: lipid posttranslational modifications. Lysosomal metabolism of lipid-modified proteins. *Journal of lipid research* **47**, 1352-1357
87. Verkruyse, L. A., and Hofmann, S. L. (1996) Lysosomal targeting of palmitoyl-protein thioesterase. *The Journal of biological chemistry* **271**, 15831-15836
88. Vesa, J., Hellsten, E., Verkruyse, L. A., Camp, L. A., Rapola, J., Santavuori, P., Hofmann, S. L., and Peltonen, L. (1995) Mutations in the palmitoyl protein thioesterase gene causing infantile neuronal ceroid lipofuscinosis. *Nature* **376**, 584-587
89. Soyombo, A. A., and Hofmann, S. L. (1997) Molecular cloning and expression of palmitoyl-protein thioesterase 2 (PPT2), a homolog of lysosomal palmitoyl-

protein thioesterase with a distinct substrate specificity. *The Journal of biological chemistry* **272**, 27456-27463

90. Shahinian, S., and Silvius, J. R. (1995) Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. *Biochemistry* **34**, 3813-3822
91. Fukata, Y., and Fukata, M. (2010) Protein palmitoylation in neuronal development and synaptic plasticity. *Nature reviews. Neuroscience* **11**, 161-175
92. Alvarez, E., Girones, N., and Davis, R. J. (1990) Inhibition of the receptor-mediated endocytosis of diferric transferrin is associated with the covalent modification of the transferrin receptor with palmitic acid. *The Journal of biological chemistry* **265**, 16644-16655
93. Abrami, L., Leppla, S. H., and van der Goot, F. G. (2006) Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. *The Journal of cell biology* **172**, 309-320
94. Kinlough, C. L., McMahan, R. J., Poland, P. A., Bruns, J. B., Harkleroad, K. L., Stremple, R. J., Kashlan, O. B., Weixel, K. M., Weisz, O. A., and Hughey, R. P. (2006) Recycling of MUC1 is dependent on its palmitoylation. *The Journal of biological chemistry* **281**, 12112-12122
95. Schweizer, A., Kornfeld, S., and Rohrer, J. (1996) Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. *The Journal of cell biology* **132**, 577-584
96. McCormick, P. J., Dumaresq-Doiron, K., Pluviose, A. S., Pichette, V., Tosato, G., and Lefrancois, S. (2008) Palmitoylation controls recycling in lysosomal sorting and trafficking. *Traffic* **9**, 1984-1997
97. Zhang, W., Tribble, R. P., and Samelson, L. E. (1998) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* **9**, 239-246

98. Gagnoux-Palacios, L., Dans, M., van't Hof, W., Mariotti, A., Pepe, A., Meneguzzi, G., Resh, M. D., and Giancotti, F. G. (2003) Compartmentalization of integrin $\alpha 6 \beta 4$ signaling in lipid rafts. *The Journal of cell biology* **162**, 1189-1196
99. Dietzen, D. J., Hastings, W. R., and Lublin, D. M. (1995) Caveolin is palmitoylated on multiple cysteine residues. Palmitoylation is not necessary for localization of caveolin to caveolae. *The Journal of biological chemistry* **270**, 6838-6842
100. Chini, B., and Parenti, M. (2009) G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats. *J Mol Endocrinol* **42**, 371-379
101. O'Brien, P. J., and Zatz, M. (1984) Acylation of bovine rhodopsin by [3H]palmitic acid. *The Journal of biological chemistry* **259**, 5054-5057
102. Moench, S. J., Moreland, J., Stewart, D. H., and Dewey, T. G. (1994) Fluorescence studies of the location and membrane accessibility of the palmitoylation sites of rhodopsin. *Biochemistry* **33**, 5791-5796
103. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**, 739-745
104. Iwanaga, T., Tsutsumi, R., Noritake, J., Fukata, Y., and Fukata, M. (2009) Dynamic protein palmitoylation in cellular signaling. *Prog Lipid Res* **48**, 117-127
105. Park, P. S., Sapra, K. T., Jastrzebska, B., Maeda, T., Maeda, A., Pulawski, W., Kono, M., Lem, J., Crouch, R. K., Filipek, S., Muller, D. J., and Palczewski, K. (2009) Modulation of molecular interactions and function by rhodopsin palmitoylation. *Biochemistry* **48**, 4294-4304
106. Wang, Z., Wen, X. H., Ablonczy, Z., Crouch, R. K., Makino, C. L., and Lem, J. (2005) Enhanced shutoff of phototransduction in transgenic mice expressing palmitoylation-deficient rhodopsin. *The Journal of biological chemistry* **280**, 24293-24300

107. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1989) Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *The Journal of biological chemistry* **264**, 7564-7569
108. Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S., and Masaki, T. (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. *The Journal of biological chemistry* **272**, 21589-21596
109. Papoucheva, E., Dumuis, A., Sebben, M., Richter, D. W., and Ponimaskin, E. G. (2004) The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *The Journal of biological chemistry* **279**, 3280-3291
110. Kennedy, M. E., and Limbird, L. E. (1993) Mutations of the alpha 2A-adrenergic receptor that eliminate detectable palmitoylation do not perturb receptor-G-protein coupling. *The Journal of biological chemistry* **268**, 8003-8011
111. Kennedy, M. E., and Limbird, L. E. (1994) Palmitoylation of the alpha 2A-adrenergic receptor. Analysis of the sequence requirements for and the dynamic properties of alpha 2A-adrenergic receptor palmitoylation. *The Journal of biological chemistry* **269**, 31915-31922
112. Hawtin, S. R., Tobin, A. B., Patel, S., and Wheatley, M. (2001) Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *The Journal of biological chemistry* **276**, 38139-38146
113. Jin, H., Xie, Z., George, S. R., and O'Dowd, B. F. (1999) Palmitoylation occurs at cysteine 347 and cysteine 351 of the dopamine D(1) receptor. *Eur J Pharmacol* **386**, 305-312
114. Pizard, A., Blaukat, A., Michineau, S., Dikic, I., Muller-Esterl, W., Alhenc-Gelas, F., and Rajerison, R. M. (2001) Palmitoylation of the human bradykinin B2 receptor influences ligand efficacy. *Biochemistry* **40**, 15743-15751
115. Munshi, U. M., Peegel, H., and Menon, K. M. (2001) Palmitoylation of the luteinizing hormone/human chorionic gonadotropin receptor regulates receptor

interaction with the arrestin-mediated internalization pathway. *European journal of biochemistry / FEBS* **268**, 1631-1639

116. Horstmeyer, A., Cramer, H., Sauer, T., Muller-Esterl, W., and Schroeder, C. (1996) Palmitoylation of endothelin receptor A. Differential modulation of signal transduction activity by post-translational modification. *The Journal of biological chemistry* **271**, 20811-20819
117. Kvachnina, E., Dumuis, A., Wlodarczyk, J., Renner, U., Cochet, M., Richter, D. W., and Ponimaskin, E. (2009) Constitutive Gs-mediated, but not G12-mediated, activity of the 5-hydroxytryptamine 5-HT7(a) receptor is modulated by the palmitoylation of its C-terminal domain. *Biochimica et biophysica acta* **1793**, 1646-1655
118. Charest, P. G., and Bouvier, M. (2003) Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient receptor endocytosis and ERK1/2 activation. *The Journal of biological chemistry* **278**, 41541-41551
119. Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J. L., Arenzana-Seisdedos, F., and Bachelier, F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *The Journal of biological chemistry* **276**, 31936-31944
120. Gao, Z., Ni, Y., Szabo, G., and Linden, J. (1999) Palmitoylation of the recombinant human A1 adenosine receptor: enhanced proteolysis of palmitoylation-deficient mutant receptors. *The Biochemical journal* **342** (Pt 2), 387-395
121. Munshi, U. M., Clouser, C. L., Peegel, H., and Menon, K. M. (2005) Evidence that palmitoylation of carboxyl terminus cysteine residues of the human luteinizing hormone receptor regulates postendocytic processing. *Molecular endocrinology* **19**, 749-758
122. Ponimaskin, E., Dumuis, A., Gaven, F., Barthet, G., Heine, M., Glebov, K., Richter, D. W., and Oppermann, M. (2005) Palmitoylation of the 5-hydroxytryptamine4a receptor regulates receptor phosphorylation, desensitization, and beta-arrestin-mediated endocytosis. *Molecular pharmacology* **67**, 1434-1443

123. Renner, U., Glebov, K., Lang, T., Papusheva, E., Balakrishnan, S., Keller, B., Richter, D. W., Jahn, R., and Ponimaskin, E. (2007) Localization of the mouse 5-hydroxytryptamine(1A) receptor in lipid microdomains depends on its palmitoylation and is involved in receptor-mediated signaling. *Molecular pharmacology* **72**, 502-513
124. Venkatesan, S., Rose, J. J., Lodge, R., Murphy, P. M., and Foley, J. F. (2003) Distinct mechanisms of agonist-induced endocytosis for human chemokine receptors CCR5 and CXCR4. *Molecular biology of the cell* **14**, 3305-3324
125. Insel, P. A., Head, B. P., Patel, H. H., Roth, D. M., Bunday, R. A., and Swaney, J. S. (2005) Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochemical Society transactions* **33**, 1131-1134
126. Zheng, H., Pearsall, E. A., Hurst, D. P., Zhang, Y., Chu, J., Zhou, Y., Reggio, P. H., Loh, H. H., and Law, P. Y. (2012) Palmitoylation and membrane cholesterol stabilize mu-opioid receptor homodimerization and G protein coupling. *BMC cell biology* **13**, 6
127. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**, 1258-1265
128. Swift, S., Leger, A. J., Talavera, J., Zhang, L., Bohm, A., and Kuliopulos, A. (2006) Role of the PAR1 receptor 8th helix in signaling: the 7-8-1 receptor activation mechanism. *The Journal of biological chemistry* **281**, 4109-4116
129. Zhang, C., Srinivasan, Y., Arlow, D. H., Fung, J. J., Palmer, D., Zheng, Y., Green, H. F., Pandey, A., Dror, R. O., Shaw, D. E., Weis, W. I., Coughlin, S. R., and Kobilka, B. K. (2012) High-resolution crystal structure of human protease-activated receptor 1. *Nature* **492**, 387-392
130. Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. R., Ijzerman, A. P., and Stevens, R. C. (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* **322**, 1211-1217

131. Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., Leslie, A. G., Tate, C. G., and Schertler, G. F. (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**, 486-491
132. Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C., and Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature* **475**, 65-70
133. Feierler, J., Wirth, M., Welte, B., Schussler, S., Jochum, M., and Faussner, A. (2011) Helix 8 plays a crucial role in bradykinin B(2) receptor trafficking and signaling. *The Journal of biological chemistry* **286**, 43282-43293
134. Gehret, A. U., Jones, B. W., Tran, P. N., Cook, L. B., Greuber, E. K., and Hinkle, P. M. (2009) Role of helix 8 of the thyrotropin-releasing hormone receptor in phosphorylation by G protein-coupled receptor kinase. *Molecular pharmacology* **77**, 288-297
135. Faussner, A., Bauer, A., Kalatskaya, I., Schussler, S., Seidl, C., Proud, D., and Jochum, M. (2005) The role of helix 8 and of the cytosolic C-termini in the internalization and signal transduction of B(1) and B(2) bradykinin receptors. *The FEBS journal* **272**, 129-140
136. Delos Santos, N. M., Gardner, L. A., White, S. W., and Bahouth, S. W. (2006) Characterization of the residues in helix 8 of the human beta1-adrenergic receptor that are involved in coupling the receptor to G proteins. *The Journal of biological chemistry* **281**, 12896-12907
137. Zhong, M., Navratil, A. M., Clay, C., and Sanborn, B. M. (2004) Residues in the hydrophilic face of putative helix 8 of oxytocin receptor are important for receptor function. *Biochemistry* **43**, 3490-3498
138. Dowal, L., Sim, D. S., Dilks, J. R., Blair, P., Beaudry, S., Denker, B. M., Koukos, G., Kuliopulos, A., and Flaumenhaft, R. (2011) Identification of an antithrombotic allosteric modulator that acts through helix 8 of PAR1. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 2951-2956

139. Adams, M. N., Christensen, M. E., He, Y., Waterhouse, N. J., and Hooper, J. D. (2011) The role of palmitoylation in signalling, cellular trafficking and plasma membrane localization of protease-activated receptor-2. *PLoS One* **6**, e28018
140. Jing, S. Q., and Trowbridge, I. S. (1990) Nonacylated human transferrin receptors are rapidly internalized and mediate iron uptake. *The Journal of biological chemistry* **265**, 11555-11559
141. Canto, I., and Trejo, J. (2013) Palmitoylation of Protease-activated Receptor-1 Regulates Adaptor Protein Complex-2 and -3 Interaction with Tyrosine-based Motifs and Endocytic Sorting. *The Journal of biological chemistry* **288**, 15900-15912
142. Canto, I., Soh, U. J., and Trejo, J. (2012) Allosteric modulation of protease-activated receptor signaling. *Mini Rev Med Chem* **12**, 804-811

Chapter 2: Characterization of PAR1 palmitoylation and other post-translational modifications

2.1 Abstract

Protease-activated receptor-1 (PAR1) is a G protein-coupled receptor (GPCR) that is irreversibly activated through proteolytic cleavage. Proteolysis of PAR1 exposes a new N-terminus that behaves as a tethered ligand that binds intramolecularly to the extracellular loops of the receptor to initiate transmembrane signaling. Due to the irreversible nature of PAR1 activation, desensitization and endocytic trafficking tightly regulate receptor signaling. Post-translational modifications such as ubiquitination and phosphorylation are involved in PAR1 internalization and down-regulation of signaling. In addition, many GPCRs are palmitoylated, which appears to have diverse functions in the regulation of receptor signaling and trafficking. PAR1 has two highly conserved C-terminal tail cysteine residues (C) that serve as sites for palmitoylation. Recent studies suggest that palmitoylation of transmembrane proteins can modulate ubiquitination, but such a relationship has not been demonstrated for a GPCR. Here we show that PAR1 is basally palmitoylated when exogenously expressed in HeLa cells at conserved cysteine residues C³⁸⁷ and C³⁸⁸. A palmitoylation-deficient PAR1 mutant, in which C³⁸⁷ and C³⁸⁸ residues are converted to alanines (A), is expressed at the cell surface and fully glycosylated. However, this mutant is defective in agonist-induced phosphorylation and ubiquitination. Results from these studies indicate that

palmitoylation of PAR1 at the C-tail domain is important for facilitating recognition by both protein kinases and ubiquitin ligases, ultimately regulating the phosphorylation and ubiquitination status of PAR1.

2.2 Introduction

Palmitoylation occurs through the covalent attachment of palmitate, a 16-carbon saturated fatty acid, to cysteine residues via a thioester linkage. This modification is a dynamic, reversible process in which the palmitoyl group is added enzymatically through palmitoyl acyl transferases (PATs) and removed by palmitoyl-protein thioesterases (PPTs) (1). Palmitoylation facilitates membrane association for some soluble proteins by increasing their hydrophobicity (2-5). In addition to membrane association, palmitoylation can regulate protein function by altering protein conformation. In the context of a G protein-coupled receptor (GPCR), palmitoylation within the carboxyl terminal tail (C-tail) is predicted to cause insertion into the lipid bilayer creating a fourth intracellular loop. Indeed, the X-ray crystal structure for rhodopsin confirmed the presence of a fourth intracellular loop that exists as an α -helix and is often referred to as the 8th helix (6). A number of GPCRs have been reported to be palmitoylated, and many of the lipidation modifications occur on the carboxyl terminus, especially on the cytoplasmic juxtamembrane cysteine residues (6,7). Defective GPCR palmitoylation has been shown to impair coupling to G proteins and to alter membrane trafficking (8). However, the functional consequences of mutating receptor palmitoylation sites differ between GPCRs, suggesting that palmitoylation has diverse functions in regulating GPCR signaling and trafficking.

The protease-activated receptor-1 (PAR1), a GPCR for thrombin, contains a set of putative palmitoylation sites in the C-tail that could be important for regulating receptor signaling. A structural model of PAR1 based on the X-ray crystal structure of rhodopsin indicates that palmitoylation of cysteine (C) residues C³⁸⁷ and C³⁸⁸ could create an 8th helix that appears to be important for receptor coupling to G α_q signaling (9). However, a crystal structure of the human PAR1 bound with vorapaxar, a PAR1 specific antagonist, does not reveal an 8th helix potentially due to a weak electron density on residues following cysteine 378 (10). It is uncertain if the lack of helix 8 is indicative of PAR1 in its native state, or whether it is an artifact of the crystallization conditions utilized in the study. Neither study confirmed that PAR1 is indeed palmitoylated. We demonstrated that PAR1 is modified with palmitoylation, and investigated whether palmitoylation functions in modulating receptor function.

Palmitoylation of GPCRs has the potential to modulate function by regulating other post-translational modifications. For example, GPCR palmitoylation can regulate receptor phosphorylation. A non-palmitoylated mutant of the β_2 -adrenergic receptor displays increased basal phosphorylation and consequently uncoupling from G α_s (11). However, studies examining a non-palmitoylated mutant of the vasopressin V_{1a} receptor demonstrated decreased phosphorylation both basally and in response to agonist (12). Our lab has previously demonstrated that phosphorylation of PAR1 is a critical modification that serves to desensitize the receptor following activation (13) and is critical for receptor ubiquitination to occur (14). Although it has been demonstrated that palmitoylation can modulate phosphorylation for some GPCRs, it is not known if palmitoylation functions in a similar manner for PAR1.

A relationship between palmitoylation and ubiquitination has been implicated for some membrane-associated proteins, however it is currently not known if such a relationship exists for a GPCR. Ubiquitination is a post-translational modification in which lysine residues are covalently modified with the 76 amino acid protein ubiquitin. When conjugated onto transmembrane proteins, including GPCRs, ubiquitin can serve as an endosomal sorting signal by facilitating interaction with ubiquitin binding domains found within many components of the endocytic sorting machinery (15). The yeast SNARE protein, mammalian anthrax toxin, and the Wnt signaling protein LRP6 are all palmitoylated, and depalmitoylation promotes protein ubiquitination and degradation (16-18). These findings raise the possibility that modification of GPCRs with palmitoylation may regulate receptor ubiquitination and consequently signaling and trafficking. To our knowledge, no studies have examined the interplay between GPCR palmitoylation and ubiquitination.

Here, we show that PAR1 is modified with palmitoylation in HeLa and endothelial cells. We also confirm that the conserved residues C³⁸⁷ and C³⁸⁸ in the PAR1 C- tail are the primary sites for palmitoylation. We also generated a mutant in which residues C³⁸⁷ and C³⁸⁸ are converted to alanines, and demonstrate that this mutation renders PAR1 palmitoylation-deficient. The palmitoylation-deficient PAR1 mutant is expressed at the cell surface in HeLa and endothelial cells, suggesting that palmitoylation is not required for biogenesis of the receptor. Additionally, we show that the palmitoylation-deficient mutant is fully glycosylated. However, the palmitoylation-deficient PAR1 mutant is defective in agonist-induced phosphorylation and ubiquitination. Taken together, these studies suggest that palmitoylation

modulates activated PAR1 phosphorylation and ubiquitination, but is not required for PAR1 glycosylation or cell surface expression.

2.3 Materials and Methods

Reagents and Antibodies – The PAR1 agonist peptides SFLLRN and TFLLRNPNDK were synthesized as the carboxyl amide and purified by reverse phase high-pressure liquid-chromatography at the Tufts University Facility (Boston, MA). Mouse monoclonal M2 anti-FLAG antibody and mouse monoclonal anti- β -actin were purchased from Sigma-Aldrich (Saint Louis, MO). Rabbit anti-FLAG polyclonal antibody was from Rockland Immunochemicals, Inc (Gilbertsville, PA). The anti-PAR1 WEDE mouse antibody was from Beckman Coulter (Fullerton, CA). The anti-PAR1 C5433 rabbit polyclonal antibody was previously described (19). Mouse monoclonal anti-ubiquitin P4D1 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse and goat anti-rabbit antibodies were from Bio-Rad Laboratories (Hercules, CA). De-lipidated fetal bovine serum was obtained from Omega Scientific (Tarzana, CA).

cDNAs and Cell Lines – A cDNA plasmid encoding an N-terminal FLAG-tagged human PAR1 cloned into pBJ vector were previously described (19,20). A PAR1 mutant in which cysteine (C) residues at positions 387 and 388 were mutated to alanines (A) was generated by quick-change site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). The following primers were utilized: 5'-GTGCCAGAGGTACGTCTACAGTATCTTAGCCGCCAAAGAAAGTTCC-3' and 5'-GGAACCTTTCTTTGGCGGCTAAGATACTGTAGACGTACCTCTGGCAC-3'. All cDNA

plasmids were subjected to dideoxy sequencing (Retrogen, San Diego, CA). HeLa cells stably expressing FLAG-tagged PAR1 CC/AA mutant were generated and maintained as previously described (20). Human umbilical vein endothelial derived EA.hy926 cells were maintained as described (21). For endothelial stable cell lines, A cDNA plasmid encoding human wild-type PAR1 with an N-terminally tagged FLAG epitope was cloned into a pLXSN vector. Site-directed mutagenesis was used to generate siRNA resistance by mutating the PAR1 siRNA binding site from 5'-AGATTAGTCTCCATCAATA-3' to 5'-AGATTAGTCTCCAAGAATA-3'. The short hairpin RNAi targeting PAR1 was previously described in Russo et al. Site-directed mutagenesis was also used to generate the siRNA resistant FLAG PAR1 CC/AA mutant, in which cysteines 387 and 388 were converted to alanines. Both constructs were confirmed with dideoxy sequencing. Retroviruses were generated in a GP2-293 packaging cell line and used to infect EA.hy926 cells that were stably transduced with PAR1 shRNA, previously described in Russo et al. Mass populations of cells expressing siRNA resistant FLAG PAR1 WT or FLAG PAR1 CC/AA in were selected in 250 μ g/ml G418.

Cell Transfections – HeLa cells were transiently transfected with FLAG-PAR1 WT and CC/AA mutant using Lipofectamine 2000 (Invitrogen), or polyethylenimine (Polysciences, Warrington PA) reagents, according to the manufacturer's instructions.

Immunoblotting – Equivalent amounts of cell lysates were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with appropriate antibodies.

Cell Surface ELISA – HeLa cells expressing FLAG-PAR1 WT or CC/AA mutant were seeded at 1.0×10^5 cells/well in 24-well plates and grown overnight. Cells were fixed with 4% paraformaldehyde for 5 min at 4°C, incubated with anti-FLAG polyclonal antibody and then with goat anti-rabbit HRP-conjugated secondary antibody for 1 h at room temperature and developed as described above. The internalization of PAR1 was measured as described (22). Briefly, HeLa cells seeded 0.8×10^5 cells/well in 24-well plates transfected with FLAG-PAR1 WT or various mutants were labeled with M1 anti-FLAG antibody at 4°C, washed and incubated in DMEM containing 0.1% BSA, 20 mM HEPES, 1 mM Ca^{2+} at 37°C. After internalization, cells were fixed and processed as described above.

PAR1 Sequence Alignment – A sequence alignment was performed by obtaining PAR1 sequences using Basic Local Alignment Search Tool (BLAST). Thirty PAR1 sequences were truncated immediately after the [N/D]PXXYL motif of the seventh transmembrane domain and aligned. The PAR1 species and NCBI Reference sequences are as follows: *H. sapiens* AAH02464; *M. mulatta*: EHH26595; *C. longicaudatus*: AAA86747; *M. musculus*: NP_034299; *R. norvegicus*: NP_037082; *S. scrofa*: NP_001231301; *B. Taurus*: NP_001096567; *B. grunniens mutus*: ELR60449; *H. glaber*: EHB00368; *M. putorius furo*: AER98213; *D. rerio*: NP_001108318; *X. laevis*: NP_001079252; *P. hamadryas*: P56488; *G. gorilla gorilla*: XP_004058710; *P. abelii*: XP_002815710; *P. troglodytes*: XP_526888; *N. leucogenys*: XP_003261537; *P. Anubis*: XP_003899888; *C. lupus familiaris*: XP_546059; *C. porcellus*: XP_003470285; *C. jacchus*: XP_002744891; *C. griseus*: XP_003498759; *S. boliviensis boliviensis*: XP_003921004; *L. Africana*:

XP_003408011; *M. domestica*: XP_001362837; *O. garnettii*: XP_003785973; *F. catus*: XP_003981132; *O. cuniculus*: XP_002723068; *O. aries*: XP_004010905; *E. caballus*: XP_001504007.

PAR1 Palmitoylation Assay – Palmitoylation of PAR1 was examined in EA.hy926 and HeLa cells. HeLa cells were seeded at 2.5×10^5 cells/well in 6-well plates and transfected with PAR1 WT or CC/AA mutant. EA.hy926 cells were seeded at 1.5×10^6 cells per 10 cm² dish. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% de-lipidated fetal bovine serum overnight and then incubated with 500 μ Ci/ml [³H]palmitic acid (Perkin Elmer, MA) for 3 h in 10% de-lipidated serum/DMEM. Cells were serum starved for 1 h and then left untreated or treated with agonist for various times at 37°C. Cells were lysed in buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, and 10 mM NaPP supplemented with protease inhibitors 100 μ g/ml PMSF, 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 10 μ g/ml benzamidine, and 1 μ g/ml soybean trypsin inhibitor. Total cell lysates were immunoprecipitated with either M2 anti-FLAG or anti-PAR1 WEDE antibody overnight at 4°C. Immunoprecipitates were resolved on 9% SDS-PAGE and gels were fixed with a 25% isopropanol/ 10% glacial acetic acid solution then incubated for 30 min in NAMP100 (GE Healthcare, NJ) or fixed with a 10% glacial acetic acid/ 30% methanol solution and then incubated with En³Hance (Perkin Elmer, MA). Gels were then dried and exposed to Amersham Hyperfilm (GE Healthcare, NJ) with intensifying screens at -80°C. Aliquots of immunoprecipitates were also resolved by SDS-PAGE, transferred to membranes and immunoblotted to detect PAR1 expression.

PAR1 Phosphorylation Assay – HeLa cells were plated in 6 well plates and transfected with 1 ug/well of FLAG-tagged PAR1 WT or CC/AA for 24 hours. Cells were labeled with 250 μ Ci/ml of [32 P]-orthophosphate (Perkin Elmer, Waltham, MA) in phosphate-free DMEM for 2 hrs at 37°C. Cells were incubated with or without 100 μ M peptide agonist for 3 min and then lysed in buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 200 μ M sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml soybean trypsin inhibitor, 1 μ g/ml pepstatin A and 1 μ g/ml benzamidine. Cell lysates were immunoprecipitated with M2 anti-FLAG antibody for 2 hrs. Immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was exposed to autoradiography to detect incorporation of [32 P]-orthophosphate. PAR1 expression and immunoprecipitation was detected by probing the membrane with a polyclonal anti-FLAG antibody.

PAR1 Ubiquitination Assay – HeLa cells stably expressing PAR1 wildtype or CC/AA mutant were plated in 10 cm dishes and grown for 48 hrs. Prior to simulation, cells were incubated in media containing 0.1% BSA, 20mM HEPES for 3 hrs. HeLa cells were stimulated with 100 μ M SFLLRN for 3 or 5 minutes, and then lysed in a buffer containing 50 mM Tris-HCl at pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% Nonidet P-40 (v/v), and 0.1% SDS (w/v). Lysis buffer was supplemented with 20 mM *N*-ethylmaleimide (NEM) and protease inhibitors, 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 10 μ g/ml benzamidine, and 1 μ g/ml soybean trypsin inhibitor. Whole cell lysates were immunoprecipitated with Wede or M2 antibody for 2 hrs. Immunoprecipitates were resolved by SDS-PAGE, transferred

to PVDF membranes, and immunoblotted with ubiquitin (P4D1) and FLAG antibodies. PAR1 shRNA EA.hy926 cells stably transduced with siRNA resistant FLAG PAR1 WT, or siRNA resistant CC/AA were plated in 10cm dishes at a density of 4.95×10^5 cells per dish. Cells were grown for 3 days, and then starved in 0.2% FBS DMEM overnight. Prior to stimulation, cells were incubated in media containing 0.1% BSA, 20mM HEPES for 3 hrs. Cells were stimulated with 100 μ M TFLLRN for 2.5 and 5 minutes and processed as described above.

2.4 Results

2.4.1 PAR1 C-terminal tail palmitoylation sites are highly conserved across multiple species

PAR1 has not been shown to be palmitoylated, although previous studies have predicted PAR1 palmitoylation to occur at C-tail cysteine residues near the seventh transmembrane domain based on molecular modeling comparisons with rhodopsin (Fig. 2.1A) (9). Furthermore, other GPCRs have been demonstrated to be palmitoylated at C-tail cysteine residues approximately 15 residues from the seventh transmembrane domain (23)(Fig. 1.6). Currently, there is no known consensus sequence for palmitoylation; therefore, we used NCBI basic local alignment search tool (BLAST) to align C-tail sequences from thirty different PAR1 species (Fig. 2.1B). We identified two highly conserved cysteine residues at positions 387 and 388 of the human PAR1 protein, both were present in twenty-four different species and could serve as potential sites for palmitoylation.

2.4.2 PAR1 is modified with palmitoylation

Although PAR1 has been predicted to be palmitoylated through molecular modeling studies based on the palmitoylated rhodopsin receptor crystal structure (9) it has yet to be demonstrated biochemically. To determine whether PAR1 is modified by palmitoylation, we directly measured [^3H]palmitate incorporation onto wildtype PAR1 and a PAR1 CC/AA mutant, in which C³⁸⁷ and C³⁸⁸ are converted to alanines. HeLa cells expressing FLAG-PAR1 WT or CC/AA mutant were labeled with [^3H]-palmitic acid, then incubated in the absence or presence of agonist peptide SFLLRN for various times at 37°C. Cells were lysed, PAR1 was immunoprecipitated, resolved by SDS-PAGE and then subjected to fluorography. In untreated control cells, wildtype PAR1 labeled with [^3H]palmitate migrated as a major species between ~50 and 75 kDa (Fig. 2.2A, lane 1), consistent with the fully glycosylated PAR1 (Fig. 2.3B, lane 1). The amount of [^3H]palmitate incorporated into PAR1 WT was unchanged after 3 or 10 min of agonist stimulation compared to untreated control cells (Fig. 2.2A, lanes 1-3). In contrast to wildtype receptor, the PAR1 CC/AA mutant failed to incorporate [^3H]palmitate under control or agonist stimulated conditions (Fig. 2.2A, lanes 4-5). These observations suggest that PAR1 is basally palmitoylated at C-tail C³⁸⁷ and C³⁸⁸ residues. However, the metabolic labeling conditions employed in these assays cannot distinguish if PAR1 is dynamically palmitoylated and de-palmitoylated at steady state.

In order to confirm that endogenous PAR1 is palmitoylated, this assay was repeated in EA.hy926 endothelial cells. Following labeling with [^3H]-palmitic acid, endogenous PAR1 was immunoprecipitated with an anti-PAR1 antibody. Endogenous

PAR1 expressed in human endothelial cells also exhibited basal palmitoylation that was not substantially altered following incubation with agonist (Fig. 2.2B, lanes 1-2). Immunoprecipitation with an IgG control antibody did not show any [^3H]palmitate signal nor PAR1 signal, indicating that the palmitoylation is specific to anti-PAR1 immunoprecipitates. Quantification confirms that the palmitoylation signal, normalized to the amount of PAR1 immunoprecipitated, is unchanged in the presence of agonist (Fig 2.2B). These studies demonstrate that endogenously expressed PAR1 is modified by palmitoylation, and confirm the PAR1 palmitoylation studies done in HeLa cells.

2.4.3 Palmitoylation-deficient PAR1 glycosylation and surface expression

HeLa cells stably expressing FLAG-tagged PAR1 WT and CC/AA mutant were generated to facilitate the characterization of receptor palmitoylation. Both FLAG-PAR1 WT and CC/AA mutant were expressed at the cell surface as assessed by ELISA (Fig. 2.3A). In addition, immunoprecipitated FLAG-PAR1 WT and CC/AA mutant migrated as prominent species between ~64 and 98 kDa (Fig. 2.3B, lanes 1-2) and were not detected in IgG immunoprecipitates (Fig. 2.3B, lanes 5-6). In order to assess if palmitoylation was required for glycosylation, we treated immunoprecipitated PAR1 WT and CC/AA receptors with the endoglycosidase PNGase F, which cleaves N-linked glycosylation from proteins. Incubation of PAR1 WT and CC/AA mutant with PNGase F resulted in a size shift to ~36 kDa (Fig. 2.3B, lanes 3-4), consistent with the predicted molecular weight of human PAR1. These findings suggest that the PAR1 CC/AA mutant is glycosylated and trafficked to the cell surface similar to wildtype receptor.

2.4.4 Palmitoylation is required for agonist-induced PAR1 phosphorylation and ubiquitination

Palmitoylation has been shown to be important for phosphorylation for some GPCRs (11,12). In order to determine if palmitoylation was required for activated PAR1 phosphorylation, HeLa cells expressing PAR1 WT or CC/AA mutant were labeled with [32 P] orthophosphate then stimulated with a peptide agonist that mimics the newly cleaved N-terminus of PAR1. Phosphorylation was assessed by detecting incorporation of [32 P] orthophosphate in immunoprecipitated receptors. PAR1 WT and CC/AA did not display any phosphorylation signal in unstimulated control conditions (Fig 2.4, lanes 1 and 3). Following a 3 minute agonist stimulation, PAR1 WT was phosphorylated, as indicated by a [32 P] signal that is consistent with the mature form of PAR1 (Fig 2.4, lane 2). The PAR1 CC/AA mutant displayed mild phosphorylation in response to agonist, but failed to reach the magnitude of phosphorylation that was exhibited by the wild type receptor (Fig 2.4, lane 4). These studies suggest that palmitoylation of PAR1 is required for agonist-induced phosphorylation.

Although it has been demonstrated that palmitoylation can modulate ubiquitination in some transmembrane proteins (16,17), it is yet to be determined if palmitoylation serves a similar function for GPCRs. We have recently shown that phosphorylation is required for agonist-induced ubiquitination to occur (14). Considering that palmitoylation is important for receptor phosphorylation (Fig 2.4), we were interested in determining if receptor ubiquitination was also affected. Stimulation of HeLa cells expressing PAR1 WT with a peptide agonist resulted in robust receptor

ubiquitination (Fig 2.5A, lanes 4-6). Interestingly, CC/AA expressing HeLa cells failed to exhibit receptor ubiquitination (Fig 2.5A, lanes 7-9). In fact, the PAR1 CC/AA ubiquitination levels were comparable to untransfected HeLa cells (Fig 2.5A, lanes 1-3). These studies suggest that palmitoylation is critical for receptor ubiquitination to occur, a process that was previously shown to be dependent upon receptor phosphorylation (14). Interestingly, palmitoylation is also required for receptor phosphorylation, potentially explaining the ubiquitination defect that we observed.

2.4.5 Palmitoylation-deficient PAR1 in endothelial cells

We have already demonstrated that endogenous PAR1 is modified by palmitoylation in endothelial cells (Fig. 2.2B). Thus, we were interested in characterizing a palmitoylation-deficient mutant in an endothelial cell system. In order to examine this, we first needed to establish a cell line that expresses the palmitoylation-deficient PAR1 mutant without any endogenous PAR1 present. We began by utilizing an EA.hy926 cell line in which endogenous PAR1 was depleted by a stably transduced PAR1 shRNA. Analysis of the PAR1 shRNA cell line with immunoprecipitation indicates that PAR1 is efficiently knocked down compared to untransformed EA.hy926 cells, which express substantially more PAR1 (Fig 2.6A, lane 2). Furthermore, PAR1 was not detected in IgG immunoprecipitates (Fig 2.6A, lanes 3-4). These studies confirmed that the PAR1 shRNA endothelial cell line would serve as a great candidate to generate PAR1 wildtype or CC/AA mutant stable cell lines.

We generated knockdown-rescue endothelial stable cell lines using PAR1 shRNA EA.hy926 cells, which are depleted of endogenous PAR1 expression. We

first approached this by generating FLAG PAR1 wildtype (WT) and palmitoylation-deficient (CC/AA) mutants that harbored mutations at the PAR1 shRNA targeting site, thus rendering them siRNA resistant. The PAR1 shRNA endothelial cells were then transduced with these siRNA resistant PAR1 WT or CC/AA mutants, and selected for stable expression. We confirmed expression of siRNA resistant receptors with immunoprecipitation, and determined that WT and CC/AA receptors migrated as a major species between ~50 and 75kDa (Fig 2.6B) consistent with endogenously expressed PAR1 (Fig 2.6A). A cell surface ELISA against the FLAG epitope indicates that receptors are expressed at the cell surface (Fig 2.6C). Furthermore, immunofluorescence staining with a FLAG antibody indicates that receptors are expressed on the cell surface as well as within internal punctate structures (Fig 2.6D).

We first utilized these knockdown-rescue stable cell lines to confirm the ubiquitination studies done in the PAR1 overexpression HeLa cell system (Fig 2.5A). In a manner similar to what was observed in HeLa cells, PAR1 WT exhibited robust agonist induced ubiquitination in endothelial cells (Fig 2.5B, lanes 1-3). However, the PAR1 CC/AA mutant failed to display agonist-induced ubiquitination (Fig. 2.5B, lanes 4-6). These studies support the role of PAR1 palmitoylation modulating receptor ubiquitination in a physiologically relevant cell type. Generation of these stable cell lines will be useful in examining signaling pathways in a physiologically relevant cell type, as well as confirming other studies done with overexpression systems.

2.5 Conclusions

Given the importance of PAR1 post-translational modifications in the fidelity of thrombin signaling, we sought to determine whether PAR1 is modified by palmitoylation and the function of such a posttranslational modification. In the present chapter, we demonstrate for the first time that PAR1 is palmitoylated on highly conserved C-tail cysteine residues. A palmitoylation-deficient PAR1 mutant is trafficked to the cell surface and fully glycosylated, indicating that palmitoylation is not required for biogenesis of a functional receptor. Additionally, PAR1 palmitoylation is important for proper receptor phosphorylation following a stimulus with a peptide agonist. In the absence of palmitoylation, PAR1 is also defective in agonist-induced ubiquitination. Taken together, these data suggest that palmitoylation of PAR1 is a crucial component for activated receptor post-translational modifications.

In addition to PAR1, several other GPCRs have been shown to undergo palmitoylation at conserved juxtamembrane C-tail cysteines, including the related PAR2 (24,25). However, not all GPCRs are palmitoylated (26) and some GPCRs contain proximal as well as distal palmitoylation sites, suggesting that multiple intracellular loops may be formed within the C-tail domain of particular GPCRs (27,28) (Fig 1.6). Palmitoylation is a dynamic and reversible posttranslational modification that can be modulated. Agonist stimulation induces an increase in palmitoylation of the β 2-adrenergic receptor (11) and other GPCRs (8), indicating that receptor palmitoylation is a regulated process. However, the thromboxane A2 receptor TP β isoform (26) and the 5-HT_{1A} serotonin receptor (29) appear to be palmitoylated constitutively and not affected by agonist stimulation. Although we failed to observe

any change in [^3H]palmitate incorporation following agonist activation of PAR1 expressed in HeLa cells or endogenous PAR1 expressed natively in endothelial cells, we cannot exclude the possibility that PAR1 is modified dynamically by palmitoylation and de-palmitoylation at steady state, since it would not be evident under the metabolic labeling conditions employed in our studies.

Palmitoylation has been shown to be important for the phosphorylation of some GPCRs (30-32). A palmitoylation-deficient 5-HT_{4a} receptor mutant has increased basal and agonist-induced phosphorylation, resulting in enhanced desensitization and β -arrestin mediated endocytosis (30). In a similar manner, a palmitoylation-deficient β 2-adrenergic receptor mutant exhibited enhanced basal phosphorylation, a consequence of exposing a PKA phosphorylation site that is adjacent to the palmitoylation site (11). Another example of enhanced basal phosphorylation in the absence of palmitoylation was observed with the adenosine A3 receptor (33). However, palmitoylation-deficiency also resulted in reduced phosphorylation for the CCR5 chemokine and V_{1a} vasopressin receptors, similar to the effect we observed with PAR1 (12,32). These studies suggest that palmitoylation of the C-tail could be important for proper GRK association. However there is also evidence that palmitoylation is not necessarily required for phosphorylation, as a palmitoylation-deficient V₂ receptor mutant is phosphorylated similarly to WT (34). Palmitoylation of GPCRs has the potential regulate receptor phosphorylation state through a diverse array of mechanisms that either induce or reduce GPCR phosphorylation. However, the effect of receptor phosphorylation in the absence of palmitoylation depends upon the specific receptor being studied.

We have demonstrated that PAR1 palmitoylation is critical for receptor ubiquitination in both HeLa and endothelial cell systems (Fig 2.5). There are a few examples in the literature of a relationship between palmitoylation and ubiquitination for transmembrane proteins (16-18,35). However, the role of palmitoylation in the context of regulating receptor ubiquitination has not been extensively examined for a GPCR. It is possible that palmitoylation could regulate the accessibility of the C-tail to ubiquitin ligases, thus allowing for proper receptor ubiquitination. Recently, our lab has demonstrated a novel role for agonist-induced ubiquitination in p38 MAPK signaling (36). Thus, the role of PAR1 palmitoylation in regulating this signaling pathway has been examined and will be discussed in Chapter 4.

Taken together, these studies suggest that the conformation of the C-tail generated by palmitoylation is critical for recognition by GRKs and ubiquitin ligases for proper agonist-induced post-translational modifications. Modifications such as ubiquitination and phosphorylation occur on the intracellular loops and C-tail of the receptor (Fig 1.4), and thus could be more sensitive to changes in C-tail conformations due to the absence of palmitoylation. These concepts could be applied towards new ideas regarding the potential for PAR1 palmitoylation to regulate accessibility of adaptor proteins to the C-tail. The absence of palmitoylation could alter the conformation of the C-tail so that it exposes amino acid residues that are normally hidden when palmitoylation is present. In the case for PAR1, amino acid residues important for PAR1 trafficking are localized near the palmitoylation sites (Fig 1.4). Thus, the function of palmitoylation in regulating PAR1 trafficking is examined and further discussed in Chapter 2.

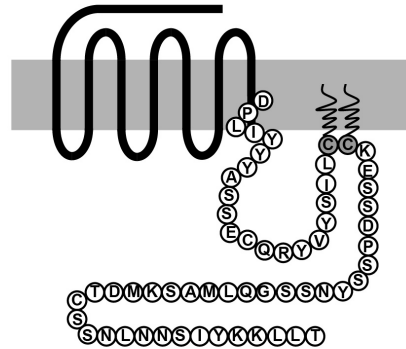
2.6 Acknowledgements

Chapter 2, in part is published as a manuscript entitled “Palmitoylation of protease-activated receptor-1 regulates adaptor protein complex-2 and -3 interaction with tyrosine-based motifs and endocytic sorting” in the *Journal of Biological Chemistry* (37). The dissertation author is the primary author who wrote the manuscript, directed experiments, and analyzed the data.

Chapter 2, in part is being prepared for publication: Canto Cordova I, Saucedo C., and Trejo J. Palmitoylation regulates PAR1 G-protein signaling pathways and thrombin-induced endothelial barrier permeability. *Journal TBD*. The dissertation author is the primary author who directed experiments and analyzed the data.

2.7 Figures

A



B

PAR1 species

H. sapiens
M. mulatta
C. longicaudatus
M. musculus
R. norvegicus
S. scrofa
B. taurus
B. grunniens mutus
H. glaber
M. putorius furo
D. rerio
X. laevis
P. hamadryas
G. gorilla gorilla
P. abelii
P. troglodytes
N. leucogenys
P. anubis
C. lupus familiaris
C. porcellus
C. jacchus
C. griseus
S. boliviensis boliviensis
L. africana
M. domestica
O. garnetii
F. catus
O. cuniculus
O. aries
E. caballus

C-tail sequences

```

..DPLIYYYASSECQRYVYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRYVYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRHLYGILCKESSD---PNSYNSTGQLMP SKM--DTCSSHLNNSIYKKLL
..DPLIYYYASSECQRHLYSILCKESSD---PNSCNSTGQLMP SKM--DTCSSHLNNSIYKKLL
..DPLIYYYASSECQKHLYSILCCRESSD---SNSCNSTGQLMP SKM--DTCSSHLNNSIYKKLL
..DPLIYYYASSECQRYLYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRHLFAILHCKESSD---PGSCNSSGQLMP SKM--DTCSSNLSSSLYKKLLT
..DPLIYYYASSECQRHLSAILHCKESSD---PGSCNSSGQLMP SKM--DTCSSNLSSSLYKKLLT
..DPLIYYYASSECQRHLYSILCKENVD---PNSYNSSGQLMASKM--DTSSTNMNNSIYKKLLT
..DPLIYYYASSECQRYLCSILCCRESSD---PSSYNSSGQLMASKM--DTCSSHLNNSIYKKLLT
..DPLIYFFGSSQCQKQVLAFLRCQGVPIERSAQFTSSTR--SSNL--ETLKSSVNSQ-YIKLM
..DPLIYYYASSQCQRYLSLLCKRKVSE---PGS--STGQLMSTAMKNDNCSTNAKSSIYKKLL
..DPLIYYYASSECQRYVYSILCKESSD---PSSNNSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRYVYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRYVYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRYVYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRYVYSILCKESSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQKHLYGILCKE GSD---PSSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRHLYSILCKENVD---PNSYNSSGQLMASKM--DTISANMNNSIYKKLLT
..DPLIYYYASSECQRYLYSILCCRESYN---PSSFNSSGQLVASKI--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRHLYGILCKESSD---PNSYNSTGQLMP SKM--DTCSSHLNNSIYKKLL
..DPLIYYYASSECQRYLYSILCCRESYD---PSSCNSSGQLVASKI--DTCSSNLNNSIYKKLLT
..DPMIYYYASSDCQRYLYSILCKESTD---PSSYNSSGQLTASKM--DTSSTTLNNSIYKKLLT
..DPLIYYYASSDCQRYLRNLLGORDSSD---PGSFNSSGQLMTRASKMDTCTNMNSIYKKLLT
..DPLIYYYASSECQRHLYSILCKESSS---ASSYNSSGQLMASKM--DTCSSNLNNSIYKKLLT
..DPLIYYYASSECQRYLYSILCKESSD---PSSYNSSGQLMASKV--DTCSSNLNSIYKKLLT
..DPLVYYYASSECQRQLRALLCCRESAD---GASYNSSGQLVPSKG--DTCSSHLAHSVYKKLLT
..DPLIYYYASSECQRHLSAVLHCKESSD---PSSCNSSGQLMP SKM--DTCSSNLSSSLYKKLLT
..DPLIYYYASSECQRHLYSIFCKESPD---PSSNNSGQLMASKM--DTCSSNLNSIYKKLL

```

Figure 2.1: PAR1 C-tail species sequence alignment. A) Snake diagram of the human PAR1 C-terminal tail (C-tail) starting from the DPXXY motif. The cysteine site(s) of palmitoylation are highlighted in gray. A list of entries of PAR1 were obtained from the NCBI data base. B) Thirty PAR1 sequences from different species were identified. The sequences were truncated immediately after the [N/D]PXXYL motif of the seventh transmembrane domain and aligned using BLAST.

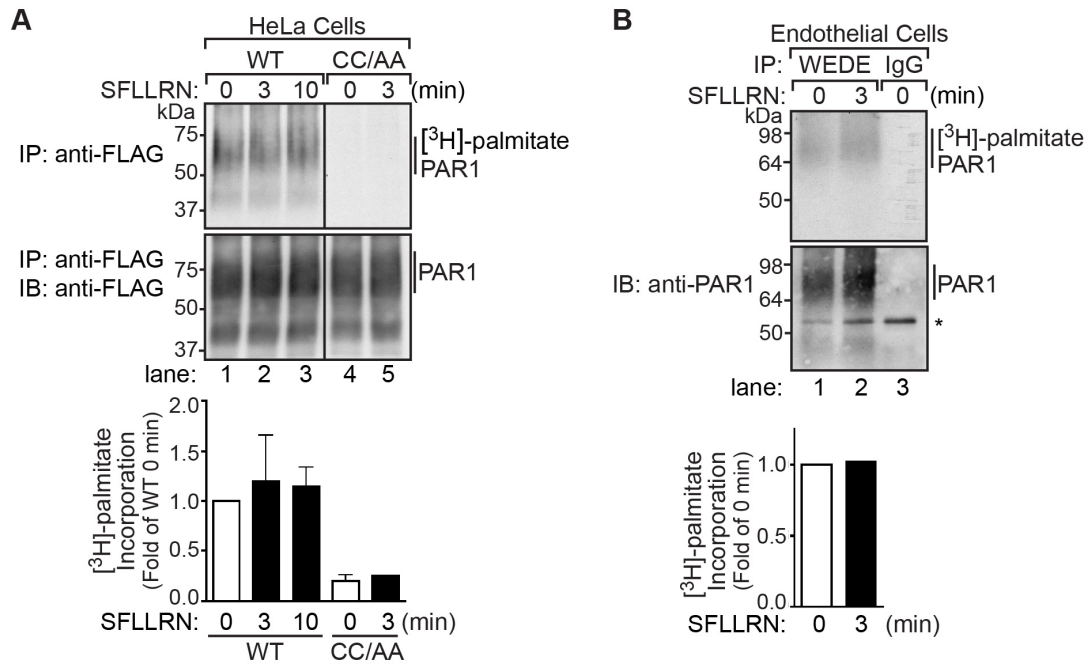


Figure 2.2: PAR1 is posttranslationally modified by palmitoylation. A) HeLa cells expressing FLAG-PAR1 wildtype (WT) or CC/AA mutant were labeled with [³H]palmitic acid and left untreated or treated with 100 μ M SFLLRN for various times at 37°C. Cells were lysed, processed and PAR1 was immunoprecipitated (IP) with M2 anti-FLAG antibody. IPs were resolved by SDS-PAGE and processed for fluorography to detect [³H]palmitate or immunoblotted with polyclonal anti-FLAG antibody to detect receptor. Three independent experiments were quantified and the amount [³H]palmitate incorporation, normalized to receptor in IPs was determined. The data shown (mean $\pm$ S.D.; $n=3$) is expressed as a fold of wildtype PAR1 0 min control. B) Human cultured endothelial EA.hy926 cells labeled with [³H]palmitic acid were stimulated with 100 μ M SFLLRN for 3 min at 37°C. Cells were lysed and immunoprecipitated with anti-PAR1 WEDE antibody and processed as described above. The asterisk indicates a non-specific band. The incorporation of [³H]palmitate was quantified, normalized to receptor in IPs and is expressed as the fold increase over untreated 0 min control.

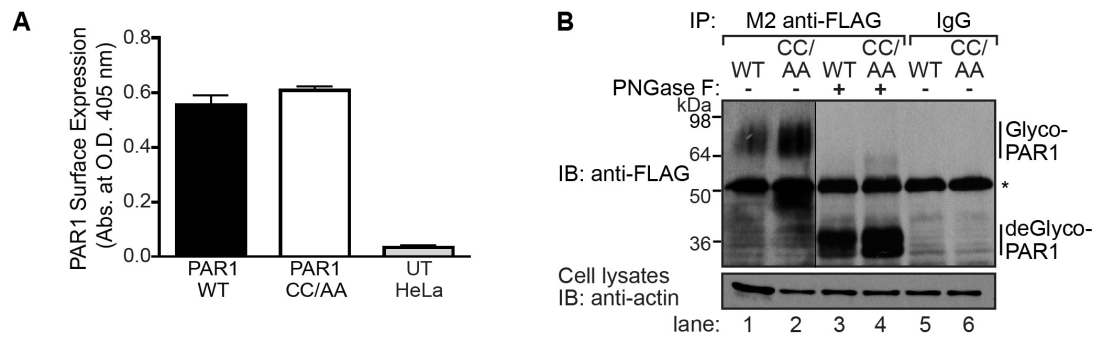


Figure 2.3: Palmitoylation is not required for cell surface expression or receptor glycosylation. A) Surface expression of FLAG-PAR1 wildtype (WT) or CC/AA mutant in HeLa cells compared to untransfected (UT) cells was quantified by ELISA. Data (mean $\pm$ S.D.) is representative of three independent experiments. B) HeLa cells expressing FLAG-PAR1 WT or CC/AA mutant were immunoprecipitated (IP) with M2 anti-FLAG antibody or IgG control. Immunoprecipitates were treated with or without PNGase F for 1 h at 37°C, resolved by SDS-PAGE and immunoblotted (IB) with rabbit polyclonal anti-FLAG antibody to detect PAR1. “Glyco-PAR1” indicates the fully glycosylated PAR1 and “deGlyco-PAR1” indicates the deglycosylated receptor following PNGase F treatment. The asterisk indicates a non-specific band detected by the anti-FLAG antibody. Cell lysates were immunoblotted for actin as a loading control.

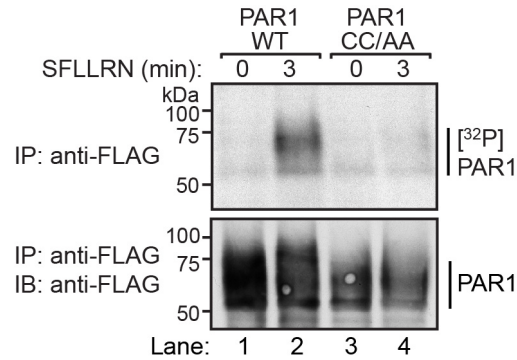


Figure 2.4: Palmitoylation is important for agonist-induced phosphorylation of PAR1. HeLa cells expressing PAR1 WT or CC/AA mutant were serum starved and labeled with $[^{32}\text{P}]$ orthophosphate. Cells were incubated in warm media with or without SFLLRN and then lysed. Total PAR1 was immunoprecipitated and resolved by SDS-PAGE, and processed for autoradiography to detect $[^{32}\text{P}]$ incorporation or immunoblotted with polyclonal anti-FLAG antibody to detect receptor

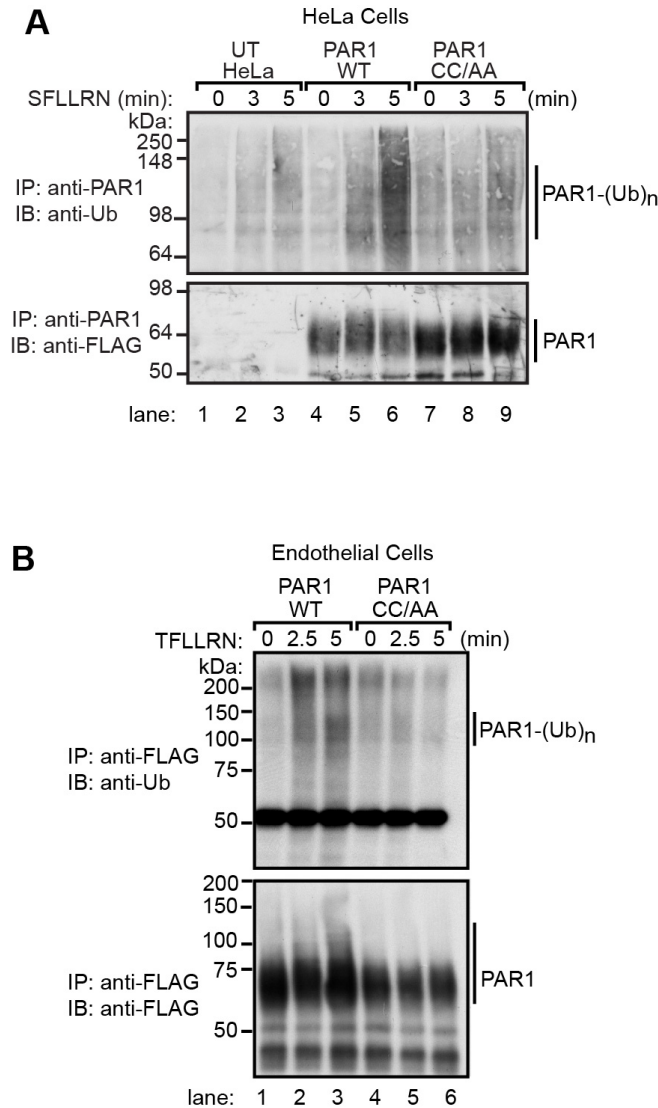


Figure 2.5: Palmitoylation is required for agonist-induced receptor ubiquitination. A) HeLa cells expressing FLAG PAR1 WT or CC/AA mutant were treated with 100 μ M SFLLRN for 3 or 5 minutes, then lysed and IPed with anti-WEDE antibody. Immunoprecipitates were resolved by SDS PAGE, transferred to PVDF membrane and immunoblotted with anti-FLAG polyclonal and P4D1 anti-ubiquitin antibodies. B) PAR1 shRNA EA.hy926 stable cell lines expressing siRNA resistant PAR1 WT or CC/AA mutant were treated with 100 μ M TFLLRN for 2.5 and 5 min, lysed and IPed with M2 anti-FLAG antibody. lysates were resolved by SDS PAGE, transferred to PVDF membranes, and immunoblotted as described in (A).

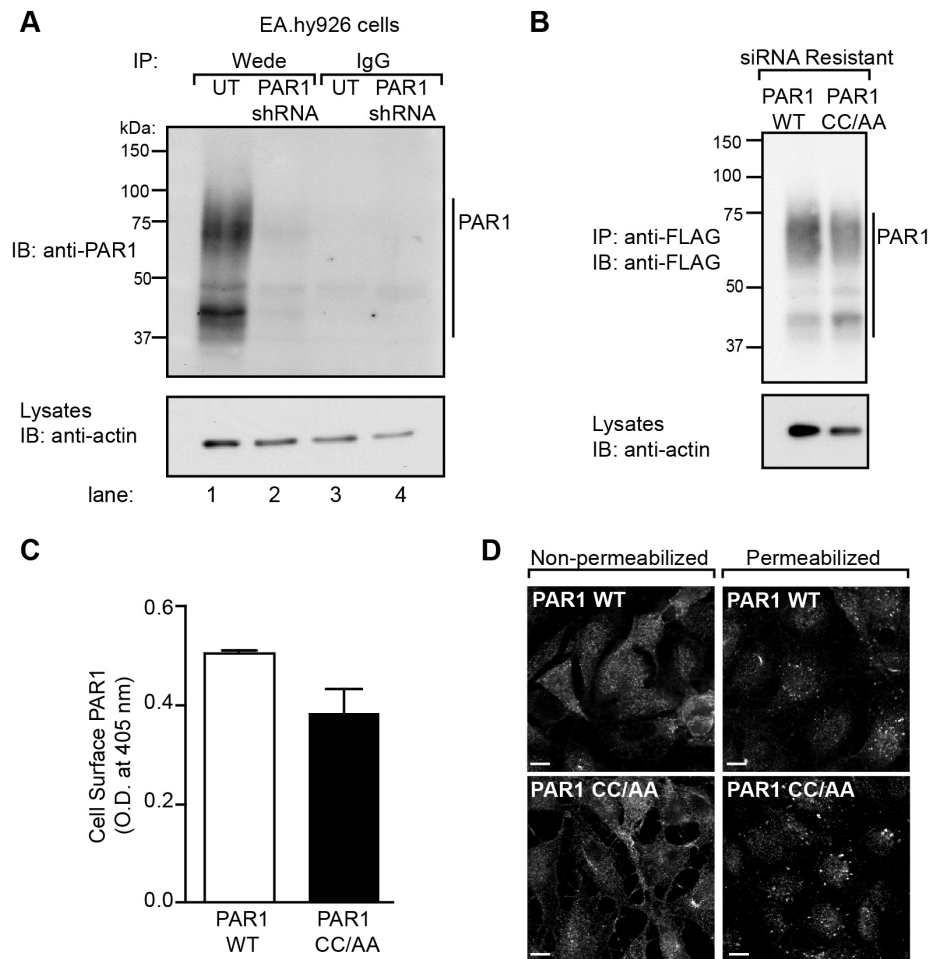


Figure 2.6: Characterization of palmitoylation-deficient PAR1 in endothelial cells. A) EA.hy926 cells were stably transduced with a PAR1 shRNA and immunoprecipitated (IP) with anti-PAR1 WEDE or IgG control. Immunoprecipitates were resolved by SDS PAGE, transferred to PVDF membrane and immunoblotted for total PAR1. Whole cell lysates were immunoblotted with anti-actin. B) PAR1 shRNA stable cell lines were transduced with siRNA resistant FLAG PAR1 wildtype (WT) or CC/AA mutant and selected for stable expression. Cells were lysed and IPed with M2 anti-FLAG antibody. IPs were resolved by SDS PAGE, transferred to PVDF membrane and immunoblotted with anti-FLAG polyclonal antibody. Total cell lysates were immunoblotted with anti-actin antibody. C) Cell surface expression of siRNA resistant PAR1 WT and CC/AA mutant stable cell lines, as quantified through cell surface ELISA with anti-FLAG antibody (mean $\pm$ SD). D) siRNA resistant FLAG PAR1 WT and CC/AA mutant EA.hy926 stable cells were fixed and treated with or without MeOH for permeabilization. Cells were stained with anti-FLAG antibody and imaged by confocal microscopy. Images shown are representative of two individual experiments (scale bar, 10 μ m).

2.8 References

1. Linder, M. E., and Deschenes, R. J. (2007) Palmitoylation: policing protein stability and traffic. *Nature reviews. Molecular cell biology* **8**, 74-84
2. Shahinian, S., and Silviu, J. R. (1995) Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. *Biochemistry* **34**, 3813-3822
3. Rocks, O., Gerauer, M., Vartak, N., Koch, S., Huang, Z. P., Pechlivanis, M., Kuhlmann, J., Brunsveld, L., Chandra, A., Ellinger, B., Waldmann, H., and Bastiaens, P. I. (2010) The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins. *Cell* **141**, 458-471
4. Rocks, O., Peyker, A., Kahms, M., Verveer, P. J., Koerner, C., Lumbierres, M., Kuhlmann, J., Waldmann, H., Wittinghofer, A., and Bastiaens, P. I. (2005) An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science* **307**, 1746-1752
5. Fukata, Y., and Fukata, M. (2010) Protein palmitoylation in neuronal development and synaptic plasticity. *Nature reviews. Neuroscience* **11**, 161-175
6. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**, 739-745
7. Iwanaga, T., Tsutsumi, R., Noritake, J., Fukata, Y., and Fukata, M. (2009) Dynamic protein palmitoylation in cellular signaling. *Prog Lipid Res* **48**, 117-127
8. Chini, B., and Parenti, M. (2009) G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats. *J Mol Endocrinol* **42**, 371-379
9. Swift, S., Leger, A. J., Talavera, J., Zhang, L., Bohm, A., and Kuliopulos, A. (2006) Role of the PAR1 receptor 8th helix in signaling: the 7-8-1 receptor activation mechanism. *The Journal of biological chemistry* **281**, 4109-4116

10. Zhang, C., Srinivasan, Y., Arlow, D. H., Fung, J. J., Palmer, D., Zheng, Y., Green, H. F., Pandey, A., Dror, R. O., Shaw, D. E., Weis, W. I., Coughlin, S. R., and Kobilka, B. K. (2012) High-resolution crystal structure of human protease-activated receptor 1. *Nature* **492**, 387-392
11. Moffett, S., Mouillac, B., Bonin, H., and Bouvier, M. (1993) Altered phosphorylation and desensitization patterns of a human beta 2-adrenergic receptor lacking the palmitoylated Cys341. *EMBO J* **12**, 349-356
12. Hawtin, S. R., Tobin, A. B., Patel, S., and Wheatley, M. (2001) Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *The Journal of biological chemistry* **276**, 38139-38146
13. Chen, C. H., Paing, M. M., and Trejo, J. (2004) Termination of protease-activated receptor-1 signaling by beta-arrestins is independent of receptor phosphorylation. *The Journal of biological chemistry* **279**, 10020-10031
14. Chen, B., Does, M. R., Grimsey, N., Canto, I., Barker, B. L., and Trejo, J. (2011) Adaptor Protein Complex-2 (AP-2) and Epsin-1 Mediate Protease-activated Receptor-1 Internalization via Phosphorylation- and Ubiquitination-dependent Sorting Signals. *The Journal of biological chemistry* **286**, 40760-40770
15. Raiborg, C., and Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458**, 445-452
16. Abrami, L., Leppla, S. H., and van der Goot, F. G. (2006) Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. *The Journal of cell biology* **172**, 309-320
17. Valdez-Taubas, J., and Pelham, H. (2005) Swf1-dependent palmitoylation of the SNARE Tlg1 prevents its ubiquitination and degradation. *EMBO J* **24**, 2524-2532
18. Abrami, L., Kunz, B., Iacovache, I., and van der Goot, F. G. (2008) Palmitoylation and ubiquitination regulate exit of the Wnt signaling protein LRP6 from the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5384-5389

19. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Mol Cell Biol* **26**, 3231-3242
20. Trejo, J., Altschuler, Y., Fu, H. W., Mostov, K. E., and Coughlin, S. R. (2000) Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *The Journal of biological chemistry* **275**, 31255-31265
21. Edgell, C. J., McDonald, C. C., and Graham, J. B. (1983) Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 3734-3737
22. Soto, A. G., and Trejo, J. (2010) N-linked glycosylation of protease-activated receptor-1 second extracellular loop: a critical determinant for ligand-induced receptor activation and internalization. *The Journal of biological chemistry* **285**, 18781-18793
23. Qanbar, R., and Bouvier, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol Ther* **97**, 1-33
24. Adams, M. N., Christensen, M. E., He, Y., Waterhouse, N. J., and Hooper, J. D. (2011) The role of palmitoylation in signalling, cellular trafficking and plasma membrane localization of protease-activated receptor-2. *PLoS One* **6**, e28018
25. Botham, A., Guo, X., Xiao, Y. P., Morice, A. H., Compton, S. J., and Sadofsky, L. R. (2011) Palmitoylation of human proteinase-activated receptor-2 differentially regulates receptor-triggered ERK1/2 activation, calcium signalling and endocytosis. *The Biochemical journal* **438**, 359-367
26. Reid, H. M., and Kinsella, B. T. (2007) Palmitoylation of the TPbeta isoform of the human thromboxane A2 receptor. Modulation of G protein: effector coupling and modes of receptor internalization. *Cellular signalling* **19**, 1056-1070
27. Zuckerman, D. M., Hicks, S. W., Charron, G., Hang, H. C., and Machamer, C. E. (2011) Differential regulation of two palmitoylation sites in the cytoplasmic

tail of the beta1-adrenergic receptor. *The Journal of biological chemistry* **286**, 19014-19023

28. Ponimaskin, E. G., Heine, M., Joubert, L., Sebben, M., Bickmeyer, U., Richter, D. W., and Dumuis, A. (2002) The 5-hydroxytryptamine(4a) receptor is palmitoylated at two different sites, and acylation is critically involved in regulation of receptor constitutive activity. *The Journal of biological chemistry* **277**, 2534-2546
29. Papoucheva, E., Dumuis, A., Sebben, M., Richter, D. W., and Ponimaskin, E. G. (2004) The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *The Journal of biological chemistry* **279**, 3280-3291
30. Ponimaskin, E., Dumuis, A., Gaven, F., Barthet, G., Heine, M., Glebov, K., Richter, D. W., and Oppermann, M. (2005) Palmitoylation of the 5-hydroxytryptamine4a receptor regulates receptor phosphorylation, desensitization, and beta-arrestin-mediated endocytosis. *Molecular pharmacology* **67**, 1434-1443
31. Gehret, A. U., Jones, B. W., Tran, P. N., Cook, L. B., Greuber, E. K., and Hinkle, P. M. (2009) Role of helix 8 of the thyrotropin-releasing hormone receptor in phosphorylation by G protein-coupled receptor kinase. *Molecular pharmacology* **77**, 288-297
32. Kraft, K., Olbrich, H., Majoul, I., Mack, M., Proudfoot, A., and Oppermann, M. (2001) Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization. *The Journal of biological chemistry* **276**, 34408-34418
33. Palmer, T. M., and Stiles, G. L. (2000) Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor. *Molecular pharmacology* **57**, 539-545
34. Sadeghi, H. M., Innamorati, G., Dagarag, M., and Birnbaumer, M. (1997) Palmitoylation of the V2 vasopressin receptor. *Molecular pharmacology* **52**, 21-29

35. Yin, H., Gui, Y., Du, G., Frohman, M. A., and Zheng, X. L. (2010) Dependence of phospholipase D1 multi-monoubiquitination on its enzymatic activity and palmitoylation. *The Journal of biological chemistry* **285**, 13580-13588
36. Grimsey, N. J., Aguilar, B., Smith, T. H., Le, P., Soohoo, A. L., Puthenveedu, M. A., Nizet, V., and Trejo, J. (2014) GPCR Ubiquitination Activates Non-canonical p38 Signaling and Regulates Endothelial Barrier Permeability. *submitted to Dev. Cell*.
37. Canto, I., and Trejo, J. (2013) Palmitoylation of Protease-activated Receptor-1 Regulates Adaptor Protein Complex-2 and -3 Interaction with Tyrosine-based Motifs and Endocytic Sorting. *The Journal of biological chemistry* **288**, 15900-15912

Chapter 3: PAR1 trafficking is regulated by palmitoylation

3.1 Abstract

Protease-activated receptor-1 (PAR1) is a G protein-coupled receptor (GPCR) for thrombin. Thrombin binds to and cleaves the N-terminus of PAR1, generating a new N-terminus that functions as a tethered ligand that cannot diffuse away. In addition to rapid desensitization, PAR1 trafficking is critical for the regulation of cellular responses. PAR1 displays constitutive and agonist-induced internalization. Constitutive internalization of unactivated PAR1 is mediated by the clathrin adaptor protein complex-2 (AP-2), which binds to a distal tyrosine-based motif localized within the C-tail domain. Once internalized, PAR1 is sorted from endosomes to lysosomes via AP-3 interaction with a second C-tail tyrosine motif proximal to the transmembrane domain. However, the regulatory processes that control adaptor protein recognition of PAR1 C-tail tyrosine-based motifs are not known. Here, we report that palmitoylation of PAR1 is critical for regulating proper utilization of tyrosine-based motifs and endocytic sorting. We show that a palmitoylation-deficient PAR1 mutant exhibits a marked increase in constitutive internalization and lysosomal degradation compared to wildtype receptor. Intriguingly, enhanced constitutive internalization of PAR1 is mediated by AP-2 and requires the proximal tyrosine-based motif, rather than the distal tyrosine motif used by wildtype receptor. Moreover, palmitoylation-deficient PAR1 displays increased degradation that is mediated by AP-3. These findings suggest that palmitoylation of PAR1 regulates proper utilization of

tyrosine-based motifs by adaptor proteins and endocytic trafficking, processes that are critical for maintaining appropriate expression of PAR1 at the cell surface.

3.2 Introduction

In the event of vascular injury and in thrombotic disease, the coagulant protease thrombin is generated and elicits cellular responses through the activation of protease-activated receptor-1 (PAR1). Thrombin cleaves the PAR1 N-terminus and unmask a cryptic ligand. This newly generated N-terminus functions as a tethered ligand that binds intramolecularly to the receptor to initiate signaling. Due to irreversible nature of PAR1 activation, signaling is tightly regulated. Activated PAR1 is rapidly phosphorylated on serine and threonine residues localized within the C-tail by G protein-coupled receptor kinases (GRKs), which promotes desensitization, a process that uncouples the receptor from G protein signaling. Activated PAR1 desensitization is also mediated by β -arrestin-1 but not β -arrestin-2 (1), which is recruited to the receptor independent of phosphorylation (2). Phosphorylation is not only important for desensitization, but is also crucial for receptor internalization (2).

PAR1 internalization is also important for regulating signaling responses. PAR1 displays constitutive and agonist-induced internalization that occur independent of β -arrestins (1). After activation, PAR1 is internalized, sorted predominantly to lysosomes and degraded, a process critical for termination of G protein signaling (3). This sorting is important in preventing activated PAR1 from returning to the surface and continuing to signal, an aberrant process that is found in invasive breast carcinoma (4). Unactivated PAR1 is constitutively internalized and recycled. This generates a pool of uncleaved PAR1 that can replenish the cell surface independent

of *de-novo* synthesis and is important for resensitization following thrombin exposure (5). The dysregulation of PAR1 trafficking contributes to the gain-of-function signaling observed in highly invasive breast carcinoma cells (6). Therefore understanding the mechanisms that regulate PAR1 trafficking are important.

Constitutive internalization of PAR1 is mediated by the clathrin adaptor protein complex-2 (AP-2) (7), a heterotetrameric complex comprised of α , β 2, μ 2 and σ adaptin subunits. AP-2 regulates internalization of transmembrane proteins via μ 2-adaptin subunit interaction with tyrosine-based YXX ϕ motifs, where Y is tyrosine, X denotes any amino acid and ϕ is a bulky hydrophobic residue (8,9). Tyrosine-based motifs can function as signals for endocytosis or lysosomal sorting depending on their location within the cytoplasmic domain relative to the plasma membrane (8,10). PAR1 contains two tyrosine-based motifs within the C-tail domain that appear to have distinct functions. We previously showed that the μ 2-adaptin subunit of AP-2 binds directly to the PAR1 C-tail distal tyrosine-based motif (YKKL) to facilitate constitutive internalization and cellular resensitization (5,7). We also showed that AP-3 regulated lysosomal degradation of activated PAR1 through interaction with a C-tail proximal tyrosine motif (YSIL) at the sorting endosome to facilitate degradation (11), a process that occurs independent of ubiquitination. Whether additional regulatory events control PAR1 sorting from endosomes to lysosomes is not known.

In addition to receptor internalization, compartmentalization of PAR1 into caveolae is critical for regulation of PAR1 signaling. PAR1 has the capacity to elicit opposing responses by two different proteases within the same cell. In endothelial cells, thrombin activation of PAR1 results in inflammatory responses. In contrast,

activation of PAR1 by activated protein C (APC) mediates anti-inflammatory responses and maintains endothelial barrier integrity (12,13). Caveolae are abundant in endothelial cells, which are cholesterol- and sphingolipid- rich membrane microdomains that form 60-80 nm invaginations through caveolin-1, a structural scaffolding protein (14). Our lab has shown that localization of PAR1 to caveolae is required for APC, but not thrombin activation of PAR1 signaling and endothelial barrier protection (12,15,16). When APC is bound to its co-receptor endothelial protein C receptor (EPCR), it directly activates PAR1 and elicits anti-inflammatory and endothelial barrier-protective responses, opposite of what is observed with thrombin-activation of PAR1 (17,18). It has been shown that palmitoylation is important for localization of some GPCRs to caveolae and lipid rafts (19,20). We sought to determine whether palmitoylation is important for targeting PAR1 to specific plasma membrane microdomains, and thereby facilitates protease-selective signaling.

Here, we demonstrate that palmitoylation is not critical for PAR1 localization to caveolae microdomains. However, we show that palmitoylation is important in regulating proper receptor expression at the cell surface. The palmitoylation-deficient PAR1 mutant exhibits an enhanced rate of constitutive internalization and lysosomal degradation compared to wildtype receptor. We further show that dysregulated trafficking of the palmitoylation-deficient PAR1 mutant is due to inappropriate utilization of the C-tail tyrosine-based motifs by the AP-2 and AP-3 sorting complexes. These studies indicate that palmitoylation of PAR1 is critical for regulating proper trafficking through the endocytic system, and defects in palmitoylation result in inappropriate PAR1 internalization from the cell surface and subsequent degradation.

3.3 Materials and Methods

Reagents and Antibodies – PAR1 peptide agonist SFLLRN was synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography at Tufts University Core Facility (Boston, MA). Rabbit polyclonal anti-FLAG antibody, mouse monoclonal M1 anti-FLAG antibody and mouse monoclonal anti- β -actin were purchased from Sigma-Aldrich (Saint Louis, MO). Anti-early endosomal antigen-1 (EEA1) antibody, anti- μ 2 adaptin AP-50 and anti- δ -subunit AP-3 antibody were obtained from BD Biosciences (San Jose, CA). Lysosomal-associated membrane protein 1 (LAMP1) antibody was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse and goat anti-rabbit antibodies were from Bio-Rad Laboratories (Hercules, CA). Alexa Fluor® 488- and 594-conjugated goat anti-mouse and anti-rabbit antibodies were obtained from Invitrogen (Carlsbad, CA).

cDNAs and Cell Lines – A cDNA plasmid encoding an N-terminal FLAG-tagged human PAR1 and tyrosine-based motif mutants cloned into pBJ vector were previously described (7,21). A PAR1 mutant in which cysteine (C) residues at positions 387 and 388 were mutated to alanines (A) was generated by quick-change site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). A PAR1 wildtype (WT) fused to yellow fluorescent protein (YFP) cloned into pRK6 vector was generously provided by Dr. Jean-Philippe Pin (Montpellier University). The PAR1 CC/AA-YFP mutant was generated by quick-change site-directed mutagenesis. A plasmid encoding μ 2-adaptin with a C-terminal *Renilla reniformis* luciferase (Rluc) cloned into pcDNA3 was generated using standard subcloning techniques. All cDNA

plasmids were subjected to dideoxy sequencing (Retrogen, San Diego, CA). HeLa cells stably expressing FLAG-tagged PAR1 CC/AA mutant were generated and maintained as previously described (21).

Cell Transfections – HeLa cells were transiently transfected with FLAG-PAR1 WT and mutants using polyethylenimine (Polysciences, Warrington PA) or JetPrime (Polyplus-transfection Inc., New York NY) reagents, according to the manufacturer's instructions. COS7 cells were transiently transfected with PAR1 WT-YFP or PAR1 CC/AA-YFP and μ 2-Rluc using FuGENE 6 (Promega, Madison WI). HeLa cells were transiently transfected with non-specific, μ 2-adaptin or δ -adaptin siRNAs using Oligofectamine (Invitrogen) or JetPrime according to the manufacturer's instructions. The non-specific siRNA 5'-CTACGTCCAGGAGCGCACC-3', and μ 2 siRNA 5'-GTGGATGCCTTTCGGGTCA-3' were synthesized by Thermo Scientific Dharmacon Inc. (Lafayette, CO). The δ -adaptin SMARTpool siRNAs were purchased from Dharmacon Inc.

Immunofluorescence Confocal Microscopy – HeLa cells expressing FLAG-PAR1 WT or mutants were prelabeled with anti-FLAG polyclonal antibody for 1 h at 4°C and incubated in media at 37°C for various times. Cells were fixed, permeabilized, and immunostained with anti-EEA1 or anti-LAMP1 antibodies followed by species-specific secondary antibodies conjugated to Alexa Fluor® 488 or 594 and imaged by confocal microscopy as described (22). Images were acquired using an Olympus DSU spinning disk confocal microscope configured with a PlanApo 60X oil objective and Hamamatsu ORCA-ER digital camera. Fluorescent images of X-Y sections at 0.28 μ m were collected using Intelligent Imaging Innovations Slidebook

4.2 Software (Denver, CO). The final composite images were created using Adobe Illustrator CS5 (San Jose, CA). Pearson's correlation coefficients (r) were determined using SlideBook 4.2 software and derived from an algorithm that provides a quantifiable analysis of degree of overlap based on pixel intensity of red and green fluorescence signals. A value of 1 indicates that fluorescent signals are perfectly correlated, whereas a value of 0 indicates that the signals are uncorrelated (23).

Antibody Uptake ELISA – The internalization of PAR1 was assessed by examining the uptake of antibody-bound to PAR1 using a sandwich ELISA as previously described (24,25). Briefly, HeLa cells stably expressing FLAG-PAR1 WT or CC/AA mutant were seeded at 1.0×10^5 cells/well in 24-well plates and grown overnight. Cells were serum-starved, labeled with M1 anti-FLAG antibody for 1 h at 4°C, washed and then incubated in media with or without agonist for varying times at 37°C. Remaining surface-bound antibody was stripped with 1 mM EDTA-PBS at 4°C. Cells were lysed and processed using a sandwich ELISA and the HRP substrate 1 Step 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Thermo Fisher, Rockford, IL) as described (24,25). After 20 min, the optical density (O.D.) of an aliquot was measured at 405 nm using a Molecular Devices SpectraMax Plus spectrophotometer (Sunnyvale, CA).

PAR1 Degradation Assays – HeLa cells seeded in 24-well plates were transiently transfected with FLAG-PAR1 WT or CC/AA mutant. Cells were serum-starved and then incubated with 10 μ M cycloheximide (CHX) for various times, lysed in 2X SDS sample buffer containing 125 mM Tris-HCl at pH 6.8, 20% v/v glycerol, 10% w/v SDS, 0.02% w/v bromophenol blue. Cell lysates were resolved by SDS-

PAGE, transferred to membranes and immunoblotted with polyclonal anti-FLAG antibody.

BRET Assays – COS7 cells were seeded at 2.5×10^5 cells/well in 6-well plates. Cells were transfected with 100 μ g of μ 2-adaptin Rluc and varying amounts of PAR1 WT-YFP or CC/AA-YFP mutant. Serum-starved cells were washed, dissociated using a non-enzymatic solution Cellstripper™ (Mediatech Inc., VA), resuspended in PBS containing 0.5 mM MgCl_2 and 0.1% glucose and counted. Cells ($\sim 4 \times 10^4$ in 80 μ l) were transferred to 96-well plates in triplicate for each condition. The fluorescence intensity of YFP was examined by excitation at 485 nm and emission at 535 nm using a Berthold Tristar LB 941 microplate reader (Berthold Technologies, GmbH & Co. Germany). The coelenterazine H (Biotium Inc., CA) substrate was added to cells at a final concentration of 5 μ M. After an 8 min delay, Rluc and YFP signals were determined using 480 nm and 530 nm filters, respectively. The BRET ratio was calculated as (emission at 530 nm)/(emission at 480 nm) and net BRET signal determined by subtracting the background BRET, which is the BRET ratio from cells expressing Rluc construct only. Total luminescence was measured by integrating the signal for 1 sec/well without filter selection. Data were fitted with non-linear regression using GraphPad Prism 4.0 software (GraphPad Inc., La Jolla, CA).

PAR1 Biotinylation Assays – HeLa cells were seeded in 6-well plates and transfected with FLAG PAR1 WT or CC/AA mutant. Serum-starved cells were labeled with 0.3 mg/ml EZ-link Sulfo-NHS-SS-biotin (Thermo Fisher, Rockford, IL) in PBS, pH 8.0 for 30 min on ice. Unbound biotin was quenched with 50 mM Tris HCl, pH 7.5, 275 mM NaCl, 6 mM KCl, and 2 mM CaCl_2 and cells were either left on ice or

warmed to 37°C. To remove the biotin label from the cell surface, cells were incubated with 50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 1% BSA in PBS, pH 8.7 for 10 min on ice, with gentle agitation, three times. Cells were washed three more times with PBS, pH 7.4 before further incubation at 37°C. Cells were lysed in buffer containing 50 mM Tris HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 supplemented with protease inhibitors as described above. Equivalent amounts of lysates were incubated with streptavidin-conjugated agarose beads (Thermo Fisher, Rockford, IL) overnight at 4°C and then washed three times with lysis buffer, eluted with 2X SDS sample buffer containing 0.2 M DTT resolved by SDS-PAGE and immunoblotted to detect PAR1.

Sucrose Fractionations – purification of caveolin enriched membrane fractions was performed as previously described (13). Briefly, EA.hy926 cells stably transduced with siRNA resistant FLAG PAR1 WT, and siRNA resistant CC/AA were plated in 10cm dishes at a density of 49.5×10^5 cells per dish. Cells were grown for 3 days and then lysed in cold 150mM sodium carbonate buffer (pH 11), supplemented with protease inhibitors: 100 µg/ml PMSF, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 µg/ml benzamidine, and 1 µg/ml soybean trypsin inhibitor. Cell lysates were subjected to 10 strokes in a Dounce homogenizer and then passed through an 18-gauge syringe 10 times. Lysates were then sonicated on ice six times in 10 sec bursts at 20% (Branson, Ultrasonics). Lysates were diluted 1:1 with 80% (wt/vol) sucrose in MES-buffered saline (MBS) and placed at the bottom of an ultracentrifuge tube. Lysates were overlaid with 6 ml of 35% (wt/vol) sucrose/MBS, followed by 4ml of 5% (wt/vol) sucrose/MBS. Lysates were centrifuged at 36,000 RPM at 4°C for 20 hrs using a Beckman L7-65 ultracentrifuge and a S41 rotor

(Beckman Coulter). 1ml gradient fractions were collected sequentially. Aliquots of fractions were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-cav1, anti-EEA, and anti-FLAG antibodies.

Data Analysis – The data were analyzed using GraphPad Prism 4.0 software. Statistical analysis was determined by performing one-way ANOVA and Dunnett's post-hoc test, or two-way ANOVA and Bonferroni post-hoc test.

3.4 Results

3.4.1 Palmitoylation is not required for PAR1 caveolae localization

Palmitoylation has been shown to be important for localization of some GPCRs to caveolae microdomains (19,20,26,27). PAR1 localization to caveolae is particularly important because PAR1 cleavage by activated protein C (APC) requires localization of endothelial protein C receptor (EPCR) to caveolin-1 enriched microdomains (15,16,18). APC-activation of PAR1 elicits endothelial barrier stability through β -arrestin mediated dishevelled-2 activation, and prevents thrombin-induced RhoA mediated barrier disruption (13). Given the physiological relevance of PAR1 localization to the caveolae, we examined if palmitoylation was important for compartmentalization of the receptor. We assessed receptor localization in EA.hy926 endothelial cells, which are enriched in caveolae microdomains. We utilized PAR1 shRNA endothelial cells that were rescued with siRNA FLAG PAR1 wildtype or CC/AA mutant and isolated caveolae fractions using sucrose density centrifugation. PAR1 WT localized to caveolae enriched fractions, as was previously reported for endogenous receptor (13) (Fig 3.1A). In a similar manner, the palmitoylation-deficient

PAR1 mutant also localized to caveolae fractions (Fig 3.1B). Taken together, these data suggest that palmitoylation is not required for PAR1 caveolae localization.

3.4.2 Palmitoylation-deficient PAR1 exhibits enhanced constitutive internalization

The trafficking behavior of palmitoylation-deficient PAR1 was examined to further characterize the function of palmitoylation. To assess constitutive internalization, FLAG-PAR1 WT and CC/AA mutant expressed in HeLa cells were labeled with M1 anti-FLAG antibody for 1 h at 4°C to ensure only receptors at the cell surface bound antibody. Unbound antibody was removed by washing and constitutive internalization of PAR1 was determined following incubation for various times at 37°C by ELISA as we previously described (25). After a 30 min incubation, ~10% of PAR1 WT was detected in an intracellular compartment (Fig. 3.2A), which is consistent with the extent of wildtype receptor constitutive internalization previously reported in these cells (25). In contrast, PAR1 CC/AA mutant exhibited enhanced constitutive internalization with ~30% receptor accumulation measured at 30 min (Fig. 3.2A). However, agonist-induced PAR1 WT and CC/AA mutant internalization was comparable (Fig. 3.2B). Immunofluorescence confocal microscopy confirmed enhanced constitutive internalization of the palmitoylation-deficient PAR1 mutant. HeLa cells expressing PAR1 WT or CC/AA mutant were labeled with antibody at 4°C and fixed (0 min) or incubated at 37°C for 10 min and then fixed, permeabilized and co-stained for EEA1, a marker of early endosomes (Fig. 3.3). Both PAR1 WT and CC/AA mutant localized to the cell surface at 0 min (Fig. 3.3). However, after a 10 min incubation at 37°C, a greater number of EEA1-positive puncta containing PAR1

CC/AA mutant were evident compared to wildtype receptor (Fig. 3.3), consistent with enhanced constitutive internalization. The difference in PAR1 CC/AA mutant colocalization with EEA1 compared to wildtype receptor was verified by determining Pearson's correlation coefficients (Fig. 3.3). These results suggest that palmitoylation is important for regulatory processes that control PAR1 constitutive internalization.

3.4.3 PAR1 CC/AA constitutive internalization is AP-2 dependent

To examine the function of AP-2 in constitutive internalization of the palmitoylation-deficient PAR1 mutant, we first examined whether AP-2 interacted with the receptor in living cells using BRET. BRET was assessed in COS7 cells transfected with a constant amount of the μ 2-adaptin fused to Rluc together with varying amounts of PAR1 WT or CC/AA mutant tagged with YFP at the C-terminus. A hyperbolic increase in BRET was detected as the ratio of PAR1 WT-YFP or CC/AA-YFP to μ 2-adaptin Rluc was increased, suggesting that receptor- μ 2-adaptin protein interaction is specific (Fig. 3.4A). In order to evaluate the relative affinity of PAR1 WT *versus* CC/AA mutant to AP-2, the BRET₅₀ values were determined and were not significantly different (Fig. 3.4A). However, the maximum BRET signal observed with PAR1 CC/AA-YFP and μ 2-adaptin Rluc was greater than that exhibited by PAR1 WT-YFP and μ 2-adaptin Rluc. These data suggest that PAR1 CC/AA mutant binds to AP-2 with a similar affinity as wildtype PAR1, but the orientation is likely different.

To further investigate AP-2 function, we examined whether constitutive internalization of palmitoylation-deficient PAR1 is AP-2 dependent like that shown for the wildtype receptor (7). HeLa cells expressing similar amounts of FLAG-PAR1 WT

or CC/AA mutant on the cell surface were transiently transfected with non-specific siRNAs or siRNAs targeting the μ 2-adaptin subunit, which disrupt AP-2 function. Cells transfected with μ 2-adaptin subunit siRNA exhibited a marked decrease in μ 2-adaptin expression compared to non-specific control transfected cells (Fig. 3.4B). As expected, PAR1 WT constitutive internalization was inhibited in cells lacking AP-2 expression compared to non-specific siRNA transfected control cells assessed after a 15 min incubation at 37°C (Fig. 3.4C), consistent with previous reports (7). PAR1 CC/AA mutant exhibited enhanced constitutive internalization compared to wildtype receptor in control siRNA transfected cells, which was virtually ablated in AP-2 deficient cells (Fig. 3.4C). Immunofluorescence confocal microscopy confirmed that AP-2 mediates constitutive internalization of PAR1 WT or CC/AA mutant. HeLa cells expressing FLAG-PAR1 WT and CC/AA mutant transfected with siRNAs, were prelabeled with anti-FLAG antibody, incubated at 37°C for 15 min, fixed and processed for microscopy. In non-specific siRNA transfected cells, PAR1 WT and CC/AA mutant localized to endocytic vesicles (Fig. 3.4D), whereas the receptors failed to internalize in cells deficient in AP-2 expression (Fig. 3.4D). These findings suggest that AP-2 regulates constitutive internalization of palmitoylation-deficient PAR1.

3.4.4 Palmitoylation is important for proper utilization of tyrosine based sorting motifs

The PAR1 C-tail domain contains two tyrosine-based YXX ϕ sorting motifs that conform to canonical AP-2 binding sites (Fig. 3.5) (8). We previously showed that the μ 2-adaptin subunit of AP-2 binds directly to the distal tyrosine YKKL motif to mediate

PAR1 constitutive internalization (7). To further define the function of AP-2 in enhanced PAR1 constitutive internalization, we generated receptor CC/AA variants harboring mutations in the proximal and distal tyrosine-based motifs (Fig. 3.5). HeLa cells expressing FLAG-PAR1 wildtype and mutants labeled with anti-FLAG antibody were incubated at 37°C for 30 min and constitutive internalization was examined by confocal microscopy and cell surface ELISA. The PAR1 CC/AA mutant exhibited a marked increase in internalization compared to wildtype receptor as assessed by examining receptor-positive vesicles that colocalized with EEA1 using confocal microscopy (Fig. 3.6A). Enhanced PAR1 CC/AA mutant constitutive internalization was confirmed by quantifying the amount of receptor remaining at the cell surface by ELISA (Fig. 3.7A). As expected, the PAR1 AKKAA mutant failed to internalize (Fig. 3.6B, 3.7B), consistent with a critical role for the distal tyrosine motif in mediating AP-2 binding and constitutive internalization of wildtype receptor (7). Remarkably, however, mutation of the distal tyrosine motif of the palmitoylation-deficient PAR1 CC/AA mutant failed to inhibit constitutive internalization (Fig. 3.6B and 3.7B). These findings suggest that in the absence of palmitoylation, the PAR1 distal tyrosine motif is no longer required for constitutive internalization, although PAR1 CC/AA+AKKAA constitutive internalization remains AP-2 dependent (Fig 3.8A).

We next examined the function of the PAR1 proximal tyrosine YSIL motif. Consistent with previous findings (7), PAR1 ASIA mutant exhibited a modest level of constitutive internalization that was comparable to wildtype receptor (Fig. 3.6C and 3.7C). In striking contrast, however, the palmitoylation-deficient PAR1 CC/AA mutant in which the proximal tyrosine motif was mutated to ASIA displayed impaired

internalization (Fig. 3.6C and 3.7C), suggesting that the proximal motif is now critical for constitutive internalization in the absence of palmitoylation. A small amount of PAR1 CC/AA+ASIA constitutive internalization was detected by immunofluorescence microscopy (Fig. 3.6C), but was completely abolished in AP-2 deficient cells (Fig. 3.8B). In addition, constitutive internalization of PAR1 CC/AA + ASIA mutant containing a defective distal motif AKKAA was virtually ablated (Fig. 3.6D and 3.7D). Together these findings indicate that palmitoylation of PAR1 is important for proper utilization of C-tail tyrosine-based sorting motifs and regulation of receptor constitutive internalization.

3.4.5 Palmitoylation deficient PAR1 is basally degraded more rapidly than wildtype PAR1

We recently showed that sorting of internalized PAR1 from early endosomes to lysosomes is mediated by AP-3 interaction with the receptor C-tail proximal tyrosine YSIL motif (11). Given the importance of PAR1 palmitoylation in regulating tyrosine-based motif accessibility, we examined its function in PAR1 lysosomal degradation. We first compared the basal rate of FLAG-PAR1 WT and CC/AA mutant degradation in HeLa cells by quantifying the amount of receptor remaining at various times after incubation with cycloheximide, a protein synthesis inhibitor. In contrast to wildtype receptor, the palmitoylation-deficient PAR1 mutant exhibited an enhanced rate of degradation with ~75% of receptor degraded at 180 min (Fig. 3.9 B and D), compared to ~25% degradation of wildtype receptor (Fig. 3.9A and D). To assess the contribution of the proximal tyrosine-based motif, we examined the degradation rate of the PAR1 CC/AA + ASIA mutant. Remarkably, mutation of the PAR1 proximal

tyrosine motif resulted in a marked decrease in basal turnover of the receptor that was comparable to wildtype PAR1 (Fig. 3.9 C and D). We next examined PAR1 WT and mutant colocalization with lysosomal-associated membrane protein 1 (LAMP1) to confirm that receptors are trafficked to lysosomes. PAR1 WT, CC/AA and CC/AA+ASIA were incubated with anti-FLAG antibody for 1 h at 4°C to prelabel the surface receptor cohort. Cells were then incubated for 30 min at 37°C in media without agonist and colocalization with LAMP1 was determined by confocal microscopy. PAR1 WT, CC/AA and CC/AA +ASIA mutant exhibited colocalization with LAMP1 (Fig. 3.10A-C). However, the extent of PAR1 CC/AA mutant colocalization with LAMP1 was greater compared to PAR1 WT and PAR1 CC/AA + ASIA mutant as determined by Pearson's correlation coefficients (Fig. 3.9D). These findings suggest that palmitoylation regulates sorting of PAR1 from endosomes to lysosomes.

We next determined whether the enhanced rate of palmitoylation-deficient PAR1 mutant degradation resulted from increased internalization or sorting of internalized receptors from endosomes to lysosomes by directly examining degradation rates of internalized receptors. In these experiments, the surface cohort of PAR1 WT or CC/AA mutant were labeled with biotin at 4°C, and either left on ice or warmed to 37°C for 30 min to promote constitutive internalization. After PAR1 internalization, biotin remaining bound to the cell surface was removed and cells were incubated for various times at 37°C. Cells were then lysed and the amount of internalized biotinylated PAR1 remaining was determined and represented as the "intracellular cohort". Both the surface cohort of PAR1 WT and CC/AA mutant

exhibited substantial biotinylation, indicated as 100% (Fig. 3.11A, lanes 1 and 7) and subsequently biotin was efficiently removed from the surface cohort with glutathione, shown as 0% (Fig. 3.11A, lanes 2 and 8). In PAR1 WT and CC/AA expressing cells labeled with biotin, incubation for 30 min at 37°C resulted in accumulation of intracellular receptor and is shown as the 0 min time point (Fig. 3.11A, lanes 3 and 9). In contrast to the intracellular pool of wildtype PAR1, the palmitoylation-deficient PAR1 CC/AA mutant displayed significant degradation after further incubation at 37°C for 30, 60 and 90 min (Fig. 3.11A and B, lanes 4-6 and 10-12). These data provide further evidence that palmitoylation of PAR1 is important for proper regulation of receptor lysosomal sorting.

To assess the function of AP-3 in PAR1 degradation, we used siRNA targeted against the δ -subunit of the AP-3 complex or nonspecific siRNA control and examined the rate of receptor degradation by immunoblotting. The loss of δ -adaptin expression results in degradation of other AP-3 components and disruption of AP-3 function (28). In non-specific siRNA transfected cells, PAR1 wildtype displayed a modest level of basal turnover (Fig. 3.12A and C, lanes 1-3) and was not significantly affected in cells deficient in δ -adaptin expression (Fig. 3.12A and C, lanes 4-6). In contrast, palmitoylation-deficient PAR1 mutant displayed an enhanced rate of turnover that was virtually abolished in cells lacking δ -adaptin expression (Fig. 3.12B and D). Taken together, these findings suggest that in the absence of palmitoylation, PAR1 exhibits enhanced lysosomal sorting that requires AP-3 function and an intact proximal tyrosine motif.

3.5 Conclusions

Palmitoylation serves distinct functions for different receptor types; however, the role of palmitoylation in regulation of PAR1 signaling or trafficking has not been previously investigated. We found that palmitoylation of PAR1 is critical for regulating appropriate adaptor protein interaction with C-tail tyrosine motifs and sorting through the endocytic pathway. We demonstrated that a palmitoylation-defective PAR1 mutant displayed enhanced constitutive internalization and an increased rate of lysosomal degradation. Moreover, AP-2 mediated enhanced internalization and interacted with the palmitoylation-deficient PAR1 mutant, but through a different tyrosine motif compared to wildtype receptor (7). The intracellular cohort of palmitoylation-deficient PAR1 also exhibited enhanced degradation, a process that required an intact tyrosine motif and AP-3. (8). In recent work, we discovered that AP-3 regulated lysosomal degradation of activated PAR1 through interaction with a C-tail proximal tyrosine motif YSIL at the sorting endosome to facilitate degradation (11). In the present study, we have now determined that AP-3-mediated lysosomal sorting of PAR1 is regulated by palmitoylation. These findings suggest that palmitoylation of PAR1 is critical for proper adaptor protein recognition of tyrosine-based motifs and endocytic sorting. Thus, palmitoylation of PAR1 is critical for retaining appropriate receptor expression at the cell surface, which is essential for important biological processes such as hemostasis and thrombosis (29) and embryonic development (30,31).

Palmitoylation has been suggested to play a role regulating accessibility of sorting motifs in GPCRs and other transmembrane proteins. Similarly to PAR1, the

thromboxane A2 receptor TP β isoform harbors a C-tail YX₃Ø motif that functions in constitutive internalization (32). Interestingly, TP β is constitutively palmitoylated at multiple C-tail cysteine residues, yet the YX₃Ø motif resides near the most proximal palmitoylated cysteine (33). Perturbation of TP β palmitoylation by mutation of the distal cysteine residues resulted in loss of constitutive internalization (33), suggesting that palmitoylation is important for the function of tyrosine motifs and constitutive internalization. This suggests that the secondary structure created by receptor palmitoylation is important for proper engagement of AP-2 with sorting motifs. In addition to PAR1 and TP β receptor, ~30 other mammalian GPCRs harbor canonical tyrosine-based motifs within their C-tail domain (34) and in some cases, nearby cysteine residues are known to be palmitoylated (35-41). Additionally, the yeast casein kinase Yck3p resides at the limiting membrane of the vacuole, an organelle equivalent to the mammalian lysosome, and requires both palmitoylation at its C-terminus and AP-3 binding to a canonical tyrosine YXXØ motif for proper sorting to the vacuole (42).

It is possible that plasma membrane insertion of palmitoylated tails on cysteine residues in the PAR1 C-tail could spatially regulate the proximity of YXXØ motifs to the plasma membrane, and thus regulate PAR1 trafficking. Interestingly, the palmitoylation sites of PAR1 are adjacent to one of the two YXXØ motifs in the C-tail. It has been suggested that purely endocytic YXXØ motifs are situated 10-40 residues away from a transmembrane domain, whereas lysosomal targeting YXXØ motifs are situated 6-9 residues away from a transmembrane (TM) domain (8). Additionally, the proximity of a tyrosine-based sorting motif to the plasma membrane has been

demonstrated to be an important feature for the proper trafficking of LAMP1 to lysosomes (10). We demonstrated that in the absence of palmitoylation, PAR1 undergoes robust constitutive internalization via the proximal YXXØ motif rather than the canonical distal YXXØ motif. It is possible that in the absence of palmitoylation, the proximal YXXØ motif is positioned 10 residues from the 7th TM domain, allowing for the engagement of AP-2 and enhanced constitutive internalization.

The studies summarized in this chapter demonstrate a novel function of palmitoylation for PAR1 trafficking. Palmitoylation of PAR1 is not required for sorting to caveolae, like other GPCRs. However, palmitoylation of the C-tail is important for proper engagement of adaptor-protein complexes with the receptor. We hypothesize that depalmitoylation of PAR1 occurs to allow AP-3 binding to the proximal tyrosine-based sorting motif to facilitate receptor sorting to the lysosome for degradation. However, we have not been able to detect a change in the palmitoylation status of the receptor using [³H]palmitic acid labeling, perhaps due to its low sensitivity. Applying other methods such as acyl-biotin exchange or click chemistry approaches might be useful in determining if PAR1 is depalmitoylated. In conclusion, GPCR palmitoylation may have a broad function in regulating adaptor protein interaction with sorting signals and likely to be important for maintaining appropriate expression of receptors on the cell surface.

3.6 Acknowledgements

Chapter 3, in part is published as a manuscript entitled “Palmitoylation of protease-activated receptor-1 regulates adaptor protein complex-2 and -3 interaction

with tyrosine-based motifs and endocytic sorting” in the *Journal of Biological Chemistry* (43). The dissertation author is the primary author who wrote the manuscript, directed experiments, and analyzed the data.

Chapter 3, in part is being prepared for publication: Canto Cordova I, Saucedo C., and Trejo J. Palmitoylation regulates PAR1 G-protein signaling pathways and thrombin-induced endothelial barrier permeability. *Journal TBD*. The dissertation author is the primary author who directed experiments and analyzed the data.

3.7 Figures

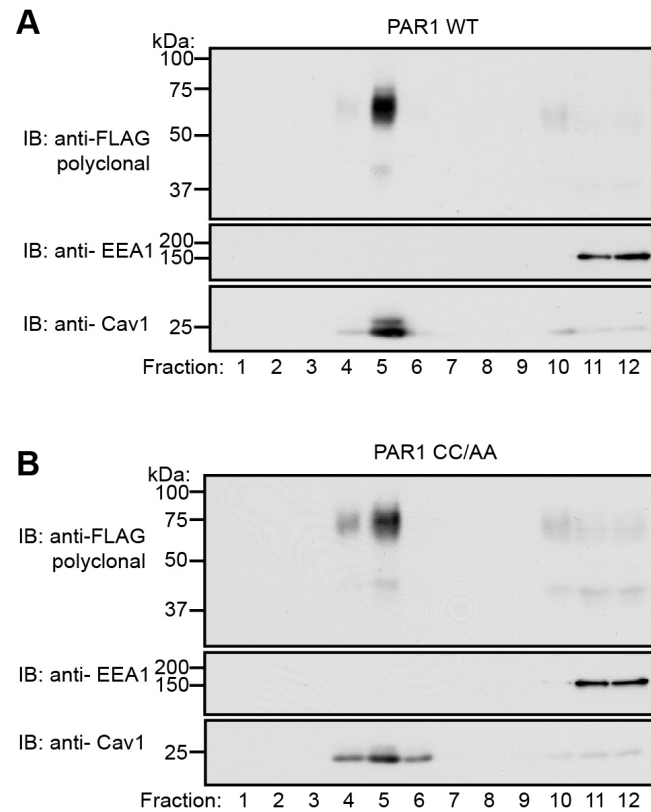


Figure 3.1: Palmitoylation is not required for PAR1 recruitment to caveolin-rich microdomains. PAR1 shRNA EA.hy926 cells stably expressing siRNA resistant PAR1 WT (A) or CC/AA mutant (B) were lysed, and caveolin-1-enriched fractions were isolated with detergent-free sucrose-gradient centrifugation. Aliquots from each of the 12 fractions were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-FLAG, anti- EEA1, and anti-caveolin-1.

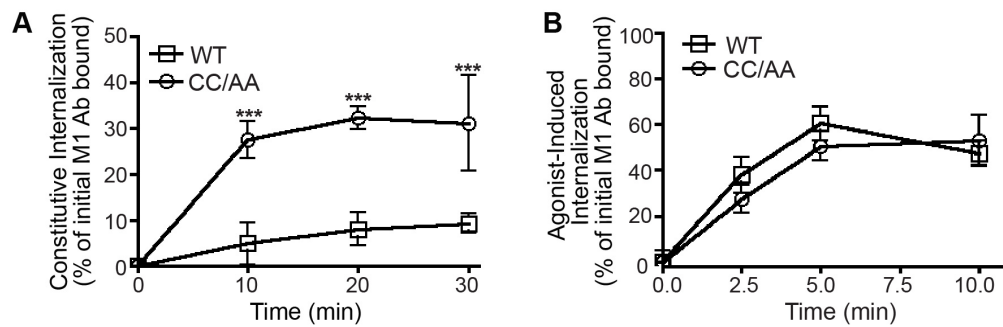


Figure 3.2: Palmitoylation-deficient PAR1 displays enhanced constitutive internalization. A) HeLa cells expressing FLAG-PAR1 WT or CC/AA mutant were prelabeled with M1 anti-FLAG at 4°C, then incubated in media without agonist for various times at 37°C. Internalized receptor bound antibody was quantified by sandwich ELISA. Data shown (mean $\pm$ S.D.; $n=3$) are expressed as a percentage of the initial receptor-bound M1 antibody and is the average of three separate experiments performed in triplicate. Differences in constitutive internalization between PAR1 WT and CC/AA mutant were significant (***, $P < 0.001$) as determined by two-way ANOVA and Bonferroni post-tests. B) HeLa cells stably expressing FLAG-PAR1 WT or CC/AA mutant were incubated with 100 μ M SFLLRN peptide agonist for various times 37°C and internalization quantified as described above. Data shown (mean $\pm$ S.D.; $n=3$) are expressed as a percentage of the initial receptor-bound M1 antibody and is representative of three individual experiments performed in triplicate.

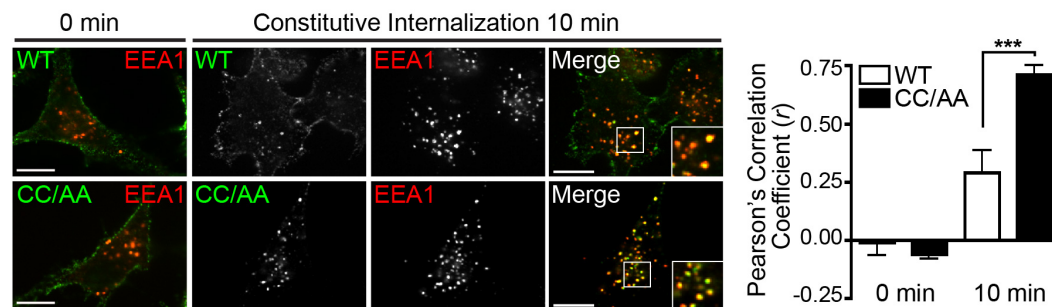


Figure 3.3: Palmitoylation deficient PAR1 is sorted to early endosomes. HeLa cells expressing FLAG-PAR1 WT or CC/AA were prelabeled with polyclonal anti-FLAG antibody at 4°C, then incubated for 30 min at 37°C. Cells were fixed, permeabilized, and co-stained for endogenous EEA1 and imaged by confocal microscopy. *Insets* are magnifications of the boxed areas. Images are representative of many cells examined in multiple independent experiments. Co-localization of PAR1 (*green*) and EEA1 (*red*) is indicated by the yellow color in the merged images and was quantified by determining Pearson's correlation coefficients (*r*) from seven different cells. The difference in PAR1 CC/AA *versus* WT colocalization with EEA1 at 10 min was statistically significant (***, $P < 0.001$) as determined by a Student's *t*-test. Scale bar, 10 mm.

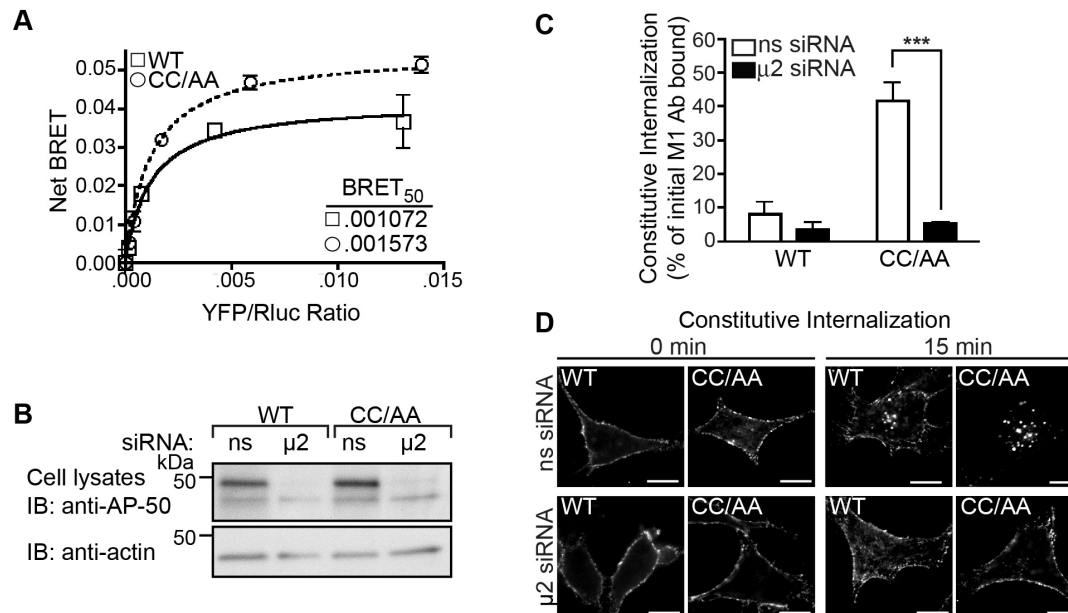


Figure 3.4: AP-2 mediates enhanced constitutive internalization of palmitoylation-deficient PAR1. A) COS7 cells were co-transfected with a fixed amount of μ 2-adaptin fused to Rluc and varying amounts of PAR1 WT-YFP or CC/AA-YFP and receptor- μ 2 interaction was assessed by BRET. The data (mean $\pm$ S.D.; $n=3$) shown are representative of three individual experiments. *Inset* shows BRET₅₀ values for PAR1 WT and CC/AA mutant. B) HeLa cells expressing FLAG-PAR1 WT or CC/AA mutant were transfected with siRNA targeting the μ 2-adaptin subunit of AP-2 or non-specific (ns) siRNA control. Cell lysates were immunoblotted for μ 2-adaptin expression, membranes were stripped and reprobed for actin. C) Cells expressing FLAG-PAR1 WT or CC/AA mutant were serum starved, prelabeled with M1 anti-FLAG antibody and then incubated for 15 min at 37°C. Internalized receptor bound antibody was quantified by sandwich ELISA. Data shown (mean $\pm$ S.D.; $n=3$) is expressed as a percentage of the initial receptor-bound M1 antibody and is representative of three separate experiments performed in triplicate. PAR1 CC/AA mutant constitutive internalization in ns-siRNA *versus* μ 2-siRNA transfected cells was significantly different (***, $P < 0.001$) as determined by two-way ANOVA and Bonferroni post-tests. D) HeLa cells expressing FLAG-PAR1 WT or CC/AA mutant were prelabeled with polyclonal anti-FLAG antibody and then incubated for 15 min at 37°C. Cells were fixed, permeabilized and immunostained for PAR1 and imaged by confocal microscopy. Scale bar, 10 μ m.

	Proximal		Distal
	<u>YXXØ</u>		<u>YXXØ</u>
PAR1 WT	..YASSECQRYVY ³⁸³ SIL ³⁸⁶ CKESSDPSSYNSSGQLMASKMDTCSSNLNNSIY ⁴²⁰ KKL ⁴²³ LT*		
PAR1 CC/AA	..YASSECQRYVY ³⁸³ SIL ³⁸⁶ AAKESSDPSSYNSSGQLMASKMDTCSSNLNNSIY ⁴²⁰ KKL ⁴²³ LT*		
	A ³⁸³ A ³⁸⁶		A ⁴²⁰ A ⁴²³ A ⁴²⁴
	“ASIA”		“AKKAA”

Figure 3.5: Palmitoylation sites are near tyrosine-based sorting motifs in the PAR1 C-tail. Alignment of PAR1 wildtype (WT) and CC/AA mutant C-tail amino acid residues. The PAR1 C-tail tyrosine-based YXXØ motifs are shown and superscripted numbers indicate the position of the critical tyrosine (Y) and leucine (L) residues that were mutated to alanine (A) to generate “ASIA” and “AKKAA” mutants. The asterisk indicates the end of the protein sequence.

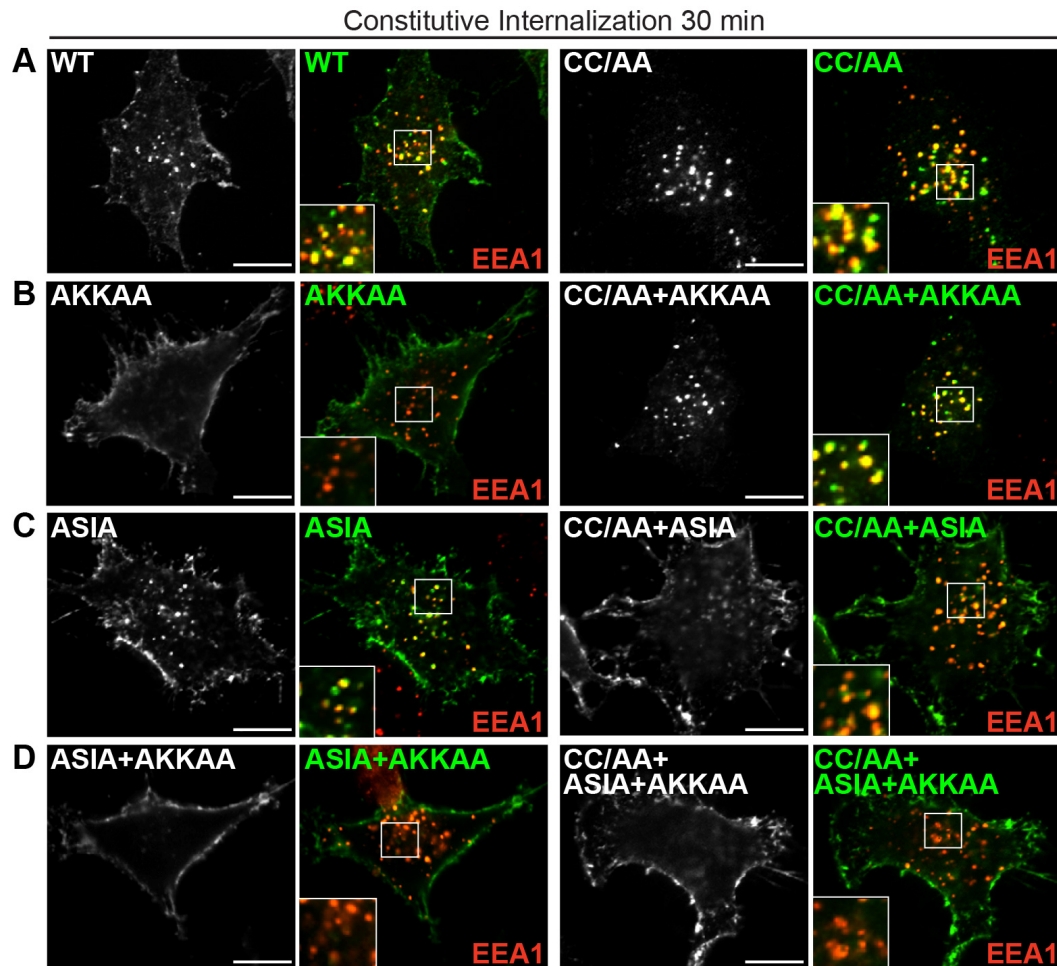


Fig 3.6: Palmitoylation of PAR1 is important for proper utilization of C-tail tyrosine-based sorting motifs. HeLa cells expressing FLAG-PAR1 WT and CC/AA mutant (A), along with tyrosine-based sorting motif mutants (B-D) were prelabeled with polyclonal anti-FLAG antibody, washed and incubated for 30 min at 37°C. Cells were fixed, permeabilized, and co-stained for EEA1, a marker of early endosomes, and imaged by confocal microscopy. PAR1 (*green*) and EEA1 (*red*) colocalization is revealed by the yellow color in the merged images. *Insets* are magnifications of the boxed areas. Images are representative of many cells examined in multiple independent experiments. Scale bar, 10 μ m.

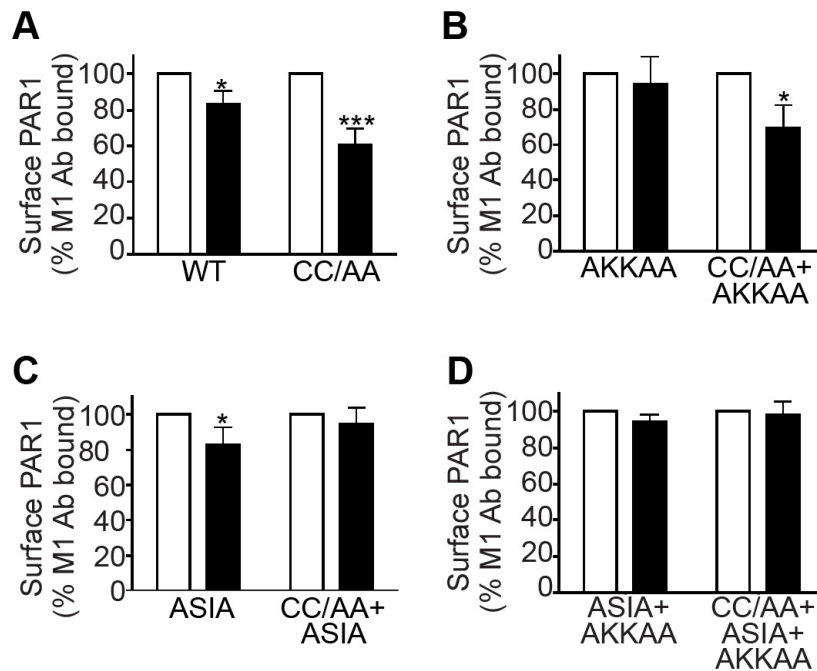


Figure 3.7: Mutagenesis of the proximal tyrosine-based sorting motif prevents PAR1 CC/AA enhanced constitutive internalization. HeLa cells expressing FLAG-PAR1 WT(A), CC/AA mutant (A) or tyrosine-based sorting motif mutants (B-D) were prelabeled with M1 anti-FLAG antibody and fixed (white bars) or incubated for 30 min at 37°C (black bars). Cells were fixed and the amount of remaining receptor-bound antibody was quantified by ELISA. The data (mean $\pm$ S.D.; $n=3$) shown is represented as the percent of initial M1 antibody bound derived from three separate experiments performed in triplicate. The differences in constitutive internalization of PAR1 WT and various mutants compared to the 0 min control were significant (*, $P < 0.05$) or (***, $P < 0.001$) as determined by two-way ANOVA and Bonferroni post-hoc tests.

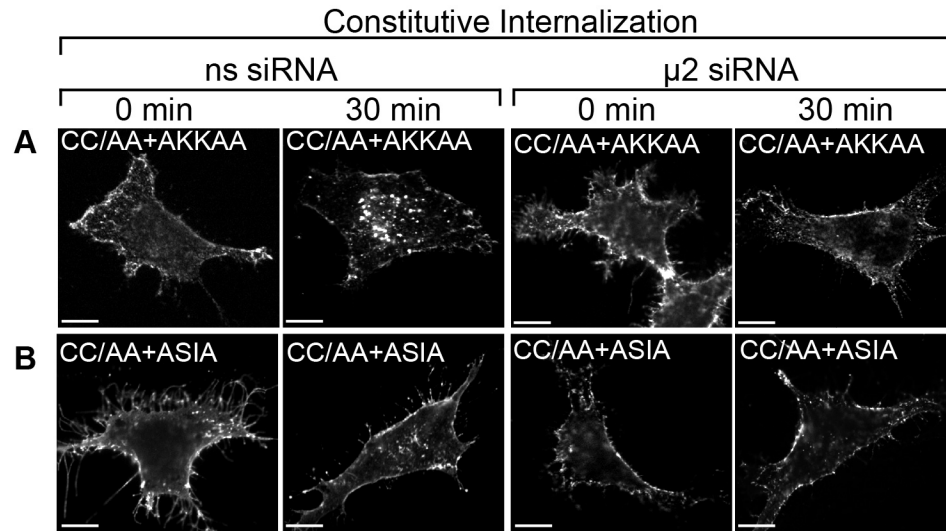


Figure 3.8: AP-2 mediates constitutive internalization of PAR1 CC/AA+AKKAA and CC/AA+ASIA mutants. HeLa cells were transfected with either FLAG-PAR1 CC/AA+AKKAA mutant (A) or PAR1 CC/AA+ASIA mutant (B) together with non-specific (ns) siRNA or siRNA targeting the μ 2-subunit of AP-2. Cells were prelabeled with polyclonal anti-FLAG antibody at 4°C and then incubated 30 min at 37°C. Cells were fixed, permeabilized and immunostained for PAR1 and imaged by confocal microscopy. The images are representative of many cells examined in multiple independent experiments. Scale bar, 10 μ m.

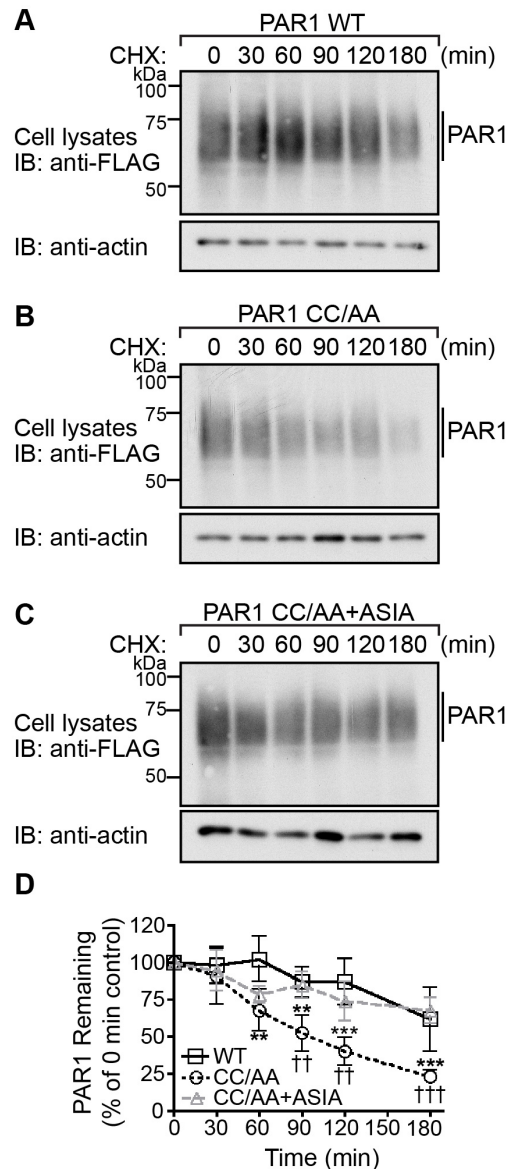


Figure 3.9: The palmitoylation-deficient PAR1 mutant exhibits enhanced degradation. HeLa cells expressing FLAG-PAR1 WT (A), CC/AA (B), or CC/AA+ASIA (C) mutants were incubated in the presence of 10 μ M cycloheximide (CHX) for various times at 37°C. Cell lysates were immunoblotted with polyclonal anti-FLAG antibody to detect PAR1 expression, then stripped and reprobed for actin. D) PAR1 WT and mutant degradation was quantified and is expressed as the percentage of remaining PAR1 compared to 0 min control (mean $\pm$ S.D.; $n=3$). The difference in the amount remaining of PAR1 WT *versus* CC/AA was statistically significant (**, $P < 0.01$; ***, $P < 0.001$) as well as PAR1 CC/AA *versus* PAR1 CC/AA+ASIA (††, $P < 0.05$; †††, $P < 0.001$). Both were determined by two-way ANOVA and Bonferroni post-hoc tests.

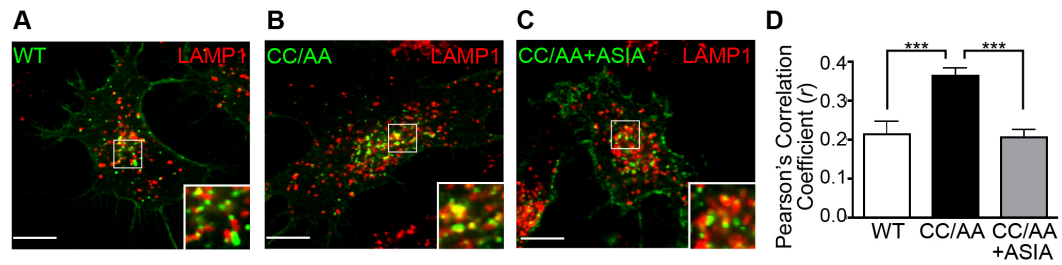


Figure 3.10: Mutagenesis of the proximal tyrosine-based sorting motif prevents PAR1 CC/AA sorting to LAMP1 positive endosomes. HeLa cells expressing FLAG-PAR1 WT (A), CC/AA (B), or CC/AA+ASIA (C) mutant were prelabeled with anti-FLAG antibody on ice for 1 h in the presence of 2 mM leupeptin. Cells were then incubated for 30 min at 37°C, fixed, permeabilized, and costained for lysosomal-associated membrane protein 1 (LAMP1), a marker of late endosomes, and imaged by confocal microscopy. The images are representative of many cells examined from three independent experiments. Scale bar, 10 μ m. PAR1 (*green*) and LAMP1 (*red*) colocalization is revealed by the yellow color in the merged images. *Insets* are magnifications of the boxed areas. D) Colocalization of PAR1 and LAMP1 was quantified by determining Pearson's correlation coefficients from seven different cells. The difference in PAR1 CC/AA *versus* WT and CC/AA+ASIA colocalization with LAMP1 was statistically significant (***, $P < 0.001$) as determined by one-way ANOVA with a Tukey post-hoc test.

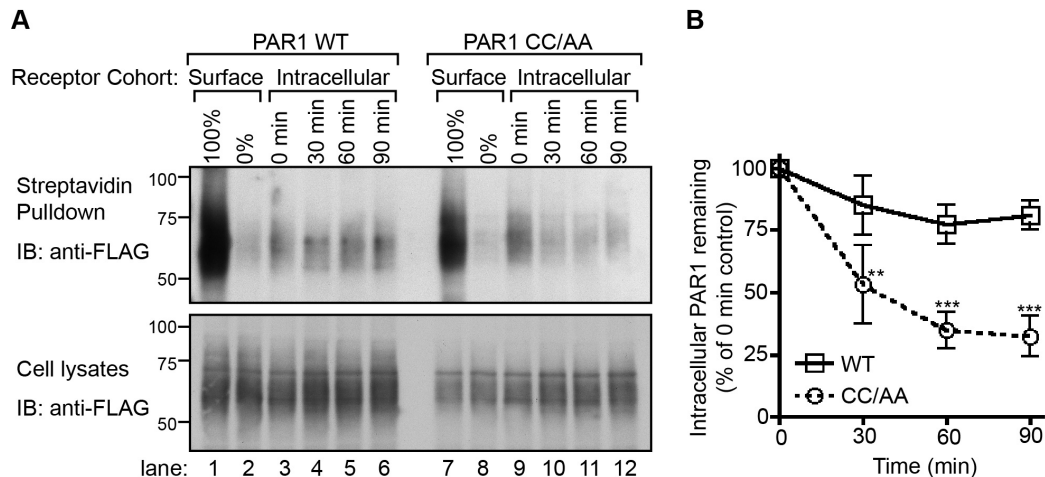


Figure 3.11: Intracellular palmitoylation-deficient PAR1 exhibits enhanced degradation. A) HeLa cells transfected with FLAG-PAR1 wildtype (WT) or CC/AA mutant were labeled with biotin at 4°C. Cells were either left at 4°C and represent the “Surface” cohort or warmed to 37°C for 30 min and represent the “Intracellular” cohort. The biotin label was stripped from the Surface cohort as is shown as 0%. After 30 min of incubation, the remaining biotin label was then stripped from the Intracellular cohort cell surface before further incubation for 30, 60, or 90 min at 37°C. Cells were lysed, biotinylated proteins isolated with streptavidin-conjugated agarose, resolved by SDS-PAGE and immunoblotted with polyclonal anti-FLAG antibody to detect PAR1. An aliquot of total cell lysates were immunoblotted with polyclonal anti-FLAG antibody to control for PAR1 expression. B) The amount of intracellular PAR1 remaining from three independent experiments was quantified and normalized to receptor present in total cell lysates. Data (mean ± S.D.; $n=3$) are expressed as a percentage of receptor remaining from the 0 min intracellular control. The difference in the amounts of internalized PAR1 CC/AA mutant remaining compared to WT at 30, 60 and 90 min was statistically significant (**, $P < 0.01$ and ***, $P < 0.001$) as determined by two-way ANOVA with a Bonferroni post-hoc test.

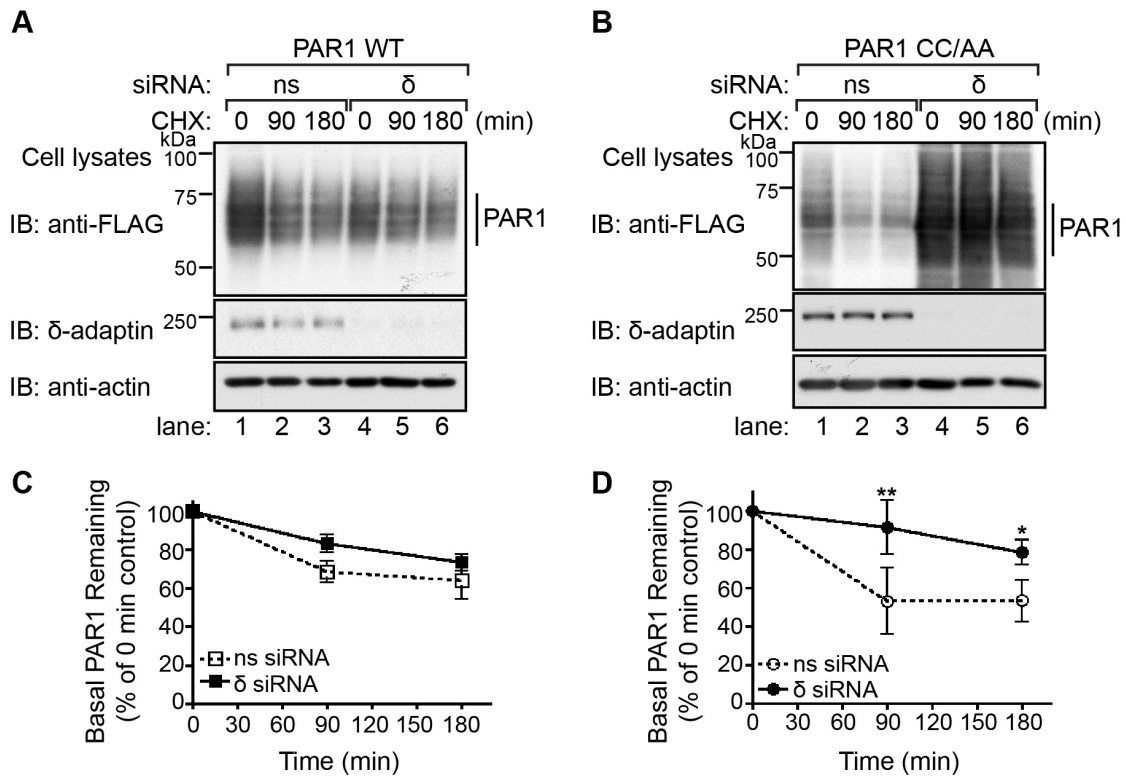


Figure 3.12: Enhanced degradation of the palmitoylation-deficient PAR1 mutant is AP-3 dependent. A) and B) HeLa cells were transfected with FLAG-PAR1 wildtype (WT) or CC/AA and either nonspecific (ns) siRNA, or siRNA targeted against the δ -subunit of AP-3. Cells were incubated in the presence of 10 μ M cycloheximide (CHX) for 90 or 180 min at 37°C, lysed and equivalent amounts of cell lysates were immunoblotted with polyclonal anti-FLAG antibody to detect PAR1. Membranes were stripped and reprobed with anti- δ -adaptin and anti-actin antibodies. C) and D) PAR1 WT and CC/AA degradation were quantified from three individual experiments. Data (mean $\pm$ S.D.; $n=3$) are expressed as a percent of total receptor remaining relative to the 0 min control. PAR1 CC/AA mutant degradation was significantly attenuated in δ -siRNA transfected cells compared to ns-siRNA treated control cells (**, $P < 0.01$ and *, $P < 0.05$) as determined by two-way ANOVA with a Bonferroni post-hoc test.

3.8 References

1. Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) beta -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *The Journal of biological chemistry* **277**, 1292-1300
2. Chen, C. H., Paing, M. M., and Trejo, J. (2004) Termination of protease-activated receptor-1 signaling by beta-arrestins is independent of receptor phosphorylation. *The Journal of biological chemistry* **279**, 10020-10031
3. Trejo, J., Hammes, S. R., and Coughlin, S. R. (1998) Termination of signaling by protease-activated receptor-1 is linked to lysosomal sorting. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13698-13702
4. Booden, M. A., Eckert, L. B., Der, C. J., and Trejo, J. (2004) Persistent signaling by dysregulated thrombin receptor trafficking promotes breast carcinoma cell invasion. *Mol Cell Biol* **24**, 1990-1999
5. Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (1994) Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. *The Journal of biological chemistry* **269**, 27719-27726
6. Arora, P., Cuevas, B. D., Russo, A., Johnson, G. L., and Trejo, J. (2008) Persistent transactivation of EGFR and ErbB2/HER2 by protease-activated receptor-1 promotes breast carcinoma cell invasion. *Oncogene* **27**, 4434-4445
7. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Mol Cell Biol* **26**, 3231-3242
8. Bonifacino, J. S., and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* **72**, 395-447

9. Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* **269**, 1872-1875
10. Rohrer, J., Schweizer, A., Russell, D., and Kornfeld, S. (1996) The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. *The Journal of cell biology* **132**, 565-576
11. Does, M. R., Paing, M. M., Lin, H., Montagne, W. A., Marchese, A., and Trejo, J. (2012) AP-3 regulates PAR1 ubiquitin-independent MVB/lysosomal sorting via an ALIX-mediated pathway. *Molecular biology of the cell*
12. Bae, J. S., Yang, L., Manithody, C., and Rezaie, A. R. (2007) The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood* **110**, 3909-3916
13. Soh, U. J., and Trejo, J. (2011) From the Cover: Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through beta-arrestin and dishevelled-2 scaffolds. *Proceedings of the National Academy of Sciences of the United States of America* **108**, E1372-1380
14. Parton, R. G., and Simons, K. (2007) The multiple faces of caveolae. *Nature reviews. Molecular cell biology* **8**, 185-194
15. Bae, J. S., Yang, L., and Rezaie, A. R. (2008) Lipid raft localization regulates the cleavage specificity of protease activated receptor 1 in endothelial cells. *Journal of thrombosis and haemostasis : JTH* **6**, 954-961
16. Russo, A., Soh, U. J., Paing, M. M., Arora, P., and Trejo, J. (2009) Caveolae are required for protease-selective signaling by protease-activated receptor-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6393-6397
17. Russo, A., Soh, U. J., and Trejo, J. (2009) Proteases display biased agonism at protease-activated receptors: location matters! *Mol Interv* **9**, 87-96

18. Riewald, M., and Ruf, W. (2005) Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. *The Journal of biological chemistry* **280**, 19808-19814
19. Kong, M. M., Verma, V., O'Dowd, B. F., and George, S. R. (2011) The role of palmitoylation in directing dopamine D1 receptor internalization through selective endocytic routes. *Biochem Biophys Res Commun* **405**, 445-449
20. Renner, U., Glebov, K., Lang, T., Papusheva, E., Balakrishnan, S., Keller, B., Richter, D. W., Jahn, R., and Ponimaskin, E. (2007) Localization of the mouse 5-hydroxytryptamine(1A) receptor in lipid microdomains depends on its palmitoylation and is involved in receptor-mediated signaling. *Molecular pharmacology* **72**, 502-513
21. Trejo, J., Altschuler, Y., Fu, H. W., Mostov, K. E., and Coughlin, S. R. (2000) Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *The Journal of biological chemistry* **275**, 31255-31265
22. Does, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., Meerloo, T., and Trejo, J. (2012) ALIX binds a YPX(3)L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *The Journal of cell biology* **197**, 407-419
23. Dunn, K. W., Kamocka, M. M., and McDonald, J. H. (2011) A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol* **300**, C723-742
24. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *The Journal of biological chemistry* **271**, 32874-32880
25. Wolfe, B. L., Marchese, A., and Trejo, J. (2007) Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. *The Journal of cell biology* **177**, 905-916
26. Insel, P. A., Head, B. P., Patel, H. H., Roth, D. M., Bunday, R. A., and Swaney, J. S. (2005) Compartmentation of G-protein-coupled receptors and

their signalling components in lipid rafts and caveolae. *Biochemical Society transactions* **33**, 1131-1134

27. Venkatesan, S., Rose, J. J., Lodge, R., Murphy, P. M., and Foley, J. F. (2003) Distinct mechanisms of agonist-induced endocytosis for human chemokine receptors CCR5 and CXCR4. *Molecular biology of the cell* **14**, 3305-3324
28. Peden, A. A., Rudge, R. E., Lui, W. W., and Robinson, M. S. (2002) Assembly and function of AP-3 complexes in cells expressing mutant subunits. *The Journal of cell biology* **156**, 327-336
29. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., and Coughlin, S. R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *The Journal of clinical investigation* **103**, 879-887
30. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V., Jr., and Coughlin, S. R. (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381**, 516-519
31. Griffin, C. T., Srinivasan, Y., Zheng, Y. W., Huang, W., and Coughlin, S. R. (2001) A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science* **293**, 1666-1670
32. Parent, J. L., Labrecque, P., Driss Rochdi, M., and Benovic, J. L. (2001) Role of the differentially spliced carboxyl terminus in thromboxane A2 receptor trafficking: identification of a distinct motif for tonic internalization. *The Journal of biological chemistry* **276**, 7079-7085
33. Reid, H. M., and Kinsella, B. T. (2007) Palmitoylation of the TPbeta isoform of the human thromboxane A2 receptor. Modulation of G protein: effector coupling and modes of receptor internalization. *Cellular signalling* **19**, 1056-1070
34. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) G protein-coupled receptor sorting to endosomes and lysosomes. *Annu Rev Pharmacol Toxicol* **48**, 601-629

35. Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J. L., Arenzana-Seisdedos, F., and Bachelier, F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *The Journal of biological chemistry* **276**, 31936-31944
36. Pizard, A., Blaukat, A., Michineau, S., Dikic, I., Muller-Esterl, W., Alhenc-Gelas, F., and Rajerison, R. M. (2001) Palmitoylation of the human bradykinin B2 receptor influences ligand efficacy. *Biochemistry* **40**, 15743-15751
37. Hayashi, M. K., and Haga, T. (1997) Palmitoylation of muscarinic acetylcholine receptor m2 subtypes: reduction in their ability to activate G proteins by mutation of a putative palmitoylation site, cysteine 457, in the carboxyl-terminal tail. *Arch Biochem Biophys* **340**, 376-382
38. Fukushima, Y., Saitoh, T., Anai, M., Ogihara, T., Inukai, K., Funaki, M., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M., Ishikawa, T., Takata, K., Nagai, R., Omata, M., and Asano, T. (2001) Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. *Biochimica et biophysica acta* **1539**, 181-191
39. Kvachnina, E., Dumuis, A., Wlodarczyk, J., Renner, U., Cochet, M., Richter, D. W., and Ponimaskin, E. (2009) Constitutive Gs-mediated, but not G12-mediated, activity of the 5-hydroxytryptamine 5-HT7(a) receptor is modulated by the palmitoylation of its C-terminal domain. *Biochimica et biophysica acta* **1793**, 1646-1655
40. Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S., and Masaki, T. (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. *The Journal of biological chemistry* **272**, 21589-21596
41. Holliday, N. D., and Cox, H. M. (2003) Control of signalling efficacy by palmitoylation of the rat Y1 receptor. *British journal of pharmacology* **139**, 501-512
42. Sun, B., Chen, L., Cao, W., Roth, A. F., and Davis, N. G. (2004) The yeast casein kinase Yck3p is palmitoylated, then sorted to the vacuolar membrane with AP-3-dependent recognition of a YXXPhi adaptin sorting signal. *Molecular biology of the cell* **15**, 1397-1406

43. Canto, I., and Trejo, J. (2013) Palmitoylation of Protease-activated Receptor-1 Regulates Adaptor Protein Complex-2 and -3 Interaction with Tyrosine-based Motifs and Endocytic Sorting. *The Journal of biological chemistry* **288**, 15900-15912

Chapter 4: The role of palmitoylation in PAR1 signaling

4.1 Abstract

Palmitoylation has diverse functions in the regulation of G protein-coupled receptor (GPCR) signaling and trafficking, yet the function of palmitoylation in signal regulation of the protease-activated receptor-1 (PAR1), a GPCR for thrombin, is not known. PAR1 is a unique GPCR that couples to multiple G-protein subtypes, but the mechanisms that regulate G-protein selectivity are not well understood. We have previously established that PAR1 is basally palmitoylated, and palmitoylation affects accessibility of tyrosine-based sorting motifs in the C-tail of the receptor. We utilized a palmitoylation-deficient mutant of PAR1 in which C-tail cysteine residues (C³⁸⁷ and C³⁸⁸) were converted to alanines (A) (PAR1 CC/AA), to examine the role of palmitoylation on PAR1 signal regulation. Similar to wildtype PAR1, the palmitoylation-deficient PAR1 mutant was capable of eliciting thrombin-induced phosphoinositide hydrolysis and ERK1/2 activation. However, thrombin-induced p38 MAPK signaling was greatly reduced in palmitoylation-deficient PAR1 mutant expressing cells. Furthermore, palmitoylation of PAR1 was also required for RhoA activation, which is likely mediated by G $\alpha_{12/13}$ (1,2) as well as an interaction with G α_i . Endothelial cells expressing PAR1 CC/AA mutant failed to display thrombin-induced barrier permeability, a phenotype that is likely due to impairment of PAR1 signaling to specific pathways observed in the absence of palmitoylation. Thus, palmitoylation is likely to stabilize the proper PAR1 C-tail conformation important for regulation of specific receptor signaling pathways.

4.2 Introduction

Activated PAR1 couples to multiple heterotrimeric G-protein subtypes including $G_{12/13}$, G_q , and $G_{i/o}$, which are comprised of α and $\beta\gamma$ subunits, and promotes diverse cellular responses in various cell types (3,4). Activated PAR1 inhibits cAMP accumulation through G_{α_i} , and stimulates phospholipase C-mediated phosphoinositide hydrolysis and calcium mobilization via G_{α_q} (5-7). G_{α_q} signaling mediates platelet aggregation and numerous endothelial cell transcriptional responses through the activation of calcium-regulated kinases and phosphatases, RasGEFs, and mitogen-activated protein (MAP) kinase cascades. PAR1 coupling to $G_{\alpha_{12/13}}$ stimulates Rho-guanine nucleotide exchange factors (GEFs) to activate RhoA, which mediates cytoskeletal changes that are involved in cell shape changes as well as cell migration and endothelial permeability. It is not clear how PAR1 selectively couples to specific G-protein subtypes within the same cell, and there is limited knowledge about the mechanisms by which activated PAR1 uncouples from these distinct G protein subtypes.

Thrombin can directly activate PAR1 on endothelial cells to evoke pro-inflammatory signaling and increase vascular permeability. Thrombin activation of PAR1 results in G_{α_q} coupling, generating second messenger responses that lead to increased intracellular Ca^{2+} and activation of kinases such as $PKC\alpha$ as well as serine/threonine phosphatases (8). $PKC\alpha$ mediated phosphorylation of p120 catenin, and dephosphorylation of VE-cadherin result in disassembly of adherens junctions and endothelial barrier permeability (9). PAR1 is also capable of coupling to $G_{\alpha_{12/13}}$ which directly activates the guanine nucleotide exchange factor (GEF) p115RhoGEF,

which in turn activates RhoA (10). Interestingly, p115RhoGEF has also been shown to be phosphorylated by PKC α , suggesting that both G $\alpha_{12/13}$ and G α_q pathways can lead to RhoA mediated endothelial barrier disruption in HUVECs (11). Following RhoA activation, Rho-associated kinase (ROCK) induces cytoskeletal reorganization and myosin contractility (12). Increased concentrations of cAMP have been associated with endothelial barrier protection via PKA activation and RhoA inhibition (13). Thrombin stimulation has been shown to result in coupling to G α_i and inhibition of cyclic AMP (cAMP) production (5,14), thus providing an additional signaling pathway by which thrombin stimulation promotes barrier disruption.

The mechanisms that govern PAR1 G protein specificity are not very well understood. It is possible that palmitoylation of the C-tail cysteine residues can influence G-protein coupling and downstream signaling by modulating the conformation of the C-tail. There are a few examples in the literature demonstrating that palmitoylation affects G-protein coupling specificity for some GPCRs. For the endothelin receptor A, palmitoylation is important for coupling to G α_q , since palmitoylation-defective mutants displayed impaired agonist-induced phospholipase C activation, but their capacity to affect agonist-induced adenylyl cyclase remained intact (15). Palmitoylation of the V₂ receptor is important for signaling to the MAPK pathway, but not for adenylyl cyclase signaling, suggesting that palmitoylation is important for interaction with β -arrestin, but not G α_s (16). Interestingly, the 5-HT_{7a} receptor is modified with palmitoylation at a proximal and distal site in the C-tail (17), suggesting that two additional intracellular loops can be formed. However, only the proximal palmitoylation site regulated 5-HT_{7a}-mediated specific G-protein pathways.

Mutagenesis of the proximal palmitoylation site increased the constitutive $G\alpha_s$ activity linked to the 5-HT_{7a} receptor, whereas constitutive $G\alpha_{12}$ activity was unaffected (17). In the studies described in this chapter, we examined if palmitoylation of PAR1 is important for thrombin-induced G protein coupling specificity by examining downstream signaling pathways.

Here we examined thrombin-induced signaling pathways of a palmitoylation-deficient PAR1 mutant (CC/AA) expressed in HeLa and endothelial cells. We previously confirmed that this mutant is expressed at the cell surface (Figs. 2.3, 2.4) and is trafficked to caveolae- enriched microdomains like wildtype PAR1 (Fig. 3.1). Remarkably, thrombin-activation of the PAR1 CC/AA mutant expressed in endothelial cells failed to increase endothelial barrier permeability in contrast to the wildtype receptor. Additionally, the PAR1 CC/AA mutant is cleaved by thrombin, indicating that this phenotype is not due to a receptor defect in thrombin cleavage. We further demonstrate that the palmitoylation-deficient PAR1 mutant is capable of inducing ERK1/2 phosphorylation, a downstream signaling effector of $G\alpha_q$ (18,19), similar to wildtype PAR1. However, palmitoylation of PAR1 is required for thrombin-induced p38 MAPK signaling pathways and $G\alpha_{12/13}$ -mediated RhoA activation, as well as $G\alpha_i$ association. Taken together, these data suggest that palmitoylation of PAR1 is important for signaling through distinct G protein subtypes that mediate endothelial barrier permeability.

4.3 Materials and Methods

Reagents and Antibodies – Human α -thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Tumor necrosis factor (TNF)- α was

purchased from PeproTech, Inc (Rocky Hill, NJ). Mouse monoclonal anti-FLAG antibody M2 and mouse anti- β -actin were purchased from Sigma Aldrich. Mouse anti-PAR1 WEDE antibody was from Beckman Coulter (Fullerton, CA). Rabbit anti-PAR1 c5433 antibody was previously described (20). Rabbit anti-FLAG polyclonal antibody was from Rockland Immunochemicals, Inc (Gilbertsville, PA). Anti-p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2), p38 MAPK, phospho-p38 MAPK, MSK1, and phospho-MSK1 antibodies were from Cell Signaling Technology (Beverly, MA). The anti-RhoA antibody is from Santa Cruz Biotechnology. Horseradish peroxidase conjugated secondary goat anti-mouse and goat anti-rabbit antibodies were obtained from Invitrogen.

cDNAs and Cell Lines – the $G\alpha_i$ -Rluc and PAR1-YFP constructs were generously provided by Dr. Jean-Philippe Pin (Montpellier University). The PAR1 CC/AA-YFP mutant was generated by quick-change site-directed mutagenesis. A cDNA plasmid encoding human wild-type PAR1 with an N-terminally tagged FLAG epitope was cloned into a pLXSN vector. Site-directed mutagenesis was used to generate siRNA resistance by mutating the PAR1 siRNA binding site from 5'-AGATTAGTCTCCATCAATA-3' to 5'-AGATTAGTCTCCAAGAATA-3'. The short hairpin RNAi targeting PAR1 was previously described (21). Site-directed mutagenesis was also used to generate the siRNA resistant FLAG PAR1 CC/AA mutant, in which cysteines 387 and 388 were converted to alanines with the following primers: 5'-GTGCCAGAGGTACGTCTACAGTATCTTAGCCGCCAAAGAAAGTTCC-3' and 5'-GGAACCTTTCTTTGGCGGCTAAGATACTGTAGACGTACCTCTGGCAC-3'. Both constructs were confirmed with dideoxy sequencing. Retroviruses were

generated in a GP2-293 packaging cell line and used to infect EA.hy926 cells that were stably transduced with PAR1 shRNA, as previously described (21). Mass populations of cells expressing siRNA resistant FLAG PAR1 wildtype (WT) or FLAG PAR1 CC/AA in were selected in 250 μ g/ml G418.

Permeability Assays – Endothelial barrier permeability was assessed by quantifying the flow of Evans blue- bound BSA through a confluent cell monolayer, as previously described (21). Briefly, siRNA resistant FLAG PAR1 WT or CC/AA mutant expressing EA.hy926 cells were plated in 12 mm transwells containing a 3 μ M pore size polycarbonate filter (Corning) at a density of 1.0×10^5 cells per well. Cells were grown for 3 days at 37°C in 10% FBS Dulbecco's Modified Eagle Medium (DMEM), supplemented with 20% pre-conditioned media. Cells were starved 24hrs prior to the experiment in 0.2% FBS DMEM. On the day of the experiment, cells were incubated with media containing 0.1% BSA, 20 mM HEPES for 3 hrs and then stimulated with 10 nM thrombin for 10min at 37°C. The media in the upper chamber was replaced with 0.67 mg/ml Evans blue diluted in growth media containing 4% BSA. The optical density (O.D.) at 605 nm was measured in a 1:2 diluted 75 μ l sample collected from the bottom chamber using a Molecular Devices Plate Reader (Sunnyvale, CA).

RhoA activation assays – GST-Rhotekin Rho-binding domain (RBD) fusion protein was transformed into BL21 *Escherichia coli*. The fusion protein was induced and purified as previously described (21). EA.hy926 cells stably transduced with siRNA resistant FLAG PAR1 WT and siRNA resistant CC/AA mutant were plated in 6 cm dishes at a density of 2.1×10^6 cells per dish and grown for 3 days. Cells were starved overnight in 0.2% FBS DMEM. Prior to stimulation, cells were incubated in

media containing 0.1% BSA, 20mM HEPES for 3 hrs. Cells were stimulated with 10nM thrombin for 2.5 and 5min, and then lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 2 mM MgCl_2 , 1% Triton X-100, 10% glycerol, 100 mM NaCl. Lysis buffer was supplemented with 1 mM DTT and protease inhibitors: 1 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin A, 10 $\mu\text{g/ml}$ benzamidine, and 1 $\mu\text{g/ml}$ soybean trypsin inhibitor. Equivalent amounts of lysates were incubated with GST Rhotekin-RBD bound to glutathione sepharose beads for 40 min at 4°C. Beads were washed with lysis buffer, and GTP-bound RhoA was eluted with 2X sample buffer [125 mM Tris-HCl at pH 6.8, 20% v/v glycerol, 10% w/v SDS, 0.2M DTT, and 0.02% w/v bromophenol blue]. Total and GTP-bound RhoA were resolved by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-RhoA antibody

BRET Assays – COS7 cells were seeded at 2.5×10^5 cells/well in 6-well plates. Cells were transfected with 100 μg of $\text{G}\alpha_i$ fused to Rluc and varying amounts of PAR1 WT-YFP or CC/AA-YFP mutant. Serum-starved cells were washed, dissociated using a non-enzymatic solution Cellstripper™ (Mediatech Inc., VA), resuspended in PBS containing 0.5 mM MgCl_2 and 0.1% glucose and counted. Cells ($\sim 4 \times 10^4$ in 80 μl) were transferred to 96-well plates in triplicate for each condition. The fluorescence intensity of YFP was examined by excitation at 485 nm and emission at 535 nm using a Berthold Tristar LB 941 microplate reader (Berthold Technologies, GmbH & Co. Germany). The coelenterazine H (Biotium Inc., CA) substrate was added to cells at a final concentration of 5 μM . After an 8min delay, total luminescence was measured by integrating the signal for 1 sec/well without filter selection. Rluc and YFP signals were determined using 480 nm and 530 nm filters,

respectively. For thrombin stimulation time course, cells were treated with 10nM thrombin or buffer control following an 8min delay after coelentrazine H addition. The BRET ratio was calculated as (emission at 530nm)/(emission at 480nm) and net BRET signal determined by subtracting the background BRET, which is the BRET ratio from cells expressing the Rluc construct only. Data were fitted with non-linear regression using GraphPad Prism 4.0 software (GraphPad Inc., La Jolla, CA).

ERK 1/2, p38, and MSK1 phosphorylation assays – FLAG PAR1 WT or CC/AA mutant expressing EA.hy926 cells were plated in 24 well plates at a density of 1.5×10^5 cells/ well. Cells were grown for 4 days, and starved 24 hrs prior to the experiment in 0.2% FBS DMEM. In a similar manner, HeLa cells transiently expressing FLAG PAR1 WT or CC/AA mutant were plated in 24 well plates at a density of 0.8×10^5 cells/well, grown for 24 hrs, and serum starved overnight. On the day of the experiment, EA.hy926 and HeLa cells were incubated for 2 hrs in media containing 0.1% BSA, 20 mM HEPES and then stimulated with 10 nM thrombin for various times at 37°C. Cells were directly lysed in 2X SDS sample containing 125 mM Tris-HCl at pH 6.8, 20% v/v glycerol, 10% w/v SDS, 0.2 M DTT, and 0.02% w/v bromophenol blue. Equivalent amounts of lysates were resolved by SDS-PAGE and immunoblotted with phospho specific antibodies for ERK 1/2, p38, and MSK1, then stripped and re-probed for total ERK1/2, total p38, and total MSK1.

Cold Cleave Assays – FLAG PAR1 WT or CC/AA mutant expressing EA.hy926 cells were plated in 6 well plates at a density of 8×10^5 cells per well. Cells were grown for 4 days and then starved overnight in 0.2% FBS DMEM. Cells were incubated for 2hrs in media containing 0.1% BSA, 20 mM HEPES at 37°C and then

placed on ice. Cells were incubated in cold 0.1% BSA, 20 mM HEPES media for 30min and then treated with cold 30nM thrombin on ice. Cells were washed with ice cold media and then incubated on ice with 0.5 U/ml hirudin for 5 min. Cells were washed with ice cold PBS and lysed in a triton lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, and 10 mM NaPP supplemented with protease inhibitors 100 μ g/ml PMSF, 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 10 μ g/ml benzamidine, and 1 μ g/ml soybean trypsin inhibitor. Whole cell lysates were immunoprecipitated with M2 FLAG antibody overnight at 4°C. Immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted to detect FLAG PAR1.

Phosphoinositide (PI) Hydrolysis – HeLa cells were seeded at 0.75×10^5 cells/well in 24-well plates and transfected with FLAG-PAR1 WT, CC/AA mutant or pBJ vector control. Cells were then labeled with 1.0 μ Ci/ml *myo*-[3 H]inositol (American Radiolabeled Chemicals, St Louis, MO) in inositol-free DMEM overnight, washed and stimulated with thrombin in the presence of 20mM LiCl for various times at 37°C. The amounts of accumulated [3 H]inositol phosphates (IPs) were measured as described (22).

4.4 Results

4.4.1 Gq signaling in PAR1 CC/AA expressing HeLa cells

To assess the function of PAR1 palmitoylation, we first compared the signaling properties of PAR1 WT *versus* CC/AA mutant in HeLa cells. Activation of PAR1 is known to stimulate phospholipase C-mediated phosphoinositide hydrolysis, a

response mediated by $G\alpha_q$ in most cell types (23,24). HeLa cells expressing similar amounts of PAR1 WT and CC/AA mutant on the cell surface (Fig. 4.1A) were labeled with *myo*-[^3H]inositol, incubated with 10 nM thrombin for various times at 37°C in the presence of 20 mM LiCl to inhibit inositol phosphate (IP) phosphatases. Following stimulation, the formation of [^3H]IPs were isolated by column chromatography and quantified by scintillation counting, as previously described (25). Thrombin-activation of PAR1 WT and CC/AA mutant resulted in a similar initial rapid increase in PI hydrolysis, followed by a slower sustained phase of [^3H]IP generation after 20 min and maximal [^3H]IP accumulation was detected at 60 min (Fig. 4.1A). No change in [^3H]IP accumulation was detected in pBJ vector transfected cells treated with thrombin for 60 min (Fig. 4.1A), indicating that thrombin signaling is PAR1 dependent. We next evaluated PAR1 WT and CC/AA capacity to couple to G protein signaling by examining the concentration effect curve of thrombin-induced PI hydrolysis (Fig. 4.1B). HeLa cells expressing PAR1 WT and CC/AA mutant labeled with *myo*-[^3H]inositol were treated with varying concentrations of thrombin for 20min at 37°C, and the generation of [^3H]IPs were measured. The effective concentration of thrombin needed to stimulate half-maximal response (EC_{50}) for PAR1 WT versus CC/AA mutant was modestly different, but significant (Fig. 4.1B), suggesting that palmitoylation can modulate receptor coupling to $G\alpha_q$ -stimulated signaling. These results are consistent with a previous report that employed a PAR1 mutant containing C³⁸⁷ and C³⁸⁸ to serine (S) mutations (26), although palmitoylation of PAR1 was not examined in this study. Together these findings suggest that palmitoylation is not

essential for PAR1-induced $G\alpha_q$ -mediated PI signaling but it can modulate the response.

4.4.2 PAR1 CC/AA elicits ERK1/2 activation in response to thrombin

It has been demonstrated that PAR1 activation by thrombin elicits ERK1/2 phosphorylation, a signaling response that is downstream of $G\alpha_q$ and $G\alpha_i$ activation (18,27). Initial studies comparing PAR1 WT versus CC/AA $G\alpha_q$ signaling in HeLa cells has revealed that the kinetics of $G\alpha_q$ signaling are indistinguishable in response to saturating doses of thrombin (Fig 4.1A). Next we examined the kinetics of ERK1/2 activation to determine whether it reflects the $G\alpha_q$ -mediated PI signaling. To test this, HeLa cells expressing comparable amounts of PAR1 WT or CC/AA mutant were stimulated with 10 nM thrombin for various times, lysed and immunoblotted with antibodies that detect ERK1/2 phosphorylation. PAR1 WT and CC/AA expressing cells elicited robust ERK1/2 phosphorylation in response to thrombin (Fig 4.2A). Quantification of the ERK1/2 phosphorylation from multiple experiments indicated that the magnitude of response is not statistically significantly different between PAR1 WT and CC/AA expressing cells (Fig 4.2B).

We utilized stable knockdown-rescue PAR1 wildtype or CC/AA in EA.hy926 endothelial stable cell lines (as previously described in Fig. 2.4) to confirm the signaling response observed in HeLa cells. Endogenous PAR1 is depleted in these cells, and serve as a good system to examine signaling pathways of the palmitoylation-deficient mutant in a physiologically relevant cell-type. Both PAR1 wildtype and CC/AA expressing endothelial cells elicited ERK 1/2 phosphorylation in

response to thrombin (Fig 4.2C), thus confirming the results we observed in HeLa cells (Fig 4.2A). Quantification of multiple experiments demonstrates that both WT and CC/AA mutant elicit a comparable magnitude of ERK1/2 activation in response to thrombin (Fig 4.2D). Taken together, these data suggests that palmitoylation of PAR1 is not essential for ERK1/2 activation, and supports our previous study demonstrating that PAR1 WT and CC/AA signal comparably to $G\alpha_q$ -stimulated PI hydrolysis (Fig 4.1).

4.4.3 Palmitoylation is required for thrombin induced barrier permeability in endothelial cells

Thrombin has been shown to induce robust endothelial barrier permeability through the activation of PAR1 (28). We investigated whether palmitoylation of PAR1 was important for this physiologically relevant response. We examined endothelial barrier permeability of a confluent monolayer of endothelial cells expressing PAR1 WT or CC/AA mutant using transwell chambers. Cells were stimulated with thrombin, and endothelial barrier permeability was assessed by quantifying the amount of Evans blue-bound BSA that passed from the upper chamber to the lower chamber. PAR1 WT expressing cells exhibited robust permeability following thrombin stimulation compared to unstimulated control cells (Fig 4.3A). Intriguingly, thrombin stimulation failed to induce barrier permeability in PAR1 CC/AA mutant expressing cells compared to unstimulated control cells (Fig 4.3A). In order to ensure that cell surface receptors were cleaved by thrombin, PAR1 WT and CC/AA expressing endothelial cells were treated with 30 nM thrombin at 4°C. Under these conditions receptors are not internalized. Glycosylated and non-glycosylated forms of PAR1 are detected in FLAG PAR1 WT and CC/AA expressing cells that were not treated with

thrombin (Fig 3.4B lanes 1 and 4). Treatment with 30 nM thrombin at 4°C shows comparable cleavage of both glycosylated and non-glycosylated receptors, as indicated by a loss of the N-terminal localized FLAG epitope (Fig 4.3B, lanes 2 and 5). Quantification of three independent experiments indicates comparable cleavage of cell surface receptors between PAR1 WT and CC/AA mutant (Fig 4.3C), indicating that the inability of PAR1 CC/AA to elicit thrombin-induced endothelial barrier permeability is not due to a defect in thrombin cleavage.

4.4.4 Thrombin-induced RhoA activation is reduced in PAR1 CC/AA expressing endothelial cells

Considering that the PAR1 CC/AA expressing endothelial cells were unresponsive to thrombin-induced endothelial barrier permeability, we sought to determine whether there were specific defects in mutant receptor signaling. We demonstrated that PAR1 CC/AA mutant is capable of activating ERK1/2 in a manner comparable to PAR1 WT in endothelial cells (Fig 4.2C and D). We next examined whether other signaling pathways that mediate thrombin-induced endothelial barrier permeability were disrupted in the absence of PAR1 palmitoylation. Thrombin activation of PAR1 results in coupling to $G_{\alpha_{12/13}}$ (29), which mediates activation of the GTPase RhoA (10). RhoA activity has been shown to regulate myosin light chain phosphorylation, and subsequently cell contractility (12). In order to determine if PAR1 palmitoylation is necessary for thrombin induced RhoA activation, we stimulated PAR1 WT or CC/AA expressing endothelial cells with 10 nM thrombin and isolated activated RhoA with GST- rhotekin binding domain (RBD) pull-downs, as previously described (21,30). Thrombin treatment resulted in robust RhoA activation in PAR1 WT expressing cells (Fig 4.4 lanes 5-6), but failed to activate RhoA as

efficiently in PAR1 CC/AA mutant expressing cells (Fig 4.4, lanes 8-9). Endothelial cells stably transduced with PAR1 shRNA failed to show any detectable RhoA activation in response to thrombin treatment (Fig 4.4, lanes 2-3). These data suggest that palmitoylation of PAR1 is important for RhoA activation, a response mediated through $G_{\alpha_{12/13}}$ coupling. Combined, these findings suggest that the failure of PAR1 CC/AA mutant to elicit thrombin-induced endothelial barrier permeability correlates with a deficiency in RhoA signaling.

4.4.5 Thrombin-induced p38 MAPK activation is reduced in PAR1 CC/AA expressing cells

Although RhoA activity is important for thrombin-induced endothelial barrier permeability, other signaling pathways contribute to disassembly of adherens junctions and endothelial barrier disruption. The MAP Kinase p38 has been shown to be involved in thrombin-induced barrier permeability (31). In addition, our group has recently shown that PAR1-induced p38 activation occurs through non-canonical autophosphorylation (32). The mechanism of thrombin-induced p38 activation is initiated through PAR1 ubiquitination, which nucleates the formation of a TAB1/TAB2/p38 signaling complex that ultimately contributes to endothelial barrier permeability. We have previously demonstrated that the PAR1 CC/AA mutant is defective in agonist-induced ubiquitination in both HeLa and endothelial cells (Fig 2.6). Thus, we assessed whether the PAR1 palmitoylation-deficient mutant was also defective in p38 MAPK activation. To test this, we stimulated PAR1 WT or CC/AA expressing endothelial cells with thrombin and immunoblotted for phosphorylated p38. PAR1 WT expressing cells exhibited p38 phosphorylation in response to thrombin as early as 2.5min (Fig 4.5A). Additionally MSK1, a downstream substrate of p38, shows similar

kinetics of activation as p38 phosphorylation (Fig 4.5A). Surprisingly, PAR1 CC/AA expressing cells did not elicit a robust p38 phosphorylation as exhibited by PAR1 WT expressing cells (Fig 4.5A). Reduced p38 activity was assessed by analyzing MSK1 phosphorylation, which was dramatically decreased in PAR1 CC/AA expressing endothelial cells (Fig 4.5A). Quantification of independent repeats indicates the magnitude of p38 (Fig 4.5B) and MSK1 (Fig 4.5C) phosphorylation is significantly greater in PAR1 WT *versus* CC/AA mutant expressing cells.

To ensure that PAR1 CC/AA expressing endothelial cells were not deficient in global p38 signaling, we treated cells with the cytokine tumor necrosis factor- α (TNF- α), which is known to activate the canonical p38 MAPK pathway via upstream MAPK kinases MKK3 and MKK6 (33,34). Stimulation with TNF- α elicited robust p38 phosphorylation in CC/AA expressing cells, which was comparable to the response seen with PAR1 WT expressing cells (Fig 4.6A). Quantification of the p38 response from independent experiments confirmed that the magnitude of p38 phosphorylation in response to TNF- α was not different between PAR1 WT versus CC/AA mutant (Fig 4.6B). Therefore, the disruption in p38 MAPK signaling exhibited by PAR1 CC/AA is not due to a global defect in p38 activation.

We next confirmed the defect in thrombin-induced p38 MAPK signaling in HeLa cells. Stimulation of HeLa cells expressing PAR1 WT or CC/AA mutant with thrombin resulted in a signaling profile similar to that observed for the endothelial cells. PAR1 WT expressing HeLa cells showed robust p38 phosphorylation, whereas PAR1 CC/AA displayed diminished a response (Fig 4.7A). Furthermore, MSK1 phosphorylation was also decreased in PAR1 CC/AA expressing cells (Fig 4.7A).

Indeed, quantification of multiple independent experiments confirmed that the magnitude of p38 phosphorylation was significantly greater in PAR1 WT than PAR1 CC/AA expressing HeLa cells (Fig 4.7B). Taken together, these data demonstrate that palmitoylation is critical for thrombin-induced p38 MAPK activation.

4.4.6 Thrombin-induced RhoA activation is not mediated by p38 MAPK

We have demonstrated that palmitoylation is critical for thrombin-induced RhoA activation and thrombin-induced p38 MAPK signaling pathways. Considering that palmitoylation is critical for PAR1 ubiquitination (Fig 2.6), and consequently p38 MAPK signaling (Fig 4.5C), we were interested in determining if p38 MAPK activity was required for RhoA activation. To examine this, we utilized the inhibitor SB203580, which binds to the nucleotide-binding pocket of p38 to inhibit p38-autophosphorylation. This inhibitor does not affect p38 phosphorylation by upstream kinases MKK3/6, and thus serves as a useful strategy to specifically inhibit non-canonical p38 signaling (35). We demonstrated that pretreatment with 1 μ M SB203580 reduced thrombin-induced p38 phosphorylation compared to untreated controls (Fig 4.8A lanes 4-6). Under these conditions, we stimulated PAR1 WT expressing endothelial cells with 10 nM thrombin for 2.5 min and isolated activated RhoA with GST-rhotekin binding domain (RBD) pull-downs. PAR1 WT expressing endothelial cells showed comparable levels of thrombin-induced RhoA activation regardless of SB20358 pretreatment (Fig 4.8B), suggesting that p38 activation is not critical for RhoA activation. Indeed, quantification of three different experiments demonstrates that RhoA activation was not statistically significantly different from untreated controls (Fig 4.8C). Taken together these data suggest that thrombin-induced p38MAPK activity is not required for RhoA activation. Therefore the defects

observed in the RhoA and p38MAPK signaling pathways, exhibited by the PAR1 CC/AA mutant are distinct.

4.4.7 Receptor palmitoylation is critical for $G\alpha_i$ association with PAR1

PAR1 couples to $G\alpha_i$ in response to thrombin, which results in decreased production of cAMP (7,14). It has been demonstrated that elevated levels of cAMP stabilize endothelial barrier permeability (36,37). Considering that PAR1 CC/AA expressing cells did not exhibit endothelial barrier permeability in response to thrombin, we determined if this could partly be due to defective $G\alpha_i$ signaling. To examine if $G\alpha_i$ coupling was defective for the palmitoylation-deficient PAR1 mutant, we examined $G\alpha_i$ interaction with the receptor in living cells using BRET. BRET was assessed in COS7 cells transfected with a constant amount of the $G\alpha_i$ fused to Rluc, together with varying amounts of PAR1 WT or CC/AA mutant tagged with YFP at the C-terminus. A hyperbolic increase in BRET was detected as the ratio of PAR1 WT-YFP to $G\alpha_i$ -Rluc increased, suggesting that PAR1 WT- $G\alpha_i$ protein interaction is specific (Fig. 4.8A). We also observed a hyperbolic increase in net BRET as the ratios of PAR1 CC/AA - $G\alpha_i$, were increased (Fig. 4.9A). However, the PAR1 CC/AA - $G\alpha_i$ net BRET values were generally lower compared to those exhibited by PAR1 WT- $G\alpha_i$ (Fig. 4.9A). In order to evaluate the relative affinity of PAR1 WT *versus* CC/AA mutant to $G\alpha_i$, the BRET₅₀ values were determined. The PAR1 CC/AA- $G\alpha_i$ BRET₅₀ value was significantly lower than the PAR1 WT- $G\alpha_i$ BRET₅₀, indicating that PAR1 has reduced affinity to $G\alpha_i$ in the absence of palmitoylation (Fig. 3.9A, *inset*). The maximum net BRET signal observed with PAR1 CC/AA-YFP and $G\alpha_i$ -Rluc was also significantly lower than that exhibited by PAR1 WT-YFP and $G\alpha_i$ -Rluc, suggesting

that the nature of the $G\alpha_i$ interaction with receptor is different (Fig. 3.8A, *inset*). Taken together, these data indicate that palmitoylation of PAR1 is important for efficient energy transfer of $G\alpha_i$ -Rluc to PAR1 YFP, suggesting that palmitoylation may induce a conformational change in the C-tail that is important for PAR1 association with $G\alpha_i$.

Although BRET examined under basal conditions suggests that PAR1 CC/AA is defective for $G\alpha_i$ association, we examined whether thrombin stimulation would result in a change of the net BRET. To examine this, we stimulated cells expressing the lowest PAR1-YFP/ $G\alpha_i$ ratio required to achieve saturation in our titration experiment (Fig. 4.9A). COS7 cells expressing PAR1 WT-YFP or CC/AA-YFP along with $G\alpha_i$ -Rluc were stimulated with 10 nM thrombin or buffer as a control. We observed a thrombin-dependent increase in net BRET between PAR1 WT and $G\alpha_i$ that was not observed in buffer treated conditions (Fig. 4.9B). In contrast, cells expressing PAR1 CC/AA and $G\alpha_i$ did not show a thrombin-dependent increase in net BRET (Fig. 4.9C). The differences observed in $G\alpha_i$ association between PAR1 WT versus PAR1 CC/AA suggest that palmitoylation could be important for thrombin-induced $G\alpha_i$ signaling.

4.5 Conclusions

Results from our studies indicate that palmitoylation of PAR1 is required for thrombin-induced endothelial barrier disruption. However, we have demonstrated that palmitoylation is not required for $G\alpha_q$ -stimulated PI hydrolysis or ERK1/2 activation, suggesting that these signaling pathways may not be essential for endothelial barrier permeability. In contrast, we observed reduced thrombin-mediated RhoA activation in

endothelial cells expressing PAR1 CC/AA mutant, suggesting that $G_{\alpha_{12/13}}$ likely contributes to endothelial barrier permeability in our cell system. In a similar manner, endothelial barrier permeability appeared to be highly dependent upon $G_{\alpha_{12/13}}$ and not on G_{α_q} for HMEC-1 cells in response to thrombin (38). However, it has been demonstrated that for other endothelial cell lines, endothelial barrier permeability is dependent on not only $G_{\alpha_{12/13}}$, but also G_{α_q} -mediated calcium mobilization (39). Release of calcium from intracellular stores can activate PKC- α , which can phosphorylate Rho- GDP guanine nucleotide dissociation inhibitor (GDI) to activate RhoA (40). PKC- α can also mediate the disruption of VE-cadherin junctions (41). In vivo studies utilizing mice with endothelial cell (EC) targeted knockouts of $G_{\alpha_{12/13}}$ or G_{α_q} expression demonstrate that PAR1-stimulated vascular leakage is attenuated in G_{α_q} , but not $G_{\alpha_{12/13}}$ deficient endothelial cells (42). Furthermore, G_{α_q} and $G_{\alpha_{12/13}}$ deficient endothelial cells are both defective in thrombin-induced myosin light chain phosphorylation, suggesting that both signaling pathways can contribute to endothelial cell contractility. (42). However, only $G_{\alpha_{12/13}}$ deficient endothelial cells are defective in thrombin-induced RhoA activation, suggesting that G_{α_q} signaling pathways can cause endothelial cell contractility independent of RhoA activation (42). This suggests that both $G_{\alpha_{12/13}}$ and G_{α_q} signaling pathways can contribute to endothelial cell contractility. Given that palmitoylation of PAR1 is not required for G_{α_q} -mediated signaling to PI hydrolysis, but it is required for RhoA activation, this suggests that the RhoA pathway is critical for thrombin-induced endothelial barrier permeability in our cell lines.

It has been demonstrated that p38 activity is strongly correlated with inflammatory signaling and endothelial barrier disruption. Most studies have focused on the canonical pathway in which p38 is activated by upstream kinases MKK3/6 (33). However it has been demonstrated that binding of transforming growth factor- β -activated protein kinase-1 binding protein-1 (TAB1) to p38 can induce a conformational change in p38 that allows for autophosphorylation and activation (35,43), thus revealing a non-canonical pathway. Our lab has recently shown that PAR1 is ubiquitinated upon agonist stimulation, and this modification serves as a nucleation for a TAB1/TAB2/p38 signaling complex on endosomes to initiate non-canonical p38 signaling (32). Furthermore, pretreatment with the p38 inhibitor SB203580 attenuated thrombin-induced barrier permeability in vitro and in vivo (31,32). We have demonstrated that palmitoylation is required for thrombin-induced p38 phosphorylation and downstream signaling. In Chapter 2, we demonstrated that palmitoylation is required for PAR1 ubiquitination to occur (Fig. 2.6). Taken together our data suggest that palmitoylation of PAR1 C-tail may stabilize a conformation that is critical for receptor ubiquitination, which ultimately affects p38 MAPK signaling and contributes to endothelial barrier disruption.

Endothelial barrier breakdown in response to thrombin is accompanied with decreases in cAMP and inactivation of Rac1 (37). Activation of cAMP with O-Me-cAMP and Rap/Epac1 signaling was shown to enhance barrier protection by activating Rac1 (36). However activation of cAMP with forskolin/rolipram also enhances barrier stabilization independently of Rac1 activation (36). It is possible that PAR1 coupling to G_{α_i} results in decreased production of cAMP, and thus

contributes to endothelial barrier disruption. Given the barrier protective phenotype exhibited by the palmitoylation-deficient PAR1 mutant, we sought to determine if this mutant is also defective in $G\alpha_i$ signaling. It has been previously demonstrated with BRET that PAR1 basally associates with $G\alpha_i$ (44). In this study, it was demonstrated that BRET between PAR1 and $G\alpha_i$ increased rapidly with thrombin stimulation, and returned to basal levels after 5 min, indicating that there is a conformational change within PAR1 that affects energy transfer from the precoupled $G\alpha_i$ -Rluc (44). We have demonstrated that palmitoylation of PAR1 is important for basal $G\alpha_i$ association. In contrast to what is observed with PAR1 WT, stimulation with thrombin failed to show an increase BRET of $G\alpha_i$ -Rluc with PAR1 CC/AA YFP. Interestingly, the previous study by Ayoub *et al.* also examined a PAR1 truncation mutant that had the YFP positioned just after the palmitoylation sites (44). This mutant did not exhibit the basal or thrombin-induced BRET that was exhibited by the WT receptor, indicating that the position of the YFP is critical for energy transfer by the $G\alpha_i$ -Rluc (44). We observed a similar phenotype when the palmitoylation site was mutated, which could indicate that the conformation of the C-tail in the absence of palmitoylation could be affecting efficient energy transfer from the $G\alpha_i$ -Rluc. Taken together, our results suggest that palmitoylation of PAR1 is required for association with $G\alpha_i$.

Palmitoylation of the PAR1 C-tail is important for RhoA activation, a downstream effector of $G\alpha_{12/13}$, and $G\alpha_i$ coupling based on BRET analysis in response to thrombin. Interestingly, $G\alpha_q$ -stimulated PI signaling appears to only be modestly affected in the absence of PAR1 palmitoylation. It has been demonstrated that the site of interaction for $G\alpha_q$ occurs in the second intracellular loop of PAR1

(45,46). Interestingly, mutagenesis of the second intracellular loop of PAR1 only affected $G\alpha_q$ coupling, but not $G\alpha_{12/13}$ or $G\alpha_o$. This suggests that G-proteins have distinct sites of interaction on the cytoplasmic regions of PAR1 (45) and may explain in part the differences we observed with the various signaling pathways. It is possible that palmitoylation deficiency affects the conformation of the PAR1 C-tail, so that it affects the ability of the receptor to associate with $G\alpha_{12/13}$ and $G\alpha_i$, but not $G\alpha_q$. In conclusion, our data suggest that a lack of palmitoylation in the PAR1 C-tail can influence receptor signaling to distinct G-protein-mediated signaling pathways (Fig 4.10) and ultimately affect thrombin-induced endothelial barrier permeability.

4.6 Acknowledgements

Chapter 4, in part is published as a manuscript entitled “Palmitoylation of protease-activated receptor-1 regulates adaptor protein complex-2 and -3 interaction with tyrosine-based motifs and endocytic sorting” in the *Journal of Biological Chemistry* (47). The dissertation author is the primary author who wrote the manuscript, directed experiments, and analyzed the data.

Chapter 4, in part is being prepared for publication: Canto Cordova I, Saucedo C., and Trejo J. Palmitoylation regulates PAR1 G-protein signaling pathways and thrombin-induced endothelial barrier permeability. *Journal TBD*. The dissertation author is the primary author who directed experiments and analyzed the data.

4.7 Figures

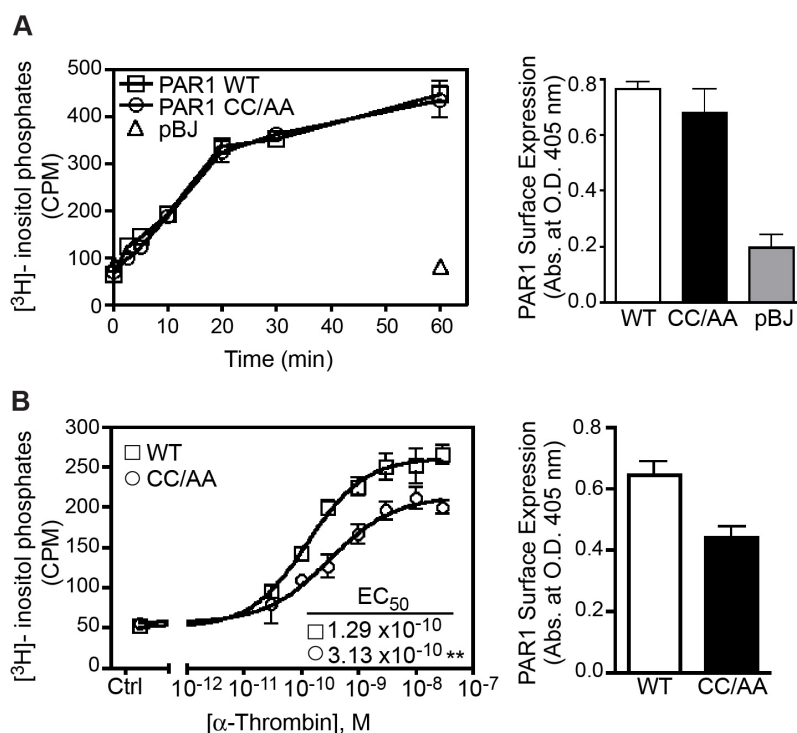


Figure 4.1: Signaling by the palmitoylation-deficient PAR1 mutant. A) HeLa cells transfected with FLAG-PAR1 wildtype (WT), CC/AA mutant, or pBJ vector were labeled with myo-[³H]inositol and stimulated with 10 nM α-thrombin for various times at 37°C. The data (mean ± S.D.; n=3) represent the accumulation of [³H]inositol phosphates in counts per minute (CPM). Cell surface expression of PAR1 WT, CC/AA, and pBJ transfected cells from the same experiment was determined by ELISA (mean ± S.D.; n=3). B) HeLa cells transfected with FLAG-PAR1 WT or CC/AA mutant labeled with myo-[³H]inositol were treated with varying concentrations α-thrombin for 20 min at 37°C. The data (mean ± S.D.; n=3) represent the accumulation of [³H]inositol phosphates expressed as CPM. The EC50 values calculated for PAR1 WT (1.29 × 10⁻¹⁰ M) versus CC/AA mutant (3.13 × 10⁻¹⁰ M) were statistically significantly different (**, *P* < 0.01). Cell surface expression of PAR1 WT and CC/AA mutant from the same experiment was determined by ELISA (mean ± S.D.; n=3).

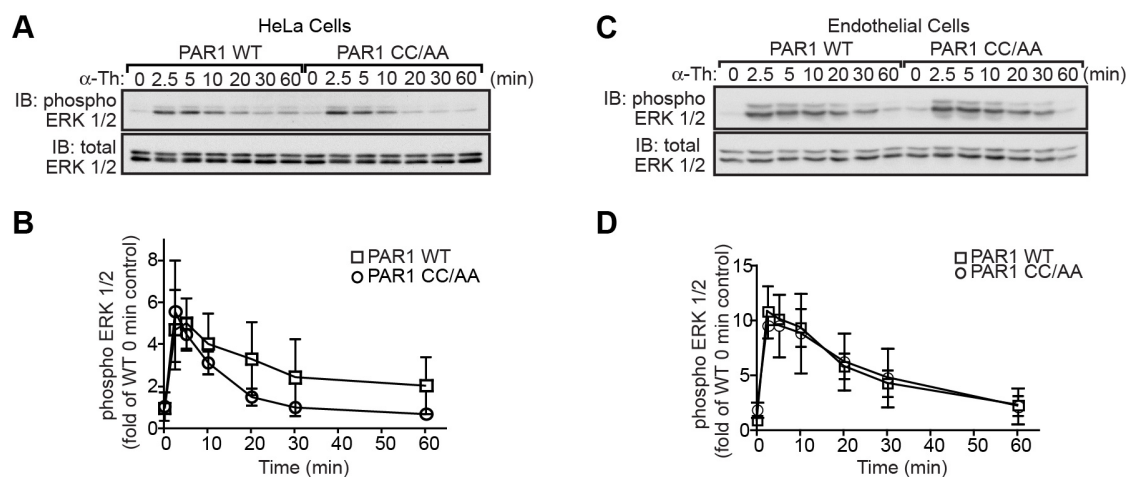


Figure 4.2: Palmitoylation-deficient PAR1 elicits ERK1/2 phosphorylation in response to thrombin. A) HeLa cells expressing PAR WT or CC/AA mutant were stimulated with 10nM thrombin for various timepoints and then lysed. Lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti- ERK1/2, then stripped and reprobed with anti-phospho ERK1/2. B) Phospho ERK signal was quantified from three independent experiments using ImageJ software and normalized to the total ERK signal. Results are plotted as a fold (mean $\pm$ SD) of the 0 min WT control. C) PAR1shRNA + siRNA resistant PAR1 WT or CC/AA expressing endothelial cells were grown to confluency and treated with 10nM thrombin for various times and then lysed and processed as described in (A). D) Phospho ERK signal from endothelial cells was quantified from three independent experiments using ImageJ software and normalized to the total ERK signal. Results are plotted as a fold (mean $\pm$ SD) of the WT control at 0 min.

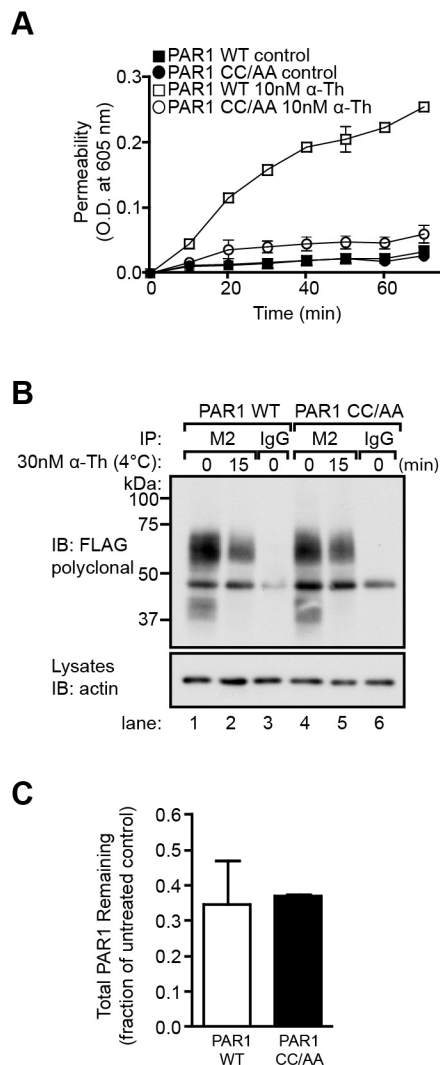


Figure 4.3: Palmitoylation is required for thrombin-induced endothelial barrier permeability. PAR1 shRNA EA.hy926 cells stably expressing siRNA resistant PAR1 WT or CC/AA mutant were grown to confluency and treated with 10 nM thrombin for 10 min at 37°C. Endothelial barrier permeability was assessed at multiple timepoints after thrombin stimulation and plotted as absorbance of Evans Blue-BSA at O.D. 605nm (mean $\pm$ SD). B) siRNA resistant PAR1 WT or CC/AA mutant EA.hy926 stable cell lines were grown to confluency and treated with or without 30nM thrombin at 4°C for 15 min, then washed, lysed and immunoprecipitated (IP) with M2 anti-FLAG antibody or IgG control. IPs were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-FLAG polyclonal antibody. Total lysates were immunoblotted with anti-actin. C) Extent of thrombin cleavage was assessed by quantifying the total levels of PAR1 in IPs using Image J. Thrombin treated samples are plotted as a fraction (mean $\pm$ S.D.) of the untreated controls.

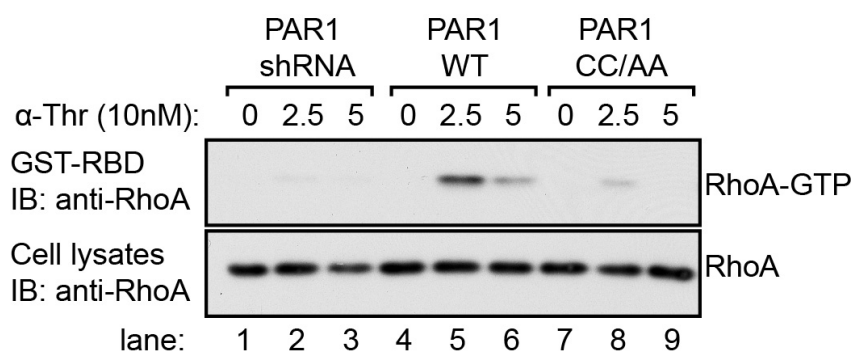


Figure 4.4: Palmitoylation is required for thrombin mediated RhoA activation.

PAR1 shRNA EA,hy926 cell lines stably expressing siRNA resistant FLAG PAR1 WT or CC/AA mutant were treated with 10 nM thrombin for 2.5 or 5 min and lysed. Equivalent amounts of lysates were incubated with GST-Rhotekin Rho-binding domain (RBD) bound Sepharose beads to isolate GTP-bound RhoA. The amount of activated RhoA was determined by immunoblotting GST pulldowns with anti-RhoA antibody. An aliquot of lysates was immunoblotted for total RhoA with anti-RhoA antibody.

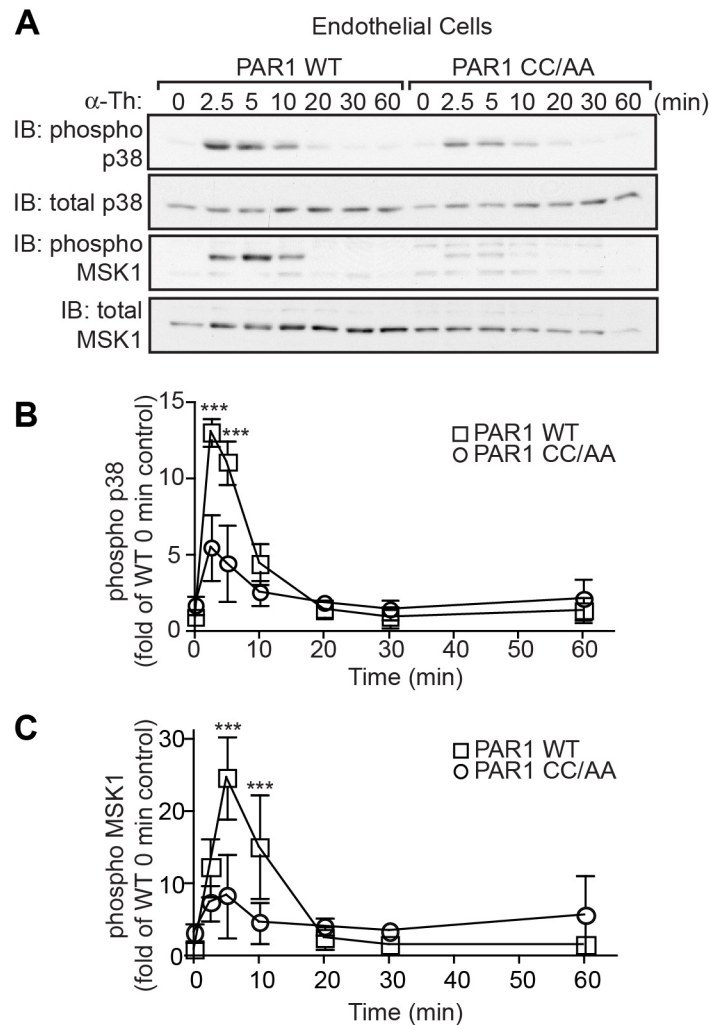


Figure 4.5: Palmitoylation is required for thrombin-induced p38 MAPK activation in endothelial cells. A) PAR1 shRNA rescued with siRNA resistant PAR1 WT or CC/AA mutant EA.hy926 stable cell lines were treated with 10nM thrombin for various times and then lysed. Lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti- p38 and anti-phospho MSK1. Blots were stripped and reprobbed with anti-phospho p38 and anti-MSK1. B) Phospho p38 and was quantified from multiple experiments using ImageJ software, normalized to the total p38 signal, and plotted as a fold of the 0 min WT control (mean $\pm$ SD). The fold of p38 phosphorylation in WT expressing cells was statistically significantly greater than CC/AA expressing cells ($***P < 0.001$) as assessed by two way ANOVA with a Bonferroni post-hoc analysis C) phospho MSK1 signal was quantified as described in (B). The fold of MSK phosphorylation was statistically significantly greater in PAR1 WT expressing endothelial cells compared to CC/AA expressing cells ($***P < 0.001$) as assessed by two way ANOVA with a Bonferroni post-hoc analysis.

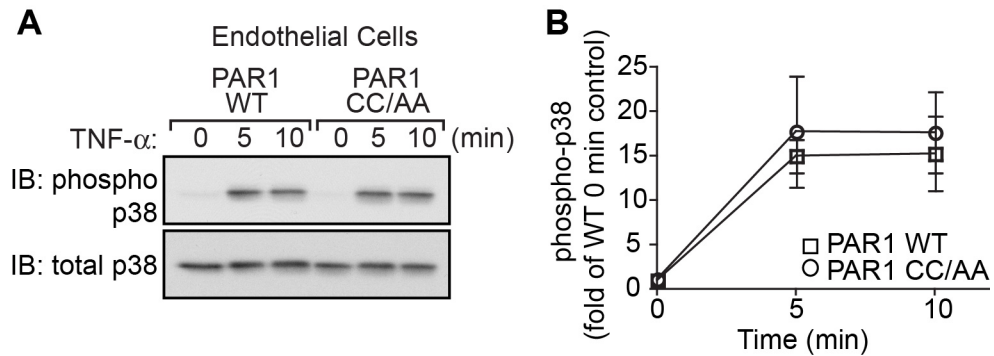


Figure 4.6: PAR1 CC/AA endothelial stable cell lines are not defective for ligand-induced p38 signaling. A) PAR1 WT or CC/AA mutant EA.hy926 stable cell lines were treated with 25ng/ml TNF- α for 5 or 10 min, and then lysed. Lysates were resolved by SDS PAGE, transferred to PVDF membranes and immunoblotted with anti-p38, then stripped and reprobed with anti- phospho p38. B) Phospho p38 signal following TNF- α treatment was quantified from three independent experiments using ImageJ software and normalized to the total p38 signal. Results are plotted as a fold (mean $\pm$ SD) of the 0 min WT control.

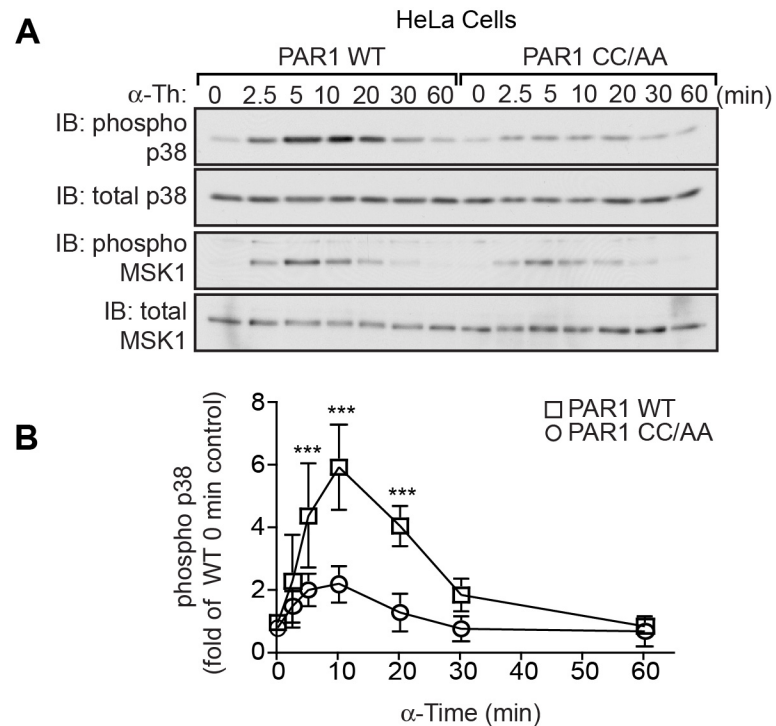


Figure 4.7: Palmitoylation is required for thrombin-induced p38 MAPK activation in HeLa cells. A) HeLa cells expressing PAR WT or CC/AA mutant were stimulated with 10 nM thrombin for various timepoints, lysed, and immunoblotted with anti- p38. Blots were stripped and reprobed with anti-phospho p38. B) Phospho p38 signal in thrombin stimulated HeLa cells was quantified from three independent experiments using ImageJ software and normalized to the total p38 signal. Results are plotted as a fold (mean $\pm$ SD) of the 0 min WT control. The fold of p38 phosphorylation in WT expressing cells was statistically significantly greater than CC/AA expressing cells ($***P < 0.001$) as assessed by two way ANOVA with a Bonferroni post-hoc analysis.

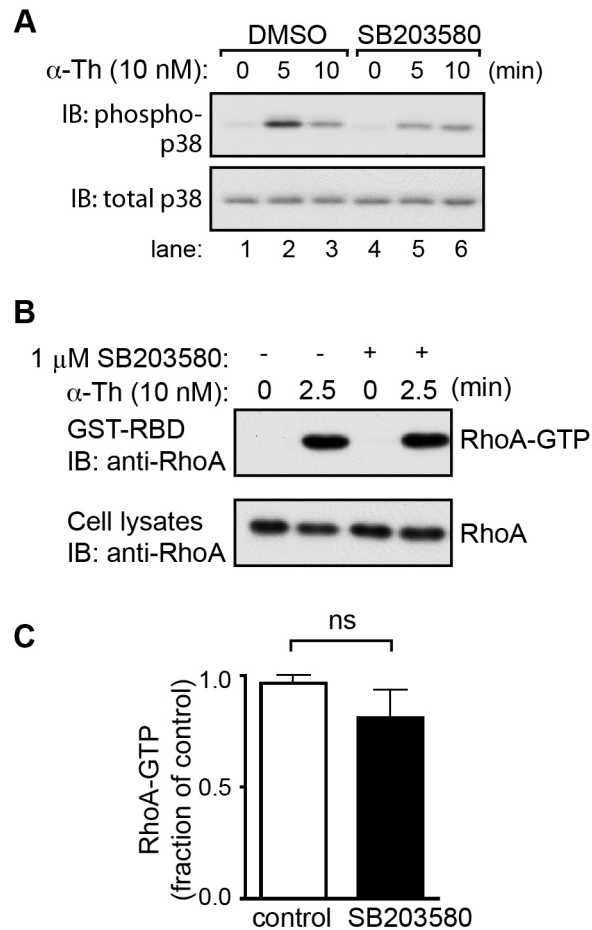


Figure 4.8: Thrombin-induced RhoA activation is not mediated by p38 MAPK. A) PAR1 WT expressing EA.hy926 cells were treated with DMSO, or pretreated with 1 μ M SB 203580, and then stimulated with 10 nM thrombin for 5 or 10 min. Cells lysates were immunoblotted for total p38, then stripped and reprobed for phospho-p38. B) PAR1 shRNA EA,hy926 cell lines stably expressing siRNA resistant FLAG PAR1 WT were pretreated with 1 μ M SB 203580, then stimulated with 10 nM thrombin for 2.5 min and lysed. Equivalent amounts of lysates were incubated with GST-Rhotekin Rho-binding domain (RBD) bound Sepharose beads to isolate GTP-bound RhoA. The amount of activated RhoA was determined by immunoblotting GST pulldowns with anti-RhoA antibody. An aliquot of lysates was immunoblotted for total RhoA with anti-RhoA antibody. C) The amount of thrombin-stimulated RhoA-GTP was quantified and normalized to total RhoA from 3 independent experiments. Data (mean $\pm$ SD) are expressed as a fraction of untreated controls.

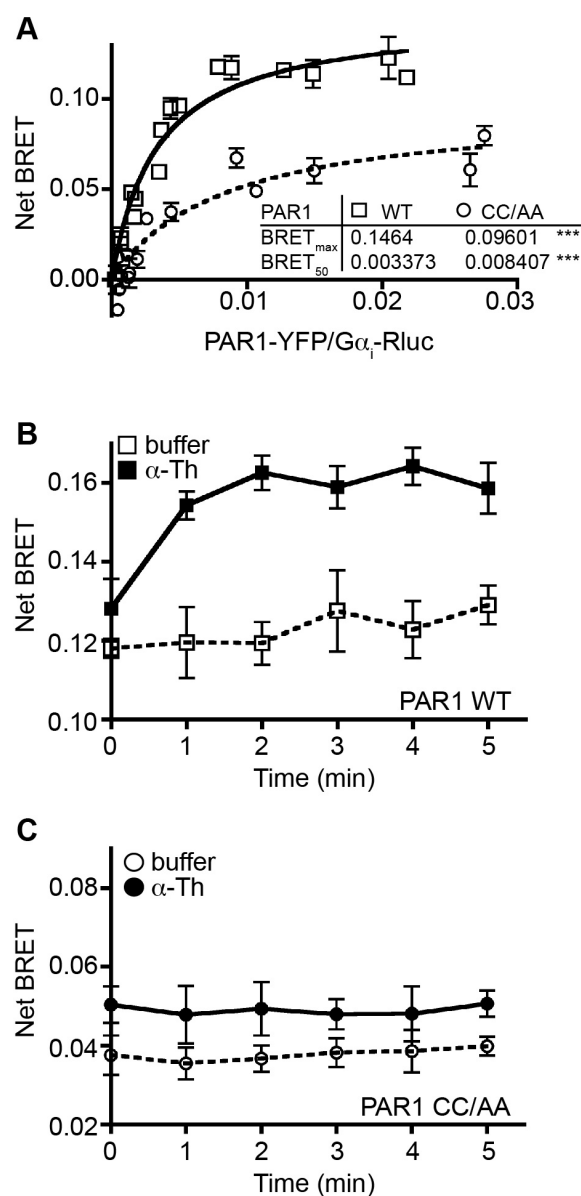


Figure 4.9: Palmitoylation of PAR1 is required for interaction with G α_i . A) COS7 cells were transfected with a fixed amount of G α_i fused to Rluc and increasing amounts of PAR1 WT-YFP or PAR1 CC/AA-YFP and receptor-G α_i interaction was assessed by BRET. The data (mean $\pm$ S.D.; $n=3$) shown are three individual experiments performed in triplicate. *Inset* shows BRET_{max} and BRET₅₀ values for PAR1 WT and CC/AA mutant. B,C) COS7 cells were transfected with PAR1 WT-YFP (B) or PAR1 CC/AA-YFP (C) along with G α_i -Rluc, then stimulated with 10 nM thrombin or buffer control. Net BRET is plotted for the first 5 min following thrombin stimulation.

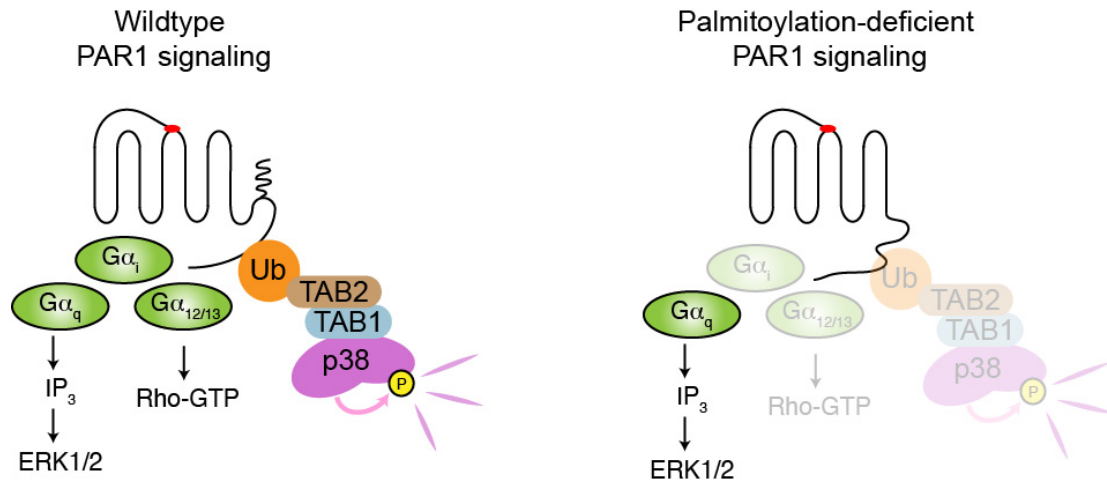


Figure 4.10: A model for the role of PAR1 palmitoylation in activated receptor signaling pathways. Activated wildtype PAR1 generates inositol phosphate (IP_3) production and activation of ERK1/2. Additionally, wildtype PAR1 displays RhoA activation, $G\alpha_i$ association, and ubiquitin-dependent p38 activation. In the absence of palmitoylation, PAR1 retains the capacity to produce IP_3 and activate ERK1/2. However, the palmitoylation-deficient PAR1 mutant displays reduced RhoA activation, ubiquitin-dependent p38 signaling, and $G\alpha_i$ association.

4.8 References

1. Klages, B., Brandt, U., Simon, M. I., Schultz, G., and Offermanns, S. (1999) Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *The Journal of cell biology* **144**, 745-754
2. Majumdar, M., Seasholtz, T. M., Buckmaster, C., Toksoz, D., and Brown, J. H. (1999) A rho exchange factor mediates thrombin and Galpha(12)-induced cytoskeletal responses. *The Journal of biological chemistry* **274**, 26815-26821
3. Coughlin, S. R. (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. *Journal of thrombosis and haemostasis : JTH* **3**, 1800-1814
4. Arora, P., Ricks, T. K., and Trejo, J. (2007) Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. *J Cell Sci* **120**, 921-928
5. Baffy, G., Yang, L., Raj, S., Manning, D. R., and Williamson, J. R. (1994) G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. *The Journal of biological chemistry* **269**, 8483-8487
6. Offermanns, S., Toombs, C. F., Hu, Y. H., and Simon, M. I. (1997) Defective platelet activation in G alpha(q)-deficient mice. *Nature* **389**, 183-186
7. Hung, D. T., Wong, Y. H., Vu, T. K., and Coughlin, S. R. (1992) The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *The Journal of biological chemistry* **267**, 20831-20834
8. Komarova, Y. A., Mehta, D., and Malik, A. B. (2007) Dual regulation of endothelial junctional permeability. *Science's STKE : signal transduction knowledge environment* **2007**, re8
9. Konstantoulaki, M., Kouklis, P., and Malik, A. B. (2003) Protein kinase C modifications of VE-cadherin, p120, and beta-catenin contribute to endothelial barrier dysregulation induced by thrombin. *American journal of physiology. Lung cellular and molecular physiology* **285**, L434-442

10. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science* **280**, 2109-2111
11. Holinstat, M., Mehta, D., Kozasa, T., Minshall, R. D., and Malik, A. B. (2003) Protein kinase Calpha-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. *The Journal of biological chemistry* **278**, 28793-28798
12. Essler, M., Amano, M., Kruse, H. J., Kaibuchi, K., Weber, P. C., and Aepfelbacher, M. (1998) Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. *The Journal of biological chemistry* **273**, 21867-21874
13. Vandenbroucke, E., Mehta, D., Minshall, R., and Malik, A. B. (2008) Regulation of endothelial junctional permeability. *Annals of the New York Academy of Sciences* **1123**, 134-145
14. Manolopoulos, V. G., Fenton, J. W., 2nd, and Lelkes, P. I. (1997) The thrombin receptor in adrenal medullary microvascular endothelial cells is negatively coupled to adenylyl cyclase through a Gi protein. *Biochimica et biophysica acta* **1356**, 321-332
15. Horstmeyer, A., Cramer, H., Sauer, T., Muller-Esterl, W., and Schroeder, C. (1996) Palmitoylation of endothelin receptor A. Differential modulation of signal transduction activity by post-translational modification. *The Journal of biological chemistry* **271**, 20811-20819
16. Charest, P. G., and Bouvier, M. (2003) Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient receptor endocytosis and ERK1/2 activation. *The Journal of biological chemistry* **278**, 41541-41551
17. Kvachnina, E., Dumuis, A., Wlodarczyk, J., Renner, U., Cochet, M., Richter, D. W., and Ponimaskin, E. (2009) Constitutive Gs-mediated, but not G12-mediated, activity of the 5-hydroxytryptamine 5-HT7(a) receptor is modulated by the palmitoylation of its C-terminal domain. *Biochimica et biophysica acta* **1793**, 1646-1655

18. Trejo, J., Connolly, A. J., and Coughlin, S. R. (1996) The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. *The Journal of biological chemistry* **271**, 21536-21541
19. Gardner, A., Phillips-Mason, P. J., Raben, D. M., and Baldassare, J. J. (2002) A novel role for Gq alpha in alpha-thrombin-mediated mitogenic signalling pathways. *Cellular signalling* **14**, 499-507
20. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Mol Cell Biol* **26**, 3231-3242
21. Russo, A., Soh, U. J., Paing, M. M., Arora, P., and Trejo, J. (2009) Caveolae are required for protease-selective signaling by protease-activated receptor-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6393-6397
22. Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J., and Trejo, J. (2002) beta -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation. *The Journal of biological chemistry* **277**, 1292-1300
23. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407**, 258-264
24. Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton, J. H. (1991) Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. *Nature* **350**, 516-518
25. Chen, C. H., Paing, M. M., and Trejo, J. (2004) Termination of protease-activated receptor-1 signaling by beta-arrestins is independent of receptor phosphorylation. *The Journal of biological chemistry* **279**, 10020-10031
26. Swift, S., Leger, A. J., Talavera, J., Zhang, L., Bohm, A., and Kuliopulos, A. (2006) Role of the PAR1 receptor 8th helix in signaling: the 7-8-1 receptor activation mechanism. *The Journal of biological chemistry* **281**, 4109-4116

27. Ellis, C. A., Malik, A. B., Gilchrist, A., Hamm, H., Sandoval, R., Voyno-Yasenetskaya, T., and Tiruppathi, C. (1999) Thrombin induces proteinase-activated receptor-1 gene expression in endothelial cells via activation of Gi-linked Ras/mitogen-activated protein kinase pathway. *The Journal of biological chemistry* **274**, 13718-13727
28. Lum, H., and Malik, A. B. (1994) Regulation of vascular endothelial barrier function. *The American journal of physiology* **267**, L223-241
29. Offermanns, S., Laugwitz, K. L., Spicher, K., and Schultz, G. (1994) G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 504-508
30. Soh, U. J., and Trejo, J. (2011) From the Cover: Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through beta-arrestin and dishevelled-2 scaffolds. *Proceedings of the National Academy of Sciences of the United States of America* **108**, E1372-1380
31. Borbiev, T., Birukova, A., Liu, F., Nurmukhambetova, S., Gerthoffer, W. T., Garcia, J. G., and Verin, A. D. (2004) p38 MAP kinase-dependent regulation of endothelial cell permeability. *American journal of physiology. Lung cellular and molecular physiology* **287**, L911-918
32. Grimsey, N. J., Aguilar, B., Smith, T. H., Le, P., Soohoo, A. L., Puthenveedu, M. A., Nizet, V., and Trejo, J. (2014) GPCR Ubiquitination Activates Non-canonical p38 Signaling and Regulates Endothelial Barrier Permeability. *submitted to Dev. Cell*.
33. Goebeler, M., Kilian, K., Gillitzer, R., Kunz, M., Yoshimura, T., Brocker, E. B., Rapp, U. R., and Ludwig, S. (1999) The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. *Blood* **93**, 857-865
34. Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J. J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R. A., and Davis, R. J. (2003) Mechanism of p38 MAP kinase activation in vivo. *Genes & development* **17**, 1969-1978

35. Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R. J., Luo, Y., and Han, J. (2002) MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. *Science* **295**, 1291-1294
36. Spindler, V., Peter, D., Harms, G. S., Asan, E., and Waschke, J. (2011) Ultrastructural analysis reveals cAMP-dependent enhancement of microvascular endothelial barrier functions via Rac1-mediated reorganization of intercellular junctions. *The American journal of pathology* **178**, 2424-2436
37. Baumer, Y., Spindler, V., Werthmann, R. C., Bunemann, M., and Waschke, J. (2009) Role of Rac 1 and cAMP in endothelial barrier stabilization and thrombin-induced barrier breakdown. *Journal of cellular physiology* **220**, 716-726
38. McLaughlin, J. N., Shen, L., Holinstat, M., Brooks, J. D., Dibenedetto, E., and Hamm, H. E. (2005) Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *The Journal of biological chemistry* **280**, 25048-25059
39. Tiruppathi, C., Minshall, R. D., Paria, B. C., Vogel, S. M., and Malik, A. B. (2002) Role of Ca²⁺ signaling in the regulation of endothelial permeability. *Vascular pharmacology* **39**, 173-185
40. Mehta, D., Rahman, A., and Malik, A. B. (2001) Protein kinase C-alpha signals rho-guanine nucleotide dissociation inhibitor phosphorylation and rho activation and regulates the endothelial cell barrier function. *The Journal of biological chemistry* **276**, 22614-22620
41. Sandoval, R., Malik, A. B., Minshall, R. D., Kouklis, P., Ellis, C. A., and Tiruppathi, C. (2001) Ca(2+) signalling and PKCalpha activate increased endothelial permeability by disassembly of VE-cadherin junctions. *The Journal of physiology* **533**, 433-445
42. Korhonen, H., Fisslthaler, B., Moers, A., Wirth, A., Habermehl, D., Wieland, T., Schutz, G., Wettschureck, N., Fleming, I., and Offermanns, S. (2009) Anaphylactic shock depends on endothelial Gq/G11. *The Journal of experimental medicine* **206**, 411-420
43. De Nicola, G. F., Martin, E. D., Chaikuad, A., Bassi, R., Clark, J., Martino, L., Verma, S., Sicard, P., Tata, R., Atkinson, R. A., Knapp, S., Conte, M. R., and

- Marber, M. S. (2013) Mechanism and consequence of the autoactivation of p38alpha mitogen-activated protein kinase promoted by TAB1. *Nature structural & molecular biology* **20**, 1182-1190
44. Ayoub, M. A., Maurel, D., Binet, V., Fink, M., Prezeau, L., Ansanay, H., and Pin, J. P. (2007) Real-time analysis of agonist-induced activation of protease-activated receptor 1/Galphai1 protein complex measured by bioluminescence resonance energy transfer in living cells. *Molecular pharmacology* **71**, 1329-1340
 45. McCoy, K. L., Gyoneva, S., Vellano, C. P., Smrcka, A. V., Traynelis, S. F., and Hepler, J. R. (2012) Protease-activated receptor 1 (PAR1) coupling to G(q/11) but not to G(i/o) or G(12/13) is mediated by discrete amino acids within the receptor second intracellular loop. *Cellular signalling* **24**, 1351-1360
 46. Verrall, S., Ishii, M., Chen, M., Wang, L., Tram, T., and Coughlin, S. R. (1997) The thrombin receptor second cytoplasmic loop confers coupling to Gq-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors. *The Journal of biological chemistry* **272**, 6898-6902
 47. Canto, I., and Trejo, J. (2013) Palmitoylation of Protease-activated Receptor-1 Regulates Adaptor Protein Complex-2 and -3 Interaction with Tyrosine-based Motifs and Endocytic Sorting. *The Journal of biological chemistry* **288**, 15900-15912

Chapter 5: Conclusions

Thrombin is the main effector protease of the coagulation cascade and elicits the majority of its cellular effects through the protease-activated receptor-1 (PAR1) (1). PAR1 has a unique mechanism of activation that involves proteolytic cleavage of the N-terminus (2). This event reveals a new N-terminus that can bind intramolecularly onto the 2nd extracellular loop of the receptor to initiate signaling through heterotrimeric G-proteins. PAR1 is capable of coupling to multiple G-protein subtypes including $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_i$ (1,3), but the mechanisms that dictate how this occurs are not clearly understood. Given the irreversible nature of PAR1 activation, signaling of the receptor is tightly regulated through desensitization and intracellular trafficking. Post-translational modifications are key regulatory mechanisms that govern PAR1 desensitization and intracellular trafficking. PAR1 has been demonstrated to be modified with phosphorylation (4), ubiquitination (4,5), and glycosylation (6). Whether PAR1 is palmitoylated, and the effect of palmitoylation on receptor signal regulation, has not been previously examined. Prior to this dissertation, there was one published study that examined the signaling of a PAR1 mutant where cysteines 387 and 388 were converted to serines (7). This study did not directly determine palmitoylation of the PAR1, but examined $G\alpha_q$ coupling of the receptor through PI hydrolysis. They demonstrated that the PAR1 CC/SS mutant was slightly less efficacious in eliciting inositol phosphate production in response to thrombin stimulation (7). However, the effect of this mutation on PAR1 signaling to other effectors was not examined. Given that the putative palmitoylation sites for

PAR1 are highly conserved (Fig. 2.1), I first sought to determine whether PAR1 was modified by palmitoylation and to assess the function of this post-translational modification on receptor signaling and trafficking. The main conclusions of this work and the significance to the field of GPCR signaling and trafficking are discussed below.

5.1 PAR1 palmitoylation and other post-translational modifications

Prior to this dissertation, there were no studies that demonstrated PAR1 was palmitoylated, although it was highly speculated (7). I first generated a mutant in which the putative palmitoylation sites, C³⁸⁷ and C³⁸⁸, were mutated to alanines. I demonstrated that PAR1 is modified with palmitoylation on these conserved cysteine residues (Fig. 2.2). Mutagenesis of these critical cysteines to alanines was sufficient to prevent palmitoylation from occurring, thus suggesting that these are the primary sites for palmitoylation. I further characterized this PAR1 mutant and showed that it is expressed at the cell surface and fully glycosylated (Fig 2.3). However, it appears as though mutant is unable to be phosphorylated (Fig. 2.5) and ubiquitinated (Fig 2.6), suggesting a role for a palmitoylated C-tail in proper recognition by GRKs and ubiquitin ligases.

Although we have reported that PAR1 is palmitoylated on conserved cysteine residues in the C-tail, it is not known if this modification is dynamic for this receptor. We have shown palmitoylation through [³H]-palmitic acid labeling, and did not detect robust changes in palmitoylation after agonist stimulation (Fig 2.2). This current method does not allow us to determine how many receptors are palmitoylated on the

cell surface, and it is not sensitive enough to detect small changes in palmitoylation. Some GPCRs have been demonstrated to have increased palmitate turnover in response to agonist (8-11) using pulse-chase [^3H]palmitic acid labeling methods. Although this method will show that a receptor is undergoing cycles of palmitoylation/depalmitoylation, it does not indicate whether the overall palmitoylation status of the receptor is increased or decreased. Acyl-biotin exchange (ABE) is another method to measure protein palmitoylation, and involves chemically blocking unmodified cysteines with N-ethylmaleimide, followed by removing palmitate on modified cysteines with hydroxylamine, and then labeling the newly freed cysteines with biotin-HPDP. The biotinylated proteins are then isolated with streptavidin beads and can be probed for a protein of interest through western blotting. Since the biotinylation exchange occurs after cells are lysed and processed, this method has the potential to show a snapshot of receptor palmitoylation at a specific time or under a specific condition. This technology is more sensitive than [^3H]-palmitic acid labeling, and has the potential to provide evidence for dynamic regulation. This method was successfully employed for the μ -opioid receptor (12) and the somatostatin receptor 5 (13). We tried to utilize this method to measure PAR1 palmitoylation, but were unsuccessful. Other methods such as labeling with chemical probes and using click chemistry have also been utilized to show palmitoylation of the μ -opioid receptor (14) and could provide another technique to determine if PAR1 palmitoylation is dynamically regulated.

Thus, we currently do not know if non-palmitoylated and palmitoylated PAR1 forms exist in a heterogeneous population, and whether this contributes to G-protein

selectivity. However, given that other GPCRs have been shown to be dynamically palmitoylated, it would be interesting to determine if this occurs for PAR1.

Additionally, we demonstrated for the first time that palmitoylation is critical for receptor ubiquitination for a GPCR. We also demonstrated that palmitoylation is critical for PAR1 phosphorylation, which has been shown to also be important for some GPCRs (15,16). The studies described in this dissertation show that palmitoylation is critical for certain aspects of PAR1 signaling and trafficking, therefore determining if PAR1 is dynamically palmitoylated could add an additional level of complexity for PAR1 signal regulation and provide new research avenues to be explored.

5.2 Palmitoylation regulates accessibility of tyrosine-based sorting motifs and PAR1 trafficking

In the absence of palmitoylation, PAR1 was constitutively internalized at a more rapid rate, yet agonist-induced internalization was unchanged (Fig. 3.2). This phenotype reflected a similar phenotype that was observed with the PAR1 ubiquitination-deficient mutant (5) and prompted us to examine the ubiquitination status of the CC/AA mutant (Fig. 2.6). Indeed, we observed that agonist-induced ubiquitination of PAR1 was ablated, but basal ubiquitination appeared intact. Previous studies have shown that mutagenesis of lysine residues K⁴²¹ and K⁴²² to alanines was sufficient to induce enhanced constitutive internalization, suggesting that occupation of the tyrosine-based sorting motif YK⁴²¹K⁴²²LL with ubiquitin sterically hinders AP-2 binding. Furthermore, it was shown that mutagenesis of the Y⁴²⁰KKL⁴²³L motif to AKKAA prevents constitutive internalization, suggesting that this is the

primary motif utilized by PAR1 (17). I determined that in the absence of palmitoylation, PAR1 displayed enhanced constitutive internalization that was AP-2 dependent (Fig. 3.4) but independent of the Y⁴²⁰KKL⁴²³ tyrosine-based sorting motif (Fig. 3.6). Since we determined that basal ubiquitination was preserved in the PAR1 CC/AA mutant, these data suggested that a lack of ubiquitination in the YKKL motif was not causing enhanced internalization of the CC/AA mutant. This result prompted us to examine if the enhanced internalization exhibited in the absence of palmitoylation is due to exposure of another tyrosine-based sorting motif localized near the palmitoylation site.

PAR1 harbors another tyrosine-based sorting motif Y³⁸³SIL³⁸⁶ near the palmitoylation sites on the C-tail. It was previously reported that this motif is not required for constitutive internalization, but is required for PAR1 degradation (18) and AP-3 binding (19). I demonstrated that mutagenesis of this motif in the palmitoylation-deficient PAR1 mutant is sufficient to revert the enhanced internalization phenotype (Fig. 3.6). This suggests that the proximal tyrosine-based sorting motif can serve as an alternative AP-2 binding site when it is at the cell surface, in the absence of palmitoylation. Interestingly, a truncation mutant that lacked the palmitoylated cysteines, but retained the YSIL motif also displayed enhanced constitutive internalization (18) suggesting that the YSIL motif can facilitate constitutive internalization in the absence of palmitoylation. I also demonstrated that the palmitoylation-deficient PAR1 mutant is rapidly degraded basally, and this phenotype is rescued with either mutagenesis of the YSIL motif (Fig. 3.9) or AP-3 knockdown (Fig. 3.12). We hypothesize that the receptor is deplamitoylated to allow for AP-3

recognition of the YSIL motif to facilitate receptor degradation following thrombin cleavage. Unfortunately, the methods utilized to detect receptor palmitoylation might not be sensitive enough to determine if the receptor is indeed dynamically palmitoylated. Taken together, the data in this dissertation suggest that palmitoylation of PAR1 can influence receptor trafficking by regulating exposure of tyrosine-based sorting motifs.

Palmitoylation has been suggested to play a role regulating accessibility of sorting motifs in other GPCRs. For example, the thromboxane A₂ receptor TP β isoform harbors a C-tail YX₃Ø motif that functions in constitutive internalization (20). Interestingly, TP β is constitutively palmitoylated at multiple C-tail cysteine residues, yet the YX₃Ø motif resides near the most proximal palmitoylated cysteine (21). Perturbation of TP β palmitoylation by mutation of the distal cysteine residues resulted in loss of constitutive internalization (21), suggesting that palmitoylation is important for the function of tyrosine motifs and constitutive internalization. Interestingly, the CCR5 receptor agonist induced internalization is predominantly dependent upon phosphorylation and modestly dependent on a di-leucine motif in helix 8 (15). It was also shown that a palmitoylation-deficient mutant displayed a defect in receptor internalization (15). Although this defect could be attributed to a defect in phosphorylation, it is also possible that the di-leucine motif is partly involved. However, the role of CCR5 palmitoylation in the regulation of the di-leucine-based sorting motif was not extensively examined.

It has been suggested that palmitoylation could be important in the regulation of linear sorting motifs for some transmembrane proteins. For example, the

transmembrane protein mucolipin-1 contains two functional acidic di-leucine based sorting motifs, one of which is localized near a palmitoylation site. Mutagenesis of the palmitoylation site reduced internalization of a mucolipin-1 C-tail chimera. These studies suggest that palmitoylation could allow for closer proximity of the di-leucine based sorting motif to the plasma membrane, where it can better associate with AP-2 for internalization (22). The yeast casein kinase Yck3p resides at the limiting membrane of the vacuole, an organelle equivalent to the mammalian lysosome, and requires both palmitoylation at its C-terminus and AP-3 binding to a canonical tyrosine YXXØ motif for proper sorting to the vacuole (23). The mannose-6-phosphate receptor is palmitoylated on two cysteine residues in the C-terminal tail, however cysteine 34 was determined to be critical for receptor localization to lysosomes (24). Interestingly, the authors proposed that palmitoylation could regulate the presentation of a downstream di-leucine sorting signal to regulate sorting, however the role of this di-leucine motif was not examined in this study.

Our studies have demonstrated a novel function for PAR1 palmitoylation in receptor trafficking by regulating accessibility of tyrosine-based sorting motifs. It is possible that palmitoylation could play a similar role in other GPCRs, as tyrosine-based sorting motifs are localized near some established palmitoylation sites for other GPCRs (Figure 5.1). Additionally, AP complexes can also bind to acidic di-leucine based motifs composed of [D/E]-X-X-L-[L/I] or [D/E]-X-X-X-L-[L/I] to facilitate receptor trafficking (25). Interestingly, some of these motifs are also located near established palmitoylation sites for some other GPCRs (Fig. 5.1). In particular, the bradykinin B₂, histamine H₂, 5-HT_{7a}, and vasopressin V₂ receptors would be good candidates to

begin examining if palmitoylation regulates receptor sorting via the adjacent linear sorting sequences (Fig 5.1). Such studies could be helpful in identifying a new role for palmitoylation in GPCR trafficking.

5.3 Palmitoylation of PAR1 specifies distinct G-protein signaling pathways

I have demonstrated that palmitoylation of PAR1 is important for some, but not all, thrombin-induced signaling pathways. Interestingly, I determined that palmitoylation of PAR1 is critical for thrombin-induced endothelial barrier permeability (Fig. 4.3). This phenotype could be attributed to defects in RhoA signaling, as RhoA activity has been shown to regulate myosin light chain phosphorylation, and subsequently cell contractility (26). Indeed, the palmitoylation-deficient PAR1 mutant failed to stimulate RhoA activation in response to thrombin (Fig 4.4). Despite this signaling defect, PAR1 CC/AA retained the capacity to elicit thrombin-induced ERK1/2 phosphorylation, comparable to wildtype PAR1 (Fig. 4.2). Thrombin-induced ERK1/2 activation is a response that is downstream $G\alpha_q$ and $G\alpha_i$ signaling pathways (27,28). Additionally, thrombin-mediated phosphoinositide hydrolysis, a signaling pathway downstream of $G\alpha_q$, was comparable between PAR1 WT and CC/AA mutant receptors. However, the palmitoylation-deficient PAR1 mutant was unable to stimulate p38 MAPK signaling to the same extent as the wildtype receptor in endothelial cells (Fig. 4.5) and HeLa cells (Fig. 4.7). Finally, I demonstrate that the palmitoylation-deficient mutant has decreased affinity for $G\alpha_i$ using BRET assays in living cells (Fig. 4.8). Taken together, these data suggest that palmitoylation of PAR1 regulates some, but not all, thrombin-induced signaling pathways. Interestingly, the

signaling pathways that are involved in endothelial barrier permeability signaling are the most drastically affected in the absence of palmitoylation.

Palmitoylation has been shown to be critical for signaling in some GPCRs. Palmitoylation-deficient rhodopsin displays a slower activation of the G-protein transducin *in vitro* (29). Palmitoylation-deficient β_2 -adrenergic receptor showed reduced stimulation of adenylyl cyclase (30), suggesting reduced coupling to $G\alpha_s$. A palmitoylation-deficient endothelin B receptor was unable to inhibit the formation of adenylyl cyclase or stimulate phospholipase C, suggesting that palmitoylation is important for coupling to $G\alpha_i$ and $G\alpha_q$ for this receptor (31). In a similar manner, GPCR palmitoylation was important for $G\alpha_i$ coupling for the 5-hydroxytryptamine-1A (5-HT_{1A}) receptor (32). Although, the chemokine CCR5 receptor retained coupling to $G\alpha_i$, the duration of the response was reduced with the palmitoylation-deficient mutant (33). These studies suggest that palmitoylation could be important to G-protein signaling in general.

There are examples where receptor palmitoylation modulates some, but not all signaling pathways for GPCRs, and could suggest a role for G-protein selectivity. For example, an endothelin receptor A (ET_A) palmitoylation-deficient mutant displays impaired agonist induced phospholipase C activation, suggesting that palmitoylation is important for coupling to $G\alpha_q$ (34). However, this mutant was also retained the ability to promote agonist induced adenylyl cyclase stimulation (34). The 5-HT_{7a} receptor is modified on multiple cysteine residues within the C-terminal tail. However, only the proximal palmitoylation site was important in regulating constitutive $G\alpha_s$ activity, as a mutation at this site increased [³⁵S] GTP γ S binding on $G\alpha_s$ subunits in

the absence of agonist (35). Interestingly, constitutive $G\alpha_{12/13}$ activity was unaffected, even if all palmitoylation sites residues were mutated (35). In a similar manner, palmitoylation of the V_2 receptor is important ERK1/2 activation, but not for the adenylate cyclase signaling, suggesting that palmitoylation is important for interaction with β -arrestin, but not $G\alpha_s$ for this receptor (36).

PAR1 couples to $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_i$ to elicit various cellular effects.

However, it is not well understood how PAR1 is able to couple to these distinct G-protein subtypes. It would be interesting to determine if palmitoylation is important for preassembly of some of these $G\alpha$ subtypes to PAR1. It has been shown that PAR1 can pre-associate with $G\alpha_{q/11}$, $G\alpha_{11/12}$, and $G\alpha_{i/0}$ families in COS7 cells (37). It has been demonstrated that PAR1 interacts with $G\alpha_q$ at the second intracellular loop (38,39). There is also evidence that intracellular loop 3 is important for $G\alpha_q$ and $G\alpha_i$ coupling (40). Thus far, I have determined that certain downstream signaling events are disrupted in the palmitoylation-deficient PAR1 mutant, but I have not demonstrated direct G-protein association. Such studies could yield interesting results, and perhaps highlight the relevance of a palmitoylated C-tail in specifying receptor-G protein interactions. Palmitoylation of GPCR C-tails are predicted to function in generating a 4th intracellular loop by inserting the palmitoyl groups into the plasma membrane. It would be interesting to determine if point mutations within the 4th intracellular loop affect G-protein affinity, and if these results could be corroborated with the palmitoylation mutation. Such studies would help elucidate if some G-protein subtypes, such as the $G\alpha_{12/13}$ or $G\alpha_i$, require the 4th intracellular loop to associate with PAR1.

5.4 Concluding Remarks

The results presented in this dissertation highlight the relevance of palmitoylation in regulating PAR1 post-translational modifications, receptor trafficking, and specific G-protein signaling pathways. Clearly this modification is important for the regulation of PAR1 levels at the cell surface and modulating a variety of cellular responses elicited by thrombin. Palmitoylation is a unique modification because of its ability to be dynamically regulated. It is possible that heterogeneous populations of palmitoylated and non-palmitoylated receptors could exist, perhaps in different cellular compartments. The palmitoylation machinery has been demonstrated to localize to multiple cellular compartments (41-43), and thus can influence receptor palmitoylation/depalmitoylation, providing an additional level of complexity to modulate signaling and trafficking. The data in this dissertation suggests that PAR1 palmitoylation is important for thrombin-induced inflammatory signaling and endothelial barrier permeability. Furthermore, G_{α_q} signaling is retained in the palmitoylation-deficient PAR1, an important signaling response for platelet aggregation. It would be interesting to identify the palmitoyl acyl transferase (PAT) that mediates PAR1 palmitoylation. Although given the diversity of palmitoylated proteins, the abundance of the PAT family, and the potential for redundancy, identifying the PAT specific for PAR1 will be challenging. We could begin with a siRNA screen targeting the 24 mammalian PATs, and searching for enhanced PAR1 constitutive internalization phenotype, or decreased p38 activity. Initial PAT candidates can be further confirmed with labeling of [^3H]palmitic acid and directly examining PAR1 palmitoylation.

For the GPCR rhodopsin, the 4th intracellular loop generated by palmitoylation is alpha-helical in structure, and has been termed the 8th helix. The conserved NPxxY³⁰⁶ motif at the end of the 7th transmembrane (TM7) domain forms a microdomain with residues of the 8th helix (H8) in the rhodopsin receptor (44). Upon activation of the receptor, Y³⁰⁶ rotates into a space opened by TM6 movement, and thus activates of the TM7-H8 (44). These studies demonstrate that movement of the 8th helix is important in GPCR activation. Furthermore, it was demonstrated that the 8th helix exhibits high mobility for the initial stages of β -arrestin binding to phosphorylated rhodopsin, and then exhibits lower mobility to facilitate high-affinity binding (45). It is interesting that the mobility of this 8th helix is very dynamic, and can be altered when other proteins are in proximity. It is tempting to speculate that the palmitoylation status allows for mobility of the 8th helix, and thus affects receptor function.

Other GPCRs have been crystallized and also have an 8th helix, although not always consistent with a palmitoylated cysteine residue anchoring the 8th helix. For example, the 8th helix was in the A_{2A} adenosine receptor crystal structure is stabilized with helix I rather than palmitoylated cysteines, as this receptor lacks a palmitoylation site (46). There have been a few other examples where an 8th helix was stabilized without palmitoylated residues (47,48). Additionally, it was demonstrated that the 4th intracellular loop is important for rhodopsin binding to G α_t , but the absence of palmitoylation did not affect binding (49). Mutagenesis studies targeting the 8th helix affect GPCR function in a variety of ways, highlighting the increasing importance of this domain. It is apparent that palmitoylation allows this domain to associate more

closely with the plasma membrane, however it is not clear if palmitoylation alone stabilizes this domain. Palmitoylation could regulate mobility of the 8th helix and thus add diversity to active conformations of the receptor.

GPCRs do not strictly have one conformational state of activation. The term “biased signaling” refers to the ability for a GPCR to undertake unique active state conformations that influence interactions with signaling proteins, ultimately leading to different relative levels of downstream signaling pathways (50,51). Some of these conformational changes are induced by different ligands, and such variability can affect G-protein coupling and cellular responses. Post-translational modifications have the potential to add another level of complexity to the signaling bias exhibited by GPCRs. Biased signaling has become an attractive concept for new therapeutics, as molecules that activate one particular pathway and not others could be useful in minimizing side-effects of a particular drug.

Small molecules can function as allosteric modulators of GPCRs by binding to distinct sites on the receptor to modulate some but not all signaling responses (52). A previous study showed that the small molecule benzimidazole derivative Q94 selectively blocks thrombin-activated PAR1 coupling to G_{α_q} signaling in mouse lung fibroblasts which inhibited proliferation and fibroblast-to-myofibroblast differentiation (53). In recent work, Dowal *et al.* identified an inhibitory molecule termed J5F that appears to act at the intracytosolic face of the receptor that requires the PAR1 putative 8th helix to inhibit G_{α_q} signaling stimulated by SFLLRN in platelets (54). Remarkably, however, the same compound J5F failed to affect SFLLRN-mediated signaling to $G_{\alpha_{12}}$ when examined in MDCK epithelial cells. J5F was also shown to

inhibit mouse PAR4 signaling based on the observed decrease in arteriolar thrombus formation using a laser induction model. Mouse PAR4 possesses a carboxyl tail cysteine residue that is not conserved in human PAR4, suggesting that palmitoylation and formation of the 8th helix may be important for J5F effects in vivo. Clearly, more work is needed to delineate the mechanisms by which activated PAR1 couples to distinct G protein subtypes, which will facilitate the development of new therapeutic reagents that can be used to selectively modulate specific signaling responses.

5.5 Acknowledgements

Chapter 5, in part, is published as a review article entitled “Allosteric Modulation of Protease-activated Receptor Signaling” in *Mini-Reviews in Medicinal Chemistry* (55). The dissertation author is the primary author of the manuscript.

5.6 Figures

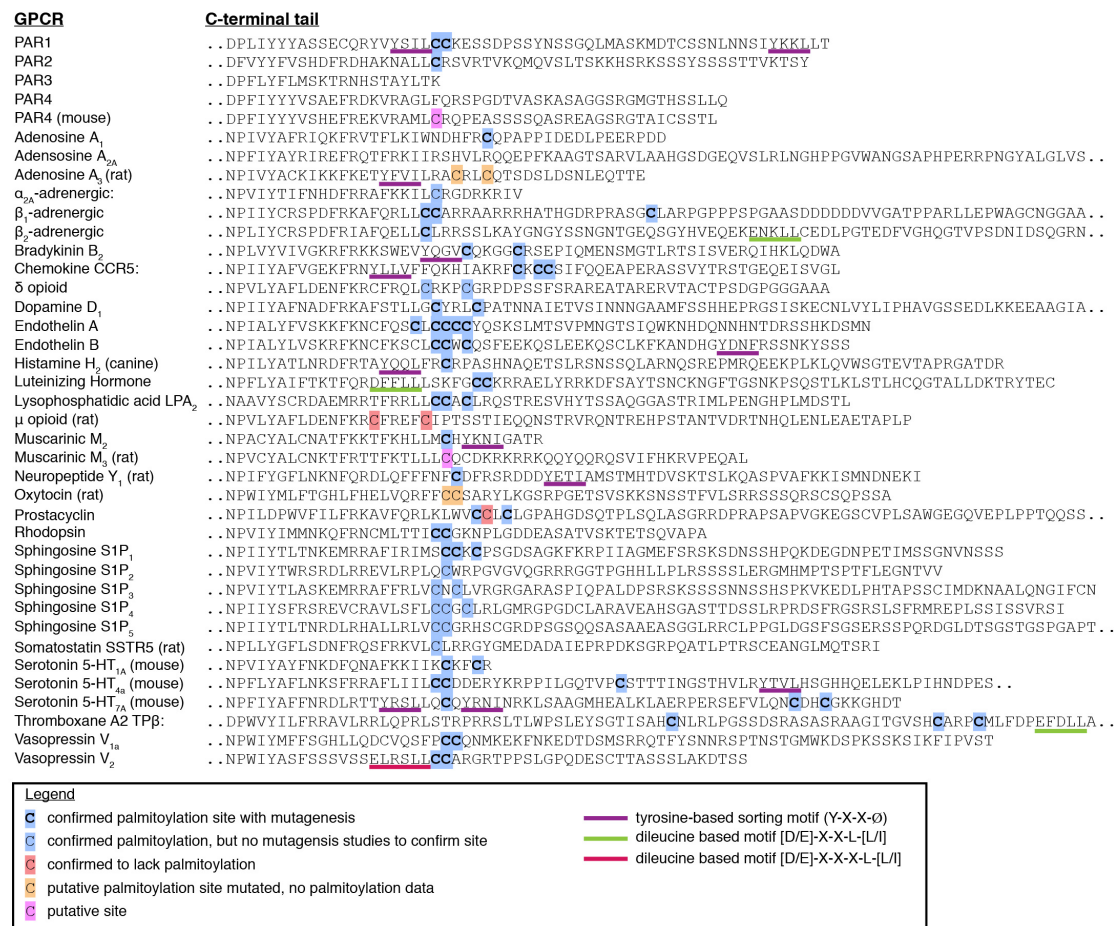


Figure 5.1: Palmitoylated cysteine residues and localization of nearby sorting motifs. Sequence alignment begins from the conserved [D/N]PXXY motif of the 7th transmembrane domain. Palmitoylated cysteine residues are highlighted in blue, red, orange, and pink according to the legend. Tyrosine-based sorting motifs are underlined in purple. Acidic di-Leucine sorting motifs are underlined in green for [D/E]-X-X-L-[L/I] and pink for [D/E]-X-X-X-L-[L/I] sequences.

5.7 References

1. Coughlin, S. R. (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. *Journal of thrombosis and haemostasis* : *JTH* **3**, 1800-1814
2. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068
3. Arora, P., Ricks, T. K., and Trejo, J. (2007) Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. *J Cell Sci* **120**, 921-928
4. Chen, B., Dores, M. R., Grimsey, N., Canto, I., Barker, B. L., and Trejo, J. (2011) Adaptor Protein Complex-2 (AP-2) and Epsin-1 Mediate Protease-activated Receptor-1 Internalization via Phosphorylation- and Ubiquitination-dependent Sorting Signals. *The Journal of biological chemistry* **286**, 40760-40770
5. Wolfe, B. L., Marchese, A., and Trejo, J. (2007) Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. *The Journal of cell biology* **177**, 905-916
6. Soto, A. G., and Trejo, J. (2010) N-linked glycosylation of protease-activated receptor-1 second extracellular loop: a critical determinant for ligand-induced receptor activation and internalization. *The Journal of biological chemistry* **285**, 18781-18793
7. Swift, S., Leger, A. J., Talavera, J., Zhang, L., Bohm, A., and Kuliopulos, A. (2006) Role of the PAR1 receptor 8th helix in signaling: the 7-8-1 receptor activation mechanism. *The Journal of biological chemistry* **281**, 4109-4116
8. Mouillac, B., Caron, M., Bonin, H., Dennis, M., and Bouvier, M. (1992) Agonist-modulated palmitoylation of beta 2-adrenergic receptor in Sf9 cells. *The Journal of biological chemistry* **267**, 21733-21737
9. Kennedy, M. E., and Limbird, L. E. (1994) Palmitoylation of the alpha 2A-adrenergic receptor. Analysis of the sequence requirements for and the

dynamic properties of alpha 2A-adrenergic receptor palmitoylation. *The Journal of biological chemistry* **269**, 31915-31922

10. Ponimaskin, E. G., Schmidt, M. F., Heine, M., Bickmeyer, U., and Richter, D. W. (2001) 5-Hydroxytryptamine 4(a) receptor expressed in Sf9 cells is palmitoylated in an agonist-dependent manner. *The Biochemical journal* **353**, 627-634
11. Zuckerman, D. M., Hicks, S. W., Charron, G., Hang, H. C., and Machamer, C. E. (2011) Differential regulation of two palmitoylation sites in the cytoplasmic tail of the beta1-adrenergic receptor. *The Journal of biological chemistry* **286**, 19014-19023
12. Zheng, H., Pearsall, E. A., Hurst, D. P., Zhang, Y., Chu, J., Zhou, Y., Reggio, P. H., Loh, H. H., and Law, P. Y. (2012) Palmitoylation and membrane cholesterol stabilize mu-opioid receptor homodimerization and G protein coupling. *BMC cell biology* **13**, 6
13. Kokkola, T., Kruse, C., Roy-Pogodzik, E. M., Pekkinen, J., Bauch, C., Honck, H. H., Hennemann, H., and Kreienkamp, H. J. (2011) Somatostatin receptor 5 is palmitoylated by the interacting ZDHHC5 palmitoyltransferase. *FEBS letters* **585**, 2665-2670
14. Ebersole, B., Petko, J., and Levenson, R. (2014) Bioorthogonal click chemistry to assay mu-opioid receptor palmitoylation using 15-hexadecynoic acid and immunoprecipitation. *Analytical biochemistry* **451**, 25-27
15. Kraft, K., Olbrich, H., Majoul, I., Mack, M., Proudfoot, A., and Oppermann, M. (2001) Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization. *The Journal of biological chemistry* **276**, 34408-34418
16. Hawtin, S. R., Tobin, A. B., Patel, S., and Wheatley, M. (2001) Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *The Journal of biological chemistry* **276**, 38139-38146
17. Paing, M. M., Johnston, C. A., Siderovski, D. P., and Trejo, J. (2006) Clathrin adaptor AP2 regulates thrombin receptor constitutive internalization and endothelial cell resensitization. *Mol Cell Biol* **26**, 3231-3242

18. Paing, M. M., Temple, B. R., and Trejo, J. (2004) A tyrosine-based sorting signal regulates intracellular trafficking of protease-activated receptor-1: multiple regulatory mechanisms for agonist-induced G protein-coupled receptor internalization. *The Journal of biological chemistry* **279**, 21938-21947
19. Does, M. R., Paing, M. M., Lin, H., Montagne, W. A., Marchese, A., and Trejo, J. (2012) AP-3 regulates PAR1 ubiquitin-independent MVB/lysosomal sorting via an ALIX-mediated pathway. *Molecular biology of the cell*
20. Parent, J. L., Labrecque, P., Driss Rochdi, M., and Benovic, J. L. (2001) Role of the differentially spliced carboxyl terminus in thromboxane A2 receptor trafficking: identification of a distinct motif for tonic internalization. *The Journal of biological chemistry* **276**, 7079-7085
21. Reid, H. M., and Kinsella, B. T. (2007) Palmitoylation of the TPbeta isoform of the human thromboxane A2 receptor. Modulation of G protein: effector coupling and modes of receptor internalization. *Cellular signalling* **19**, 1056-1070
22. Vergarajauregui, S., and Puertollano, R. (2006) Two di-leucine motifs regulate trafficking of mucolipin-1 to lysosomes. *Traffic* **7**, 337-353
23. Sun, B., Chen, L., Cao, W., Roth, A. F., and Davis, N. G. (2004) The yeast casein kinase Yck3p is palmitoylated, then sorted to the vacuolar membrane with AP-3-dependent recognition of a YXXPhi adaptin sorting signal. *Molecular biology of the cell* **15**, 1397-1406
24. Schweizer, A., Kornfeld, S., and Rohrer, J. (1996) Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. *The Journal of cell biology* **132**, 577-584
25. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) G protein-coupled receptor sorting to endosomes and lysosomes. *Annu Rev Pharmacol Toxicol* **48**, 601-629
26. Essler, M., Amano, M., Kruse, H. J., Kaibuchi, K., Weber, P. C., and Aepfelbacher, M. (1998) Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. *The Journal of biological chemistry* **273**, 21867-21874

27. Ellis, C. A., Malik, A. B., Gilchrist, A., Hamm, H., Sandoval, R., Voyno-Yasenetskaya, T., and Tiruppathi, C. (1999) Thrombin induces proteinase-activated receptor-1 gene expression in endothelial cells via activation of Gi-linked Ras/mitogen-activated protein kinase pathway. *The Journal of biological chemistry* **274**, 13718-13727
28. Trejo, J., Connolly, A. J., and Coughlin, S. R. (1996) The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. *The Journal of biological chemistry* **271**, 21536-21541
29. Park, P. S., Sapra, K. T., Jastrzebska, B., Maeda, T., Maeda, A., Pulawski, W., Kono, M., Lem, J., Crouch, R. K., Filipek, S., Muller, D. J., and Palczewski, K. (2009) Modulation of molecular interactions and function by rhodopsin palmitylation. *Biochemistry* **48**, 4294-4304
30. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1989) Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *The Journal of biological chemistry* **264**, 7564-7569
31. Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S., and Masaki, T. (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. *The Journal of biological chemistry* **272**, 21589-21596
32. Papoucheva, E., Dumuis, A., Sebben, M., Richter, D. W., and Ponimaskin, E. G. (2004) The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *The Journal of biological chemistry* **279**, 3280-3291
33. Blanpain, C., Wittamer, V., Vanderwinden, J. M., Boom, A., Renneboog, B., Lee, B., Le Poul, E., El Asmar, L., Govaerts, C., Vassart, G., Doms, R. W., and Parmentier, M. (2001) Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. *The Journal of biological chemistry* **276**, 23795-23804
34. Horstmeyer, A., Cramer, H., Sauer, T., Muller-Esterl, W., and Schroeder, C. (1996) Palmitoylation of endothelin receptor A. Differential modulation of

signal transduction activity by post-translational modification. *The Journal of biological chemistry* **271**, 20811-20819

35. Kvachnina, E., Dumuis, A., Wlodarczyk, J., Renner, U., Cochet, M., Richter, D. W., and Ponimaskin, E. (2009) Constitutive Gs-mediated, but not G12-mediated, activity of the 5-hydroxytryptamine 5-HT7(a) receptor is modulated by the palmitoylation of its C-terminal domain. *Biochimica et biophysica acta* **1793**, 1646-1655
36. Charest, P. G., and Bouvier, M. (2003) Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient receptor endocytosis and ERK1/2 activation. *The Journal of biological chemistry* **278**, 41541-41551
37. McCoy, K. L., Traynelis, S. F., and Hepler, J. R. (2010) PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. *Molecular pharmacology* **77**, 1005-1015
38. McCoy, K. L., Gyoneva, S., Vellano, C. P., Smrcka, A. V., Traynelis, S. F., and Hepler, J. R. (2012) Protease-activated receptor 1 (PAR1) coupling to G(q/11) but not to G(i/o) or G(12/13) is mediated by discrete amino acids within the receptor second intracellular loop. *Cellular signalling* **24**, 1351-1360
39. Verrall, S., Ishii, M., Chen, M., Wang, L., Tram, T., and Coughlin, S. R. (1997) The thrombin receptor second cytoplasmic loop confers coupling to Gq-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors. *The Journal of biological chemistry* **272**, 6898-6902
40. Covic, L., Gresser, A. L., Talavera, J., Swift, S., and Kuliopulos, A. (2002) Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 643-648
41. Ohno, Y., Kihara, A., Sano, T., and Igarashi, Y. (2006) Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochimica et biophysica acta* **1761**, 474-483

42. Roth, A. F., Wan, J., Bailey, A. O., Sun, B., Kuchar, J. A., Green, W. N., Phinney, B. S., Yates, J. R., 3rd, and Davis, N. G. (2006) Global analysis of protein palmitoylation in yeast. *Cell* **125**, 1003-1013
43. Rocks, O., Gerauer, M., Vartak, N., Koch, S., Huang, Z. P., Pechlivanis, M., Kuhlmann, J., Brunsveld, L., Chandra, A., Ellinger, B., Waldmann, H., and Bastiaens, P. I. (2010) The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins. *Cell* **141**, 458-471
44. Hofmann, K. P., Scheerer, P., Hildebrand, P. W., Choe, H. W., Park, J. H., Heck, M., and Ernst, O. P. (2009) A G protein-coupled receptor at work: the rhodopsin model. *Trends in biochemical sciences* **34**, 540-552
45. Kirchberg, K., Kim, T. Y., Moller, M., Skegro, D., Dasara Raju, G., Granzin, J., Buldt, G., Schlesinger, R., and Alexiev, U. (2011) Conformational dynamics of helix 8 in the GPCR rhodopsin controls arrestin activation in the desensitization process. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18690-18695
46. Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. R., Ijzerman, A. P., and Stevens, R. C. (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* **322**, 1211-1217
47. Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., Leslie, A. G., Tate, C. G., and Schertler, G. F. (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**, 486-491
48. Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C., and Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature* **475**, 65-70
49. Marin, E. P., Krishna, A. G., Zvyaga, T. A., Isele, J., Siebert, F., and Sakmar, T. P. (2000) The amino terminus of the fourth cytoplasmic loop of rhodopsin modulates rhodopsin-transducin interaction. *The Journal of biological chemistry* **275**, 1930-1936

50. Kenakin, T., and Christopoulos, A. (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nature reviews. Drug discovery* **12**, 205-216
51. Wisler, J. W., Xiao, K., Thomsen, A. R., and Lefkowitz, R. J. (2014) Recent developments in biased agonism. *Current opinion in cell biology* **27**, 18-24
52. Kenakin, T. (2010) Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**, 296-302
53. Deng, X., Mercer, P. F., Scotton, C. J., Gilchrist, A., and Chambers, R. C. (2008) Thrombin induces fibroblast CCL2/JE production and release via coupling of PAR1 to Galphaq and cooperation between ERK1/2 and Rho kinase signaling pathways. *Molecular biology of the cell* **19**, 2520-2533
54. Dowal, L., Sim, D. S., Dilks, J. R., Blair, P., Beaudry, S., Denker, B. M., Koukos, G., Kuliopulos, A., and Flaumenhaft, R. (2011) Identification of an antithrombotic allosteric modulator that acts through helix 8 of PAR1. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 2951-2956
55. Canto, I., Soh, U. J., and Trejo, J. (2012) Allosteric modulation of protease-activated receptor signaling. *Mini Rev Med Chem* **12**, 804-811