THE UNIVERSITY OF HULL

THE ROLE OF C-TERMINAL CYSTEINES IN REGULATING HUMAN PROTEINASE-ACTIVATED RECEPTOR-1 FUNCTION

Being a Thesis submitted for the Degree of Doctor of Philosophy

In the Division of Cardiovascular & Respiratory Studies, University of Hull

By

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Abstract

Proteinase-activated receptors (PARs) are a novel group of G-protein coupled receptors (GPCRs). The most striking evidence to distinguish them from other GPCRs is that they carry their own tethered ligand within extracellular N-terminus. To activate the receptors, the tethered ligand is exposed by proteolytic cleavage which subsequently binds to the extracellular loop 2 to trigger receptor function. Four members have been identified so far in this group—PAR_1, PAR_2, PAR_3 and PAR_4.

Palmitoylation is the reversible covalent attachment of fatty acids to the cysteine residues of some GPCRs via a thioester linkage; resulting in a fourth intracellular loop. Although recent evidence has suggested that palmitoylation can have a profound effect on GPCR function such as cell surface expression and receptor signalling, the role of palmitoylation in regulating PAR function is currently unknown. This study focused on the role of putative palmitoylation region of $hPAR_1$--C-terminal cysteines (C387and C388) in regulating receptor function.

Wild type $hPAR_1$ (wt-$hPAR_1$) and $hPAR_1$ mutants ($hPAR_1$C387A, $hPAR_1$C388A and $hPAR_1$C387AC388A) were constructed and permanently expressed in Kirsten virus sarcoma transformed rat kidney epithelial cells (KNRK). $hPAR_1$C387A and $hPAR_1$C388A displayed similar cell surface expression (~80%) to that of wt-$hPAR_1$, but $hPAR_1$C387AC388A displayed only ~40% cell surface expression. $hPAR_1$C387A, $hPAR_1$C388A and wt-$hPAR_1$ displayed similar sensitivity in calcium signalling towards selective PAR_1 agonists—thrombin and TFLLR-NH_2. Surprisingly, $hPAR_1$C387AC388A failed to generate a calcium signal to either PAR_1 agonists. The reduced cell surface expression of $hPAR_1$C387A388A was not responsible for the lack of calcium signal since a wt-$hPAR_1$ cell line with similar cell surface expression to $hPAR_1$C387AC388A displayed robust responses to both thrombin and TFLLR-NH_2. $hPAR_1$C387AC388A was also unable to trigger ERK1/2 phosphorylation in response to either PAR_1 agonists. In agonist triggered internalisation experiments all mutant receptors internalised in response to thrombin and TFLLR, except for wt-$hPAR_1$ which only internalised in response to thrombin. Therefore, we conclude that putative palmitoylation sites within $hPAR_1$ regulate receptor expression, agonist triggered internalisation and are critical for $hPAR_1$ coupling to calcium and ERK1/2.

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The thesis is delicated to my parents,
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Publications

Paper

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Conference Abstracts

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Declaration

I hereby declare that the thesis entitled "The role of C-terminal cysteines in regulating human proteinase-activated receptor-1 ($hPAR_1$) function" has not been submitted for a degree, diploma or any other qualification at any other university. This thesis is the result of my own work and does not include any work that is the outcome of collaboration.

Xiaodan Guo

Abbreviations

CAB	Calcium assay buffer
cDNA	Complementary DNA
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
ECL2	Extracellular loop 2
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
EV	Empty vector
eYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Fluo-3 AM	Fluo-3 acetoxymethyl ester
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	Guanosine triphosphate
hPAR	Human proteinase-activated receptor
IP_3	Inositol triphosphate
JNK	Jun amino-terminal kinase
KNRK	Kirsten virus sarcoma transfected normal rat kidney
LH/CG	luteinizing hormone/chorionic gonadotropin
MAPK	Mitogen-activated protein kinase
MAPKK/MKK/MEK	Mitogen-activated protein kinase kinase
MAPKKK/MEKK	Mitogen-activated protein kinase kinase kinase

mPAR	Mouse proteinase-activated receptor
mRNA	Messenger ribonucleic acid
PAR	Proteinase-activated receptor
PAR-AP	PAR-activating peptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI-3 kinase	Phosphatidylinositide-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PSG	Penicillin/streptomycin/L-glutamine
RT	Room temperature
S.E.	Standard error
wt	wild type
$5\text{-}HT_{4a}$	$5\text{-Hydroxytryptamine}_{4a}$

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1 | INTRODUCTION

1. Introduction

1.1 G-protein coupled receptors

G-protein coupled receptors (GPCRs) constitute the largest family of cell surface receptors comprising approximately 3% of the human genome (Ostrom and Insel, 2004). More than 800 human GPCRs have been identified since the first GPCR was cloned in 1986 (Ostrom and Insel, 2004, Gether, 2000, Oldham and Hamm, 2008). GPCRs are characterised as seven transmembrane spanning proteins with an extracellular N-terminus and a cytoplasmic C-terminus. They transmit a diverse range of extracellular signals into eukaryotic cells mainly by activating heterotrimetic guanine nucleotide binding proteins, which contain a guanosine diphosphate (GDP) bound α subunit and an inseparable complex of β and γ subunits (Gether, 2000, Oldham and Hamm, 2008). Signal transduction begins with the binding of an extracellular ligand to the receptor, the activated receptor then couples to a G protein, resulting in the exchange of GDP to GTP and subsequently the dissociation between α subunit and the βγ complex (Gether, 2000, Oldham and Hamm, 2008). Both α subunit and βγ complex in turn are able to regulate a wide variety of downstream effectors (Ostrom and Insel, 2004, Gether, 2000, Oldham and Hamm, 2008).

Based on phylogenetic analysis, GPCRs existing in humans are grouped into five major classes, named rhodopsin, secretin, glutamate, adhension and frizzled/taste2 (Oldham and Hamm, 2008, Fredriksson et al., 2003).

The secretin family consists of a variety of receptors for peptide hormones and neuropeptides (Gether, 2000). They are characterized by a 60~80 amino acid long

N-terminus containing conserved cysteine bridges (Gether, 2000, Fredriksson et al., 2003). The adhesion receptors were first termed as a group of the secretin family due to their structural similarities (Foord et al., 2005). Recently, the phylogenetic analysis by Fredriksson *et al* indicated a clear distinction between them, and thus adhension receptors are classified as an independent class, distinct from the secretin family (Fredriksson et al., 2003). The main characteristic of the adhesion family is their long N termini that contain adhesion-like motifs, such as epidermal growth factor-like and mucin-like repeats (Fredriksson et al., 2003, Yona et al., 2008). Members of the glutamate family have a so-called 'Venus flytrap' N terminus, which is composed of two lobes separated by a cavity where ligands bind (Fredriksson et al., 2003, Oldham and Hamm, 2008). The Frizzled/Taste2 family includes two distinct clusters the frizzled receptors and the taste2 recetors (Fredriksson et al., 2003). Frizzled receptors respond to signals from secreted glycoproteins termed Wnt and their 200 amino acid long N-termini with conserved cysteines are believed to participate in Wnt binding (Fredriksson et al., 2003). Taste2 receptors share several consensus sequences with frizzled receptors, but have a very short N terminus that is unlikely to contain a ligand binding site (Fredriksson et al., 2003).

The rhodopsin family, also called class A, is the largest group of GPCRs and the most studied (Fredriksson et al., 2003, Gether, 2000). The most distinct feature differentiating the rhodopsin family from other families is their short N-termini (Fredriksson et al., 2003). The overall homology among all the receptors in this family is low, but they still have several highly conserved residues such as a DRY motif at the cytoplasmic side of transmembrane domain 3, and an NSxxNPxxY motif in

transmembrane domain 7 (Fredriksson et al., 2003, Gether, 2000, Oldham and Hamm, 2008). Moreover, most of the rhodopsin family receptors contain a disulfide bridge connecting the second and third extracellular loop, and a putative palmitoylation site in the C-terminus (Gether, 2000).

The rhodopsin family receptors respond to diverse types of ligands, and thus have different modes in ligand binding (Gether, 2000, Oldham and Hamm, 2008). For example, amine neurotransmitters are activated by ligand binding within the crevice formed by transmembrane domains, whereas glycoprotein hormones such as follicle stimulating hormone, luteinizing hormone and thyroid stimulating hormone activate the receptors by initially binding to the extracellular N-terminus, leading to a liganded N-terminal segment which makes a secondary contact with the extracellular loops (Gether, 2000).

1.2 Proteinase-activated receptors

Proteinase-activated receptors (PARs) are a novel group of the rhodopsin family of GPCRs. They have the typical GPCR structure: seven transmembrane domains, three intracellular loops, three extracellular loops, an extracellular N-terminus and an intracellular C-terminal tail. However, PARs are characterised by a distinctive mechanism of activation. They carry their own ligand in the extracellular N-terminus instead of requiring exogenous ligand binding. Proteolytic cleavage of the N-terminal of PARs releases a tethered ligand sequence which can activate the receptors to initiate signalling. Four members of PARs have been identified in humans so far--PAR_1, PAR_2 PAR_3, and PAR_4.

1.2.1 Cloning of PARs

1.2.1.1 PAR_1

Thrombin has been recognized as a key effector proteinase in the coagulation process for a number of decades. It was originally identified as a trypsin-like serine proteinase which converts the soluble fibrinogen into insoluble strands of fibrin in the bloodstream, and produced through the proteolytic activation of prothrombin by the action of factor Xa (Davie et al., 1991, Stubbs and Bode, 1993). Tissue factor, an integral membrane protein, is the initiating factor in the coagulation cascade, is expressed by the cell types that under normal condition do not access the bloodstream (Drake et al., 1989). Disruption of vascular integrity allows circulating coagulation factors to contact extravascular tissue factor, and then trigger thrombin generation (Coughlin, 2005). Thrombin was thus identified as a component of a coagulation process linking tissue damage to wound repair (Coughlin, 2005). Thrombin is also found to be perhaps the most potent physiologic activator of platelet aggregation, and thrombin-induced platelet aggregation is critical for hemostasis and thrombosis (Berndt et al., 1986, Eidt et al., 1988). In addition, several studies suggested that thrombin was a potent agonist for a number of biological responses, and had direct cellular effects on various cell types such as monocytes, smooth muscle cells or endothelial cells (Bar-Shavit et al., 1983, Bizios et al., 1986). These observations led to the search for a thrombin receptor. Although classical radioligand binding studies with modified thrombin had identified several candidate throumbin-binding proteins, no functional thrombin receptors had been identified until 1991 (Gronke et al., 1987).

In 1991, the first functional thrombin receptor, which subsequently termed as PAR_1,

was identified by two laboratories. Vu et al expressed mRNA isolated from human thrombin responsive megakaryocytic HEL and Dami cells in *Xenopus* oocytes, and then identified a functional clone using a size-selected cDNA library (Vu et al., 1991a). The other group cloned the thrombin receptor from CCL39 hamster lung fibroblasts by selective PCR amplification of mRNA sequences that displayed homology to the transmembrane domains of GPCR (Rasmussen et al., 1991, Vu et al., 1991a). The study by Vu *et al* (1991) revealed the cDNA sequence encoded a 425 amino acid protein, and the hydropathy analysis revealed the protein included an amino-terminal signal sequence and seven hydrophobic transmembrane domains of a typical GPCR (Vu et al., 1991a). A potential thrombin cleavage site ($LDPR^{41}\downarrow S^{42}FLLRN$, where ↓ is the cleavage site) was identified within the 75 amino acid long N-terminus, and the first six residues of newly created amino terminus SFLLRN would mediate the receptor activation (Vu et al., 1991a). Moreover, genomic analysis indicated that the human PAR_1 ($hPAR_1$) gene was localised to region q13 of chromosome 5 and consisted of two exons separated by a 22kb intron (Bahou et al., 1993b, Schmidt et al., 1996). The majority of the protein including the thrombin cleavage site is encoded in the larger second exon (Schmidt et al., 1996).

1.2.1.2 PAR_2

PAR_2 was first identified in 1994 by screening a mouse genomic library with a mixture of two oligonucleotide primers corresponding to transmembrane regions of the bovine substance K receptor (Nystedt et al., 1994, Nystedt et al., 1995b) Subsequently, the human PAR_2 ($hPAR_2$) was cloned in the next year by the same group (Nystedt et al.,

1995). The deduced $hPAR_2$ sequence revealed that the protein was 397 amino acids long and 83% similar to the mouse PAR_2 with many conservative substitutions (Nystedt et al., 1995, Bohm et al., 1996b). The putative cleavage sites ($SKGR^{34}\downarrow S^{35}LIG$) for both receptors were conserved, however, the exposed tethered ligands were different (SLIGKV in human and SLIGRL in mice) (Nystedt et al., 1994, Bohm et al., 1996b). The first four residues are identical but the lysine and valine residues at position 5 and 6 in the tether ligand of $hPAR_2$ have been replaced by arginine and leucine residues in $mPAR_2$ (Bohm et al., 1996b, Nystedt et al., 1995). Similar to the organization of $hPAR_1$, the $hPAR_2$ gene is divided into two exons with the cleavage site encoded in the larger second exon, and is also mapped to chromosomal region 5q13, where the $hPAR_1$ receptor was previously shown to be located (Nystedt et al., 1995).

1.2.1.3 PAR_3

In PAR_1 knockout mice, the platelets remained responsive to thrombin whereas thrombin responses were not detectable in the fibroblasts (Connolly et al., 1996). The evidence strongly suggested the existence of a second thrombin receptor in mouse platelets (Connolly et al., 1996). cDNA of a new thrombin receptor designated PAR_3, was then yielded from human and mice using a PCR based strategy in 1997 (Ishihara et al., 1997). The deduced $hPAR_3$ protein sequence is 374 amino acids long with a 27% sequence homology to $hPAR_1$ and a 28% sequence homology to $hPAR_2$ (Ishihara et al., 1997). The thrombin cleavage site of $hPAR_3$ is located at $TLPIK^{38}\downarrow T^{39}FRGAP$ with a thrombin-binding site ($S^{47}FEEFP^{52}$) immediately downstream in the N-terminus

(Ishihara et al., 1997). Genomic analysis identified that the $hPAR_3$ gene, like $hPAR_1$ and $hPAR_2$, displayed a two-exon structure in which the second exon contained the majority of the protein and the cleavage site, additionally, the $hPAR_3$ gene was located to the same hPAR gene cluster at 5q13 as $hPAR_1$ and $hPAR_2$ (Schmidt et al., 1998).

1.2.1.4 PAR_4

Studies in platelets from PAR_1 and PAR_3 deficient mice suggested the existence of yet another thrombin receptor (Connolly et al., 1996, Kahn et al., 1998). PAR_4 as a fourth member of the PAR family was identified by searching an expressed sequence tag database by two separate laboratories (Xu et al., 1998, Kahn et al., 1998). The protein consists of 385 amino acids with a proteinase cleavage site ($PAPR^{47}\downarrow G^{48}YPGQV$) in the N-terminus (Xu et al., 1998). PAR_4 shares 33% sequence identity with the other PAR family members, but its extracelluar N-terminus and intracellular C-terminus have little or no sequence similarity to the corresponding regions of the other hPARs (Xu et al., 1998). When expressed in *Xenopus oocyte*, or COS cells, PAR_4 was responsive to thrombin, but the activation required higher thrombin concentrations than PAR_1 and PAR_3 (Xu et al., 1998). This is probably due to the absence of the highly acidic thrombin-binding site observed in the N-terminus of PAR_1 and PAR_3 (Xu et al., 1998). The PAR_4 gene chromosomal localisation was identified as 19p12 (Xu et al., 1998). This location was different from the PAR gene cluster located at chromosome 5q13 and suggested differences in the evolutionary development of PAR_4 (Xu et al., 1998).

1.2.2 Mechanism of activation

Unlike other GPCRs, PARs contain their own ligand within the extracellular N-terminus. Proteinases cleave PARs at a specific enzymatic site in the extracellular N-terminus; the unmasking of new N-terminal residues serves as a tethered ligand and binds to the extracellular domain 2 and subsequently triggers receptor function (Fig 1.1) (Hollenberg and Compton, 2002, Dery et al., 1998, Macfarlane et al., 2001). In addition, it is found that with the exception of PAR_3, the short synthetic peptides, (only five or six animo acids in length) derived from the tethered ligand sequences can also activate the receptors without proteolytic cleavage by direct binding to the extracellular loop 2 (Fig 1.1) (Hollenberg and Compton, 2002, Dery et al., 1998, Macfarlane et al., 2001). These peptides are so-called PAR-activating peptides (PAR-APs). However, PAR-APs show lower potency than proteinases in receptor activation. This may be due to the inefficient presentation of these peptides to the binding domain of the receptor in comparison to the tethered ligand (Ossovskaya and Bunnett, 2004, Blackhart et al., 2000).

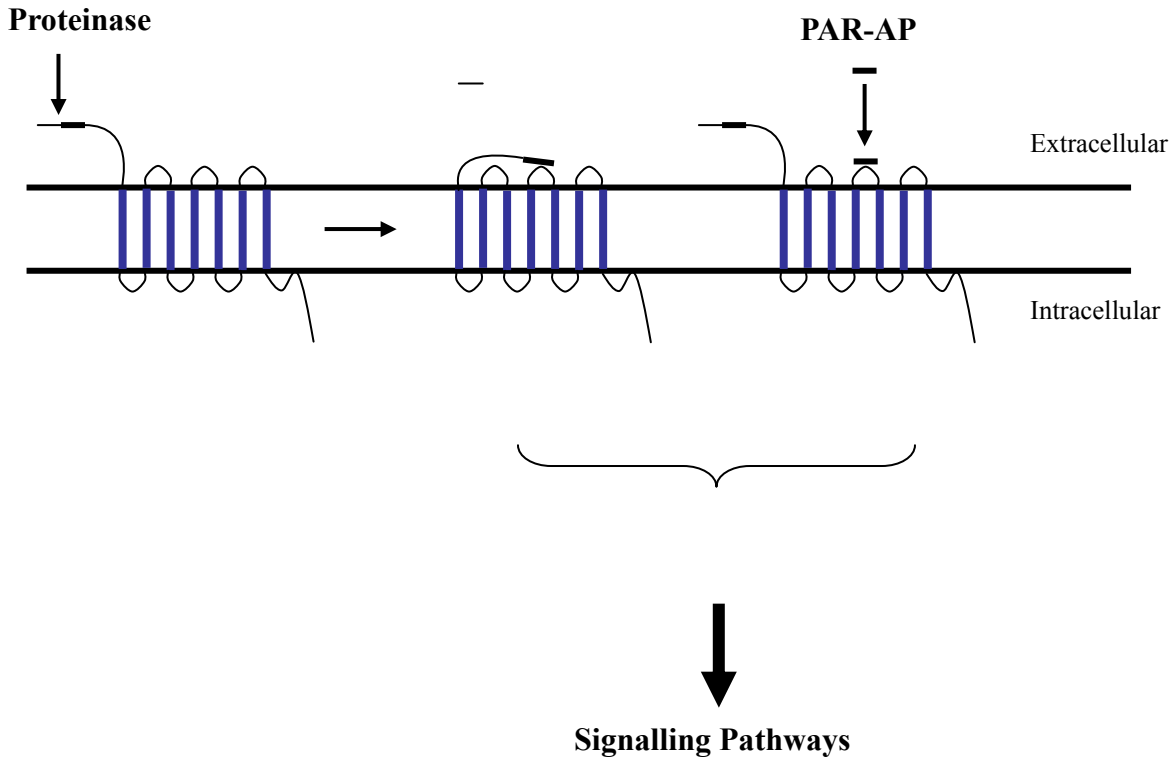


Figure 1.1, PAR activation mechanism by tethered ligands and synthetic agonist peptides.

1.2.2.1 PAR_1

The mechanism of activation of PAR_1 has been extensively investigated and well defined. PAR_1 is activated by thrombin and contains a thrombin cleavage recognition and cleavage site ($LDPR^{41}\downarrow S^{42}FLLRN$) in the N-terminal domain. Immediately following the cleavage site, there is a highly acidic domain ($D^{51}KYEPFWEDEE$) resembling a thrombin binding site in the leech anticoagulant hirudin, which can effectively bind to thrombin's anion-binding exosite (Vu et al., 1991a), and thus this domain is a so-called hirudin-like site. The importance of the thrombin cleavage recognition sequence was indicated by domain swap experiments in which the amino acids LDPR were replaced with the sequence DDDK, the recognition site for the

proteinase enterokinase (Vu et al., 1991b). The resulting PAR_1 mutant completely switched the receptor specificity, abolished thrombin responses and responded to enterokinase (Vu et al., 1991b). Further experiments using antibodies blocking the thrombin cleavage site also abolished the receptor activation (Bahou et al., 1993a, Chen et al., 1994). The deletion of the hirudin-like site in PAR_1 reduced the capacity of thrombin to activate PAR_1, whereas substitution of this domain with the corresponding hirudin C-terminal thrombin binding domain restored the full ability of thrombin to activate PAR_1 (Vu et al., 1991b). Moreover, γ-thrombin lacking the anion-binding exosite is 100-fold less potent than thrombin in PAR_1 activation (Bouton et al., 1995). This site is essential for high affinity binding and activating potency of thrombin towards PAR_1. Therefore, it suggested that thrombin interacts with PAR_1 through the recognition sequence LDPR/S and hirudin-like sequence DKYEPFWEDEE, and then cleaves the receptor at the Arg-Ser peptide bond to activate the receptor. To investigate the interaction between the tethered ligand and the body of the receptor, substitution of *Xenopus* PAR_1 receptor with cognate human receptor sequence suggested that two regions contritubed to the tethered ligand binding specificity: residues 82-90 in the amino-terminal and residues 259-262 in the second extracellular loop (ECL2) (Nanevicz et al., 1995). In addition, the transmembrane 7^{th} helix and 8^{th} helix in the C-terminal domain anchored by the 7^{th} transmembrane helix and putative palmitoylation sites have been indicated to be critical in activation of G_q signalling, and this result supported a 7-8-1 receptor activation mechanism for PAR_1 activation of G_q, whereby the 8th helix interacts with transmembrane domain 7 and intracellular loop 1 during signal transference from receptors to G proteins (Swift et al., 2006).

The short synthetic peptide corresponding to the $hPAR_1$ tethered ligand, SFLLR can activate PAR_1 without proteolytical cleavage. However, SFLLR was also shown to have a cross-activation with $hPAR_2$ (Blackhart et al., 1996). Substitution of the first serine residue with a threonine residue made TFLLR highly specific to PAR_1 (Hollenberg et al., 1997), indicating that Phe^2, Leu^4 and Arg^5 are important residues for full PAR_1 activation (Scarborough et al., 1992). Further, a series of PAR_1-APs have been synthesized with enhanced agonist potencies. For example, a designed peptide Ala-(pF)Phe-Arg-Cha-hArg-Tyr-NH_2 shows 1000-fold higher agonist potency than SFLLR (Feng et al., 1995). However, although PAR_1-AP can elicit full response from PAR_1, it does not mimic the activation of the receptor by thrombin in all respects (Blackhart et al., 2000). Studies have shown that there are profound differences in the activation mechanism of PAR_1 by the tethered ligand and free peptide (Blackhart et al., 2000). Mutations capable of virtually eliminating a response to the PAR_1-AP have little effect on the activation of the receptor by thrombin. For example, the deletion mutant lacking amino acids 68-93 of the N-termini has completely lost the ability to respond to PAR_1-AP yet still retains the ability to generate a maximal response to thrombin at a 10-fold higher concentration. PAR_1 also shows the functional selectivity of G protein signalling by thrombin and PAR_1-APs (McLaughlin et al., 2005). Compared with stimulation by thrombin, peptide induced activation alters PAR_1-G protein coupling to favor G_q binding over $G_{12/13}$ in human microvascular cells (McLaughlin et al., 2005).

There is evidence that PAR_1 activation can be intermolecular between adjacent PAR_1 receptors, and intramolecular liganding is the dominant mode of thrombin receptor activation (Chen et al., 1994). Coexpression of a PAR_1 mutant lacking a functional

tethered ligand domain with PAR_1 mutant that contained a functional tethered ligand but unable to signal, still resulted in thrombin signalling (Chen et al., 1994).

1.2.2.2 PAR_2

PAR_2 was identified as a trypsin receptor and can be activated by trypsin cleavage at $SKGR^{34}\downarrow S^{35}LIG$ in the N-terminal domain (Bohm et al., 1996b, Lerner et al., 1996, Nystedt et al., 1994, Bohm et al., 1996a). Similar to PAR_1, the ECL2 of PAR_2 was shown to be a critical determinant for PAR_2 responsiveness to the tethered ligand. A PAR_1-PAR_2 chimera in which the ECL2 of PAR_1 was replaced by the cognate segment of PAR_2 gained more than 10-fold responsiveness to PAR_2 peptide (Lerner et al., 1996). This study also revealed that extracellular N-terminus and ECL3 participate in agonist recognition. Moreover, Al-Ani et al indicated that the first tethered ligand dipeptide $S^{35}L$ and the acidic tripeptide $P^{231}EE$ in ECL2 perform an important role in PAR_2 activation (Al-Ani et al., 1999, Al-Ani et al., 2004).

In contrast to PAR_1, where thrombin attaches to the hirudin-like domain of the receptor during the activation, there is no evidence to suggest that proteinases interact with PAR_2 at sites other than the cleavage site. The synthetic peptide corresponding to the tethered ligand (SLIGRL in mouse, SLIGKV in human) activates PAR_2 by the same ligand mechanism as PAR_1 (Bohm et al., 1996b, Nystedt et al., 1994). The $hPAR_2$-AP SLIGKV is very specific but has a relatively low potency, interestingly the $mPAR_2$-AP SLIGRL is shown to be a higher potency agonist for $hPAR_2$ than the native peptide (Blackhart et al., 1996). Recently, several synthetic pepetides have been designed for PAR_2 with a high degree of agonist potency (Maryanoff et al., 2001, Kawabata et al.,

2004a, McGuire et al., 2004, Kanke et al., 2005, Seitzberg et al., 2008).

PAR_2 can also be transactivated by cleaved PAR_1 intermolecularly (O'Brien et al., 2000). O'Brien and colleagues showed that the cleaved PAR_1 can donate its tethered ligand to PAR_2 and transactive PAR_2 under the conditions in which PAR_1 has a mutation in the second extracellular loop and thus can not signal (O'Brien et al., 2000). This may explain why PAR_1-AP is able to activate PAR_2, while PAR_2-AP failed to activate PAR_1 (Blackhart et al., 1996).

1.2.2.3 PAR_3

PAR_3 the second thrombin receptor was revealed to have a number of structural and functional similarities to PAR_1. Its N-terminus contains a hirudin-like domain (FEEP) and a thrombin cleavage site $LPIK^{38}\downarrow T^{39}FRG$ (Ishihara et al., 1997). Both mutation of thrombin cleavage site and mutation of hirudin-like domain prevent receptor cleavage by thrombin (Ishihara et al., 1997). After cleavage by thrombin, the exposed amino terminus is presumed to interact with the receptor as a tethered ligand (Ishihara et al., 1997). However, the synthetic peptide representing the tethered ligand sequence of PAR_3 failed to activate PAR_3 but was shown to activate PAR_1 and PAR_2 instead (Ishihara et al., 1997, Hansen et al., 2004, Kaufmann et al., 2005). Curiously, $mPAR_3$ itself is unable to mediate transmembrane signalling even when overexpressed, but instead functions as a cofactor for $mPAR_4$ activation by thrombin (Nakanishi-Matsui et al., 2000).

1.2.2.4 PAR_4

Both thrombin and trypsin cleave PAR_4 at the N-terminal site $PAPR^{47}\downarrow G^{48}YPGQV$ and unmask a new amino terminus which serves as a tethered ligand to activate the receptor (Xu et al., 1998). Mutation of the cleavage site results in a receptor that is unresponsive to proteinases (Xu et al., 1998). PAR_4 lacks the hirudin-like site, and is therefore less responsive to thrombin in comparison to PAR_1 (Xu et al., 1998). However, it has been reported that PAR_4 uses anionic residues (Asp^{57}, Asp^{59}, Glu^{62} and Asp^{65}) in the exodomain to extend contacts with thrombin during activation (Nieman, 2008). The mutation of all the four anionic residues resulted in a receptor which responded to thrombin with a 6-fold higher EC_{50} value compared to the wild type PAR_4, Asp^{59} having the greatest influence (Nieman, 2008). The synthetic peptides based on the proteolytically tethered ligand sequence (GYPGQV in human, GYPGKF in mouse) can fully activate the receptor (Xu et al., 1998). Nonetheless like PAR_1, the $hPAR_4$-AP, GYPGKF, is less efficient than thrombin at activating PAR_4, the analogy AYPGKF has been developed with ~10 fold greater potency than GYPGKF and also relatively specific for PAR_4 (Faruqi et al., 2000).

1.2.2.5 Receptor dimerisation

The ligand-activated G-protein coupled receptors are initially considered as monomers that interact with their specific G proteins (Bulenger et al., 2005). However, more and more evidence is suggesting many GPCRs are capable of forming and functioning within dimers or larger oligometric complexes (Bulenger et al., 2005). A significant

finding was the metaboryopic $GABA_B$ receptor which exists as an obligatory heterodimer (Marshall et al., 1999). Although it was first believed to be an exception, a large number of resonance energy transfer-base studies subsequently showed that GPCRs could form both homo and hetero-dimers in living cells. Receptor dimerisation has been shown to modulate GPCRs function such as signalling, trafficking, internalisation and desensitisation.

Recently, a nuclear magnetic resonance structure of the thrombin-cleaved PAR_1 exodomain displays that PAR_1 bound to anion-binding exosite of thrombin leaves the active site of thrombin still capable of interacting with other large macromolecules such as PAR_4 (Seeley et al., 2003). PAR_3 has been shown to act as a high affinity thrombin binder for adjacent PAR_4 in mouse platelets (Nakanishi-Matsui et al., 2000). These observations suggest the existence of PAR dimerisation. A study using coimmunoprecipitation and flurorescence resonance energy transfer has shown that PAR_1 and PAR_4 formed a heterodimer in human platelets and fibroblasts, and coexpression of PAR_1 with PAR_4 enhanced PAR_4 cleavage and activation (Leger et al., 2006). Furthermore, PAR_3 has been demonstrated to form heterodimers with PAR_1 affecting PAR_1/G_{13} coupling in endothelial cells and thus regulates PAR_1 signalling (McLaughlin et al., 2007).

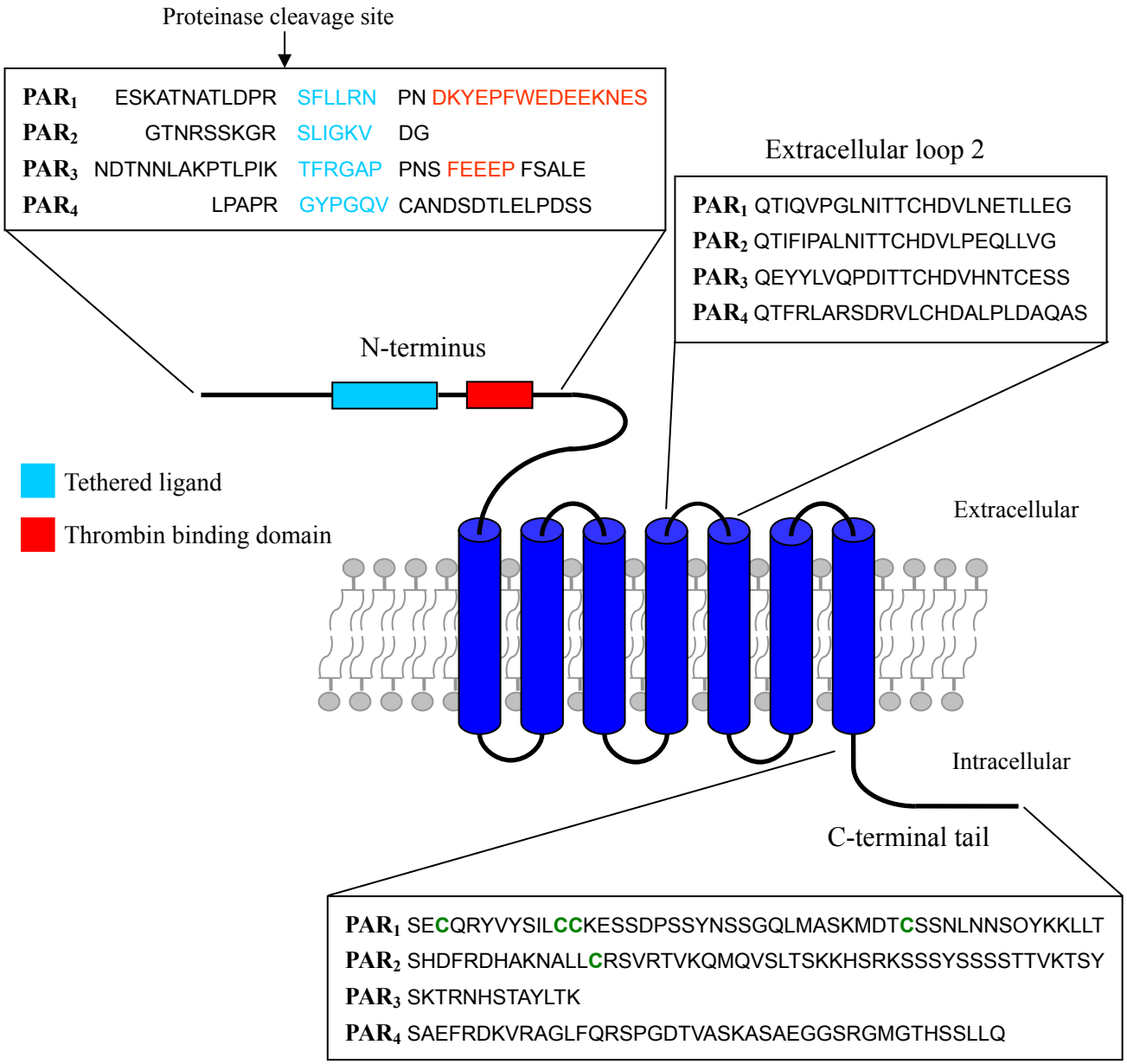


Figure 1.2 Summary of domains thought to be important in PAR signalling. Adapted from (Macfarlane et al., 2001, Ossovskaya and Bunnett, 2004)

1.2.3 PAR mediated intracellular signalling

As described above, Mammalian G proteins are membrane-bound heterotrimeric proteins that receive signals from GPCRs and then transduce signals to intracellular effectors. They are made up of α, β and γ subunits, and are divided into four classes based on the sequence of the Gα subunit—G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Oldham and Hamm, 2008, Wettschureck and Offermanns, 2005). The different downstream effectors activated by Gα are dependent on Gα subtype, for example, G_s α subunits activate adenylyl cyclase, $G_{i/o}$ α subunits inhibit adenylyl cyclase and $G_{q/11}$ α subunits activate phospholipase C (Cabrera-Vera et al., 2003). Most GPCRs can activate more than one G protein subtype, and thus the activation of a single GPCR can trigger several signal transduction pathways. One downstream pathway of G proteins is the activation of phospholipase C (PLC), resulting in the phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG), IP_3 in turn mediates the calcium release from endoplasmic reticulum, while DAG activates protein kinase C (PKC) (Cabrera-Vera et al., 2003).

Activation of G proteins can also activate the mitogen-activated protein kinase (MAPK) pathway. The activation of MAPK occurs through the sequential phosphorylation cascade; at least two upstream kinases are required--mitogen-activated protein kinase kinase kinase (MAPKKK or MEKK) and mitogen-activated protein kinase kinase (MAPKK, MKK or MEK) (Krishna and Narang, 2008, Chang and Karin, 2001). In mammals, six distinct MAPK groups have been reported, extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38 proteins ERK7/8, ERK3/4 and ERK5 (Krishna and Narang, 2008). Each group is activated by specific

MAPKKs such as ERK1/2 which is activated by MEK1/2, ERK5 is activated by MEK5 and p38 is activated by MKK3/6, however, each MAPKK is able to be phosphorlyated by more than one MAPKKK that enhances the complexity and diversity of MAPK signalling system (Chang and Karin, 2001).

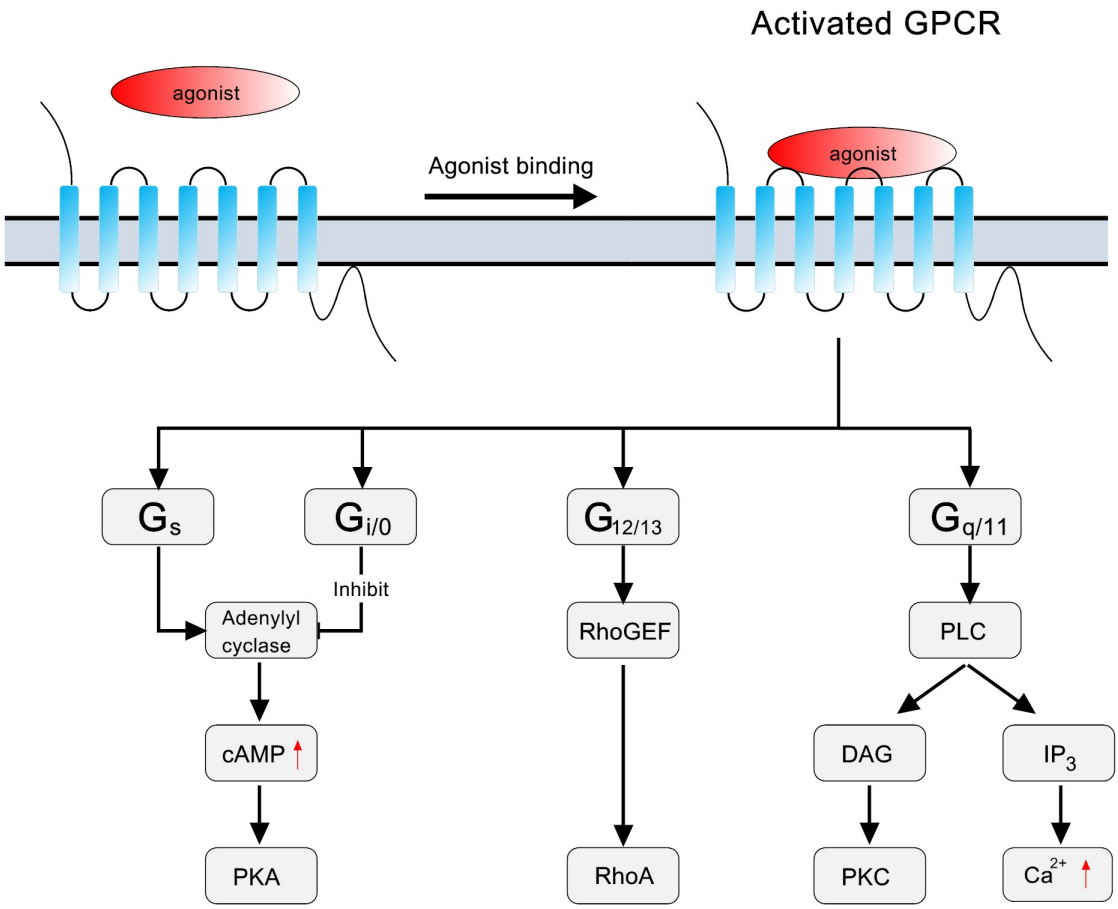


Figure 1.3, An outline of GPCR signalling pathways. Activated GPCR can couple to different G protein subtypes and trigger different signalling pathways. cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; RhoGEF, Rho guanine nucleotide exchange factor; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol triphosphate; PKC, protein kinase C.

1.2.3.1 PAR_1

PAR_1 is shown to couple with multiple G proteins, including members of $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ families (Macfarlane et al., 2001, Steinhoff et al., 2005).

The PAR_1 interaction with G_i proteins, resulting in the inhibition of adenylyl cyclase and formation of cAMP have been reported in multiple cell types such as HEL cells (Brass et al., 1991), osteosarocoma cells (Babich et al., 1990), vascular smooth muscle cells (Kanthou et al., 1996), fibroblasts (Hung et al., 1992a), platelets (Kim et al., 2002), astrocytes (Wang et al., 2002), endothelial cells (Vanhauwe et al., 2002) and olfactory sensory neurons (Olianas et al., 2007). Coupling of PAR_1 to $G_{q/11}$, leading to the activation of PLC and calcium mobilization was revealed through the use of G protein specific antibodies inhibiting PAR_1 mediated calcium signalling in CCL-39 fibroblast cells and through co-immunoprecipitation of $G_{q/11}$ with PAR_1 (Baffy et al., 1994, Ogino et al., 1996). Further evidence for PAR_1 interaction with $G_{q/11}$ has been reported in astrocytes and olfactory sensory neurons (Wang et al., 2002, LaMorte et al., 1993). Studies also show that PAR_1 couples to $G_{12/13}$ in thrombin stimulated platelets and couples to G_{12} in astrocytoma cells (Offermanns et al., 1994, Post et al., 1996, Aragay et al., 1995). PAR_1 is also shown to activate multiple downstream signalling pathways including ERK1/2 MAPK via activation of PI-3 kinase (Malarkey et al., 1995) and Src family tyrosine kinases (Sabri et al., 2002, Darmoul et al., 2004).

1.2.3.2 PAR_2

There is no direct evidence linking PAR_2 to G protein activation, however, numerous

studies show that PAR_2 activation leads to increased second messenger responses. Trypsin and PAR_2-AP stimulate calcium mobilization in PAR_2 transfected cell lines, suggesting that coupling and signalling through $G_{q/11}$ and possibly $G_{i/o}$ (Macfarlane et al., 2001). Coupling of PAR_2 with $G_{12/13}$ has not been reported.

1.2.3.3 PAR_3 and PAR_4

PAR_4 is reported to couple to G_q not G_i in platelets (Faruqi et al., 2000). The activation of PAR_4 has been reported to result in calcium signalling (Kahn et al., 1998, Xu et al., 1998, Camerer et al., 2002) and PAR_4 activation in vascular smooth muscle cells stimulated MAPK signalling (Bretschneider et al., 2001). Additionally PAR_4 mediated Src dependant p38 phosphorylation and activation of ERK and PLC has been demonstrated in PAR_1 knockout mice derived cardiomyocytes (Sabri et al., 2003).

1.2.4 Termination of signalling

The activation of PARs is an irreversible proteolytic mechanism, the generated tethered ligand cannot diffuse away from the receptor, and thus activation would result in a prolonged signalling event. However, the signalling mediated by PARs is transient in nature, efficient mechanisms must exist for termination of PARs signalling (Trejo, 2003, Hollenberg and Compton, 2002). GPCR signalling is terminated by rapid phosphorylation of activated receptors by G protein receptor kinases and/or other kinases, followed by β-arrestin binding to GRK phosphoylated receptors, which disrupts the interaction between receptors and G proteins (Arora et al., 2007, Traynelis and Trejo,

2007). Subsequently, GPCR internalization occurs to remove activated receptor from G protein and signalling effectors at the plasma membrane. Studies on PARs have reported that sequences within the intracellular receptor domains and the C-terminal tail regulate receptor desensitisation and internalisation (Macfarlane *et al.*, 2001; Hollenberg & Compton, 2002; Trejo, 2003).

1.2.4.1 Desensitisation

Activated PAR_1 is desensitised by rapid phosphorylation at the carboxy terminus which is mediated by G protein receptor kinases (GRKs). GRK3 and GRK5 are shown to be two principal mediators in this phosphorylation. Overexpression of GRK3 in Xenpus oocytes enhanced PAR_1 phosphorylation and blocked receptor mediated calcium signalling, whereas GRK2 is considerably less effective (Ishii et al., 1994). *In vivo*, studies in transgenic mice over expressing myocardial GRK3 have confirmed the importance of GRK3 in PAR_1 desensitisation (Iaccarino et al., 1998). A separate study in endothelial cells showed that GRK5 overexpression markedly increased the phosphorylation of PAR_1 (Tiruppathi et al., 2000). β-arrestins are also involved in PAR_1 desensitisation through phosphorylating PAR_1 C-terminal tail, but they do not show the involvement in receptor internalisation (Paing et al., 2002). Desensitisation of PAR_1 is markedly impaired in mouse embryonic fibroblasts lacking β-arrestin 1 and β-arrestin 2 (Paing et al., 2002). In addition, a recent study showed that β-arrestins were able to bind and desensitise the activated PAR_1 independent of GRK phosphorylation at the C-terminus (Chen et al., 2004).

Protein kinase C appears to be the primary mediator of PAR_2 C-terminal

phosphorylation and desensitisation rather than GRK (Bohm et al., 1996a). Desensitisation and internalisation of activated PAR_2 is shown to be dependent on β-arrestins but occur independently of the C-terminal tail (Stalheim et al., 2005, DeFea et al., 2000). There have been no studies in mechanisms of PAR_3 desensitisation. The study in PAR_4 has shown that PAR_4 desensitises slower than PAR_1 perhaps due to the lack of C-terminal phosphorylation sites (Shapiro et al., 2000).

1.2.4.2 Internalisation

Unlike most GPCRs, which are internalised, dephosphorylated and then return to the cell surface, PARs are internalised and directed to lysosomes for degradation. Activated PAR_1 is internalised by a clathrin and dynamin dependent pathway to coated pits (Dery et al., 1998, Trejo, 2003). Dominant-negative dynamin and clathrin mutants both blocked PAR_1 internalisation (Trejo et al., 2000). Phosphorylation at the C-terminus plays an important role in PAR_1 internalisation. A receptor with alanine substitutions at all phosphorylation sites in the C-terminus failed to internalise (Hammes et al., 1999). Moreover, evidence revealed that PAR_1 internalisation was independent of β-arrestin which play a critical role in internalisation of many GPCRs (Paing et al., 2002). Interestingly, uncleaved PAR_1 in endothelial cells has been reported to cycle constitutively between the cell surface and the intracellular compartment (Paing et al, 2006).

Like PAR_1, internalisation of PAR_2 is mediated by clathrin and dynamin, disruption or inhibition of clathrin function has been shown to affect receptor endocytosis (Dery et al., 1999). Unlike PAR_1, however, β-arrestins were revealed to play a critical role for PAR_2

internalization. PAR_2 internalization is completely abolished in cells lacking arrestins (Stalheim et al., 2005, Kumar et al., 2007). A mutant PAR_2 which is unable to interact with β-arrestins failed to internalise (DeFea et al., 2000). Phosphorylation is an important mediator in PAR_1 internalization, whereas mutations of potential phosphorylation sites in C-tail of PAR_4 have no effect in receptor internalization (Shapiro et al, 2000). This may explain why agonist-induced internalization of PAR_4 is much slower than that of PAR_1.

1.2.4.3 Disarming and amputation of tethered ligand by proteinase

Some proteinases can disable PARs by cleaving and removing the tethered ligand so that the generated receptors are unresponsive to activating proteinases. Neutrophil enzymes such as cathepsin G, elastase and proteinase 3 can cleave PAR_1 at distinct cleavage sites within the N-terminus and remove the tethered ligand domain, and thereby abolish the signalling to thrombin (Renesto et al., 1997, Molino et al., 1995). The amputated PAR_1 is still responsive to PAR_1AP suggesting that the binding domain in the second extracellular loop is preserved (Renesto et al., 1997). Chymase, a mast cell proteinase was also shown to inhibit thrombin signalling of keratinocytes (Schechter et al., 1998). Trypsin has also been reported to eliminate the response of PAR_1 to thrombin in endothelial cells (Nakayama et al., 2003, Kawabata et al., 1999). However, studies in human embryonic kidney cells have revealed that trypsin activates PAR_1 at a high concentration, but disarms PAR_1 at a low concentration (0.5-10 nM) (Kawabata et al., 1999). Plasmin, a proteinase of the coagulation cascade, is also shown to activate and inactivate PAR_1 depending on the concentration (Kuliopulos et

al., 1999).

Elastase and cathepsin G have been revealed to disarm PAR_2 by proteolysis of the extracellular domain downstream of the activation site, rendering the receptors unresponsive to the activating proteinases (Uehara et al., 2003). Cathepsin-G and elastase have also been shown to abolish thrombin signalling in PAR_3 transfected cells (Cumashi et al., 2001).

1.2.4.4 Recycling

Due to the irreversible activation mechanism, most activated PARs are destined for lysosomal degradation. However, studies showed that a fraction of activated PAR_1 recycle back to the cell surface after internalisation (Hein et al., 1994). It was observed that more than 85% of PAR_1 receptors on megakaryoblastic human erythroleukemia (HEL) were rapidly internalised after activation, and with approximately 25% of the internalised receptors returned to the cell surface (Hoxie et al., 1993). This observation was subsequently confirmed by using monoclonal antibodies with different epitopes (Brass et al., 1994). Over a three-hour period following thrombin stimulation, the cell surface receptors reacted with the antibodies recognizing the retained portions of the receptor N terminus, but not with the antibodies binding to the cleavage site of PAR_1 (Brass et al., 1994). Interestingly, the recycled receptors also failed to respond to thrombin and to self-activate, but remain responsive to PAR_1-AP SFLLRN (Brass et al., 1994).

1.3 Post-translational modification of GPCRs

Following translation many proteins can undergo chemical modifications termed post-translational modifications. There are a number of different post-translational modifications such as phosphorylation, glycosylation, methylation and acylation. Palmitoylation is one of the most common post-translational lipid modifications in eukaryotic cells. It is the reversible covalent attachment of a 16-carbon saturated palmitic acid to one or more cysteine residues of the protein via a thioester linkage. GPCRs are also subject to this post-translational addition of palmitic acid, and it results in the formation of an additional (fourth) intracellular loop with the plasma membrane. The following section will focus on palmitoylation, its regulation and the role it plays in GPCR signalling events.

1.3.1 Palmitoylation of GPCRs

The palmitoylation of a GPCR was first demonstrated in bovine rhodopsin receptors in 1984, and subsequently the palmitoylation site was determined (O'Brien and Zatz, 1984, Ovchinnikov et al., 1988). Typical palmitoylation sites are cysteine residues in the C-terminal tail of the protein, located 10 to 14 amino acids downstream of the last transmembrane domain (Escriba et al., 2006). These cysteine residues have been found to be conserved in about 80% of all GPCRs (Escriba et al., 2006). Nonetheless, some studies suggested that alternative palmitoylation sites also exist, even for those receptors which are already palmitoylated at their C-tail (Escriba et al., 2006, Qanbar and Bouvier, 2003). For example, Mutation of all the cysteines in the C-terminus of the

μ opioid receptor failed to affect [^{3}H] palmitic acid incorporation supporting the idea that palmitoylation site may not be limited to the C-terminus of the proteins (Chen et al., 1998). The study involving the substitution of C-terminal cysteines of the V_{1a} vasopressin receptor also indicated an additional palmitoylation site at another locus in the receptor (Hawtin et al., 2001).

For some plasma membrane proteins, studies have suggested that palmitoylation could serve a role in the processing and targeting of the protein to the correct membrane site. For example, palmitoylation-deficient CCR5 receptor resulted in both accumulation in intracellular stores and a profound decrease of membrane expression of the receptor (Percherancier et al., 2001). Lack of the palmitoylation site in bovine opsin expressed in COS cells led to the retention of the mutant receptors in the endoplasmic reticulum (Karnik et al., 1993). Similar results were also shown in the mutant luteinizing hormone/chorionic gonadotropin (LH/CG) receptor which has alanines instead of two cysteines in the C-terminus (Zhu et al., 1995). However, this is not the case for all GPCR proteins. The palmitoylation-deficient human β_2-adrenergic receptor has similar receptor cell surface expression as the wild type (O'Dowd et al., 1989).

In addition to receptor cell surface expression, palmitoylation is believed to have an impact on the GPCR signalling functions such as G protein coupling, internalisation and phosphorylation. The elimination of a palmitoylation site seems to exert a different influence on G protein coupling in terms of the type of receptor and G protein subtype. Loss of palmitoylation in LH/hCG receptor does not affect the efficiency of coupling to G_s protein (Kawate and Menon, 1994). Substitution of the palmitoylated cysteine by a glycine in β_2-adrenergic receptor exhibited a drastically reduced ability to interact

with G_s protein, while no significant change in coupling to G_i protein was detected in palmitoylation-deficient α_{2A}-adrenergic receptor (O'Dowd et al., 1989, Kennedy and Limbird, 1993). Palmitoylation of 5-Hydroxytryptamine receptor is critical for the coupling of the receptor with G_i protein and effector signalling (Papoucheva et al., 2004). Moreover, CCR5 receptor palmitoylation sites were not required for G_i-mediated signalling but necessary for the efficient coupling to some other G protein subtypes (Blanpain et al., 2001). Human endothelin B contains three palmitoylation sites—cysteine 402, 403 and 405 (C402, 403 and 405). Without palmitoylation, the receptor failed to interact with either G_q or G_i, but with the first cysteine (C402) restored, the receptor was able to couple with G_q but not G_i (Okamoto et al., 1997). However, studies of human A_1 adenosine receptor indicated that palmitoylation has little effect on receptor-G protein coupling, agonist-induce internalisation (Gao et al., 1999).

An investigation of the β_2-adrenergic receptor also showed the palmitoylation mutant was highly phosphorylated and failed to undergo further phosphorylation when exposed to agonists, whereas the additional mutation of phosphorylation sites in the C-tail restored normal phosphorylation and receptor-G_s coupling, suggesting that the palmitoylation state regulates the β_2-adrenergic receptor phosphorylation (Moffett et al., 2001, Moffett et al., 1993, Moffett et al., 1996). The palmitoylation-deficient LH/hCG mutants have shown a higher ligand-induced internalisation rate than wild type, while the palmitoylation-defective Vasopressin V_{1a} receptor exhibited decreased agonist-induced phosphorylation compared to wild type (Hawtin et al., 2001, Kawate and Menon, 1994, Munshi et al., 2005). Inhibition of CCR5 palmitoylation profoundly

reduced ligand-induced receptor phosphorylation, desensitisation and internalisation (Kraft et al., 2001, Oppermann, 2004). The non-palmitoylated 5-Hydroxytryptamine$_{4a}$ (5-HT$_{4a}$) receptor mutant exhibited enhanced receptor phosphorylation under both basal and agonist stimulated conditions and more effective desensitisation and internalisation (Ponimaskin et al., 2005, Ponimaskin et al., 2002). *In vivo*, the palmitoylation-less rhodopsin in transgenic knock in mice has been shown to become phosphorylated at a faster rate upon exposure to light resulting in a decreased sensitivity to light (Wang et al., 2005).

1.3.2 Palmitoylation and PARs

Palmitoylation appears to facilitate the expression of functional receptors on the cell surface and modulate receptor functions. In the PAR family, only PAR$_1$ and PAR$_2$ have potential typical palmitoylation sites on their C-tail, there is no cysteine residue in the C-terminus of either PAR$_3$ or PAR$_4$ (Figure 1.2, highlighted with green). The cysteine located at 361 of hPAR$_2$ C-terminus was proved to be the palmitoylation site of hPAR$_2$ using autoradiography (Botham, 2007). The lack of the palmitoylation site caused decreased calcium signalling, but greater and prolonged ERK signalling towards agonists (Botham, 2007). The palmitoylation-deficient hPAR$_2$ mutant also displayed a greater cell surface expression compared to wild type, and faster internalisation kinetics in response to the proteinase agonist, but not the peptide agonist (Botham, 2007). But little work has been done on the palmitoylation state of PAR$_1$. There are four cysteines located on the C-terminus of PAR$_1$—C378, C387, C388 and C411 (Figure 1.4). C387 and C388 are located 12 and 13 amino acids away from the last

transmembrane domain which are typical palmitoylation sites of GPCR. Using a computer model based on the 2.8 Å X-ray structure of rhodopsin, these two cysteines were predicted to be dual palmitoylation sites of PAR_1 (Figure 1.4) (Swift et al., 2006). However, the role of these two cysteines in regulating PAR_1 function is still unclear.

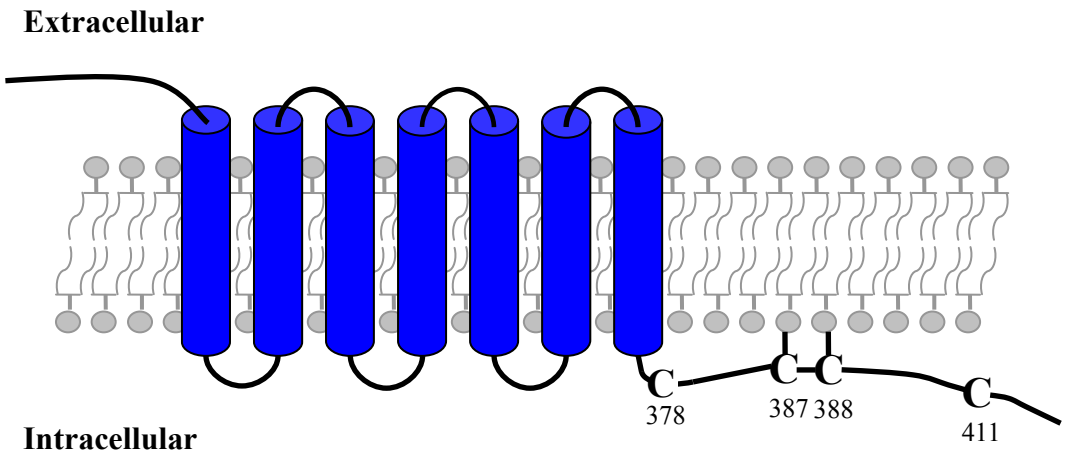


Figure 1.4, $hPAR_1$ structure showing the cysteines located at the C-terminus. There are four cysteins in the C-tail of $hPAR_1$, respectively located 3, 12, 13 and 36 amino acids away from the 7th transmembrane domain.

1.4 Physiological role of PAR_2, PAR_3 and PAR_4

PARs are widely distributed in a number of different cell types in various systems. Studies using enzyme activators, PAR-AP and PAR-gene deficient mice provide strong evidence that PARs play a critical role in regulation of various physiological and pathophysiological functions in mammals including humans (Steinhoff et al., 2005). PARs are thus considered as a potential drug target.

PAR_1 has been detected in a variety of tissues, and will be described in detail in the next section.

PAR_2 has been found to be expressed in several human tissues including brain, heart,

kidney, stomach, pancreas, liver, small intestine and colon, and has been implicated in numerous different physiological and pathophysiological events (Cicala, 2002, Steinhoff et al., 2005). For example, PAR_2 is highly expressed in vascular endothelial cells and smooth muscle cells. Activation of PAR_2 by PAR_2-AP causes the nitric oxide and prostaglandin mediated vasodilation in pre-contracted rat aorta, porcine coronary artery and in human *in vivo*, but no vascular contraction (al-Ani et al., 1995, Magazine et al., 1996, Robin et al., 2003, Hamilton and Cocks, 2000, Hamilton et al., 2001b, Hamilton et al., 2002). Studies have also revealed that PAR_2 activation stimulated proliferation of cultured endothelial cells and smooth muscle cells (Mirza et al., 1996, Bretschneider et al., 1999, Koo et al., 2002). Moreover, the functional expression of PAR_2 is increased in human arterial and venous conduits after exposure to inflammatory stimuli (Ritchie et al., 2007, Ballerio et al., 2007, Hamilton et al., 2001a). The injection of PAR_2-AP in rat hind paw lead to significant oedema and granulocyte infiltration also suggesting the role of PAR_2 in mediating inflammatory responses (Vergnolle et al., 1999a).

Human PAR_3 is expressed in bone marrow, heart, brain, small intestine, airway smooth muscle, vascular endothelium and astrocytes etc (Ishihara et al., 1997, Hauck et al., 1999, Bartha et al., 2000). However, no expression of the receptor on human platelets was observed, which differs from that observed with the murine receptor. In murine platelets, which express PAR_3 and PAR_4, PAR_3 does not mediate thrombin signalling but instead functions as a cofactor to enhance PAR_4 activation by thrombin (Nakanishi-Matsui et al., 2000). $mPAR_3$ blocking antibodies and $mPAR_3$-deficient mice have been shown to inhibit mouse platelet activation at low but not high thrombin

concentrations (Kahn et al., 1998, Ishihara et al., 1998), whereas mPAR$_4$-deficient mice platelets completely failed to trigger platelet aggregation even at high thrombin concentrations (Sambrano et al., 2001).

PAR$_4$ has been identified and cloned from human, mouse and rat tissues. In human, Nothern blot analysis showed PAR$_4$ mRNA in brain, testes, placenta, lung, liver, pancreas, thyroid, skeletal muscle, and small intestine (Xu et al., 1998, Striggow et al., 2001, Steinhoff et al., 2005). Expression of PAR$_4$ can also be found on human platelets, lung endothelial cells and vascular smooth muscle cells (Kahn et al., 1999, Fujiwara et al., 2005, Bretschneider et al., 2001). Although PAR$_1$ is the predominant thrombin mediator in human platelets, activation of PAR$_4$ on human platelets is sufficient to trigger calcium mobilization, platelet secretion and aggregation (Kahn et al., 1999). However, inhibition of PAR$_4$ using a blocking antibody against the thrombin binding site of PAR$_4$ had no effect in platelet activation (Kahn et al., 1999). PAR$_4$ has been also reported as a receptor for cathepsin G as well as thrombin in human platelets (Sambrano et al., 2000). More recently it has been reported that PAR$_4$ activation induced nitric oxide production in vascular endothelial cells (Momota et al., 2006) and contributed to vascular smooth muscle proliferation (Bretschneider et al., 2001). The involvement of PAR$_4$ in inflammatory responses was also revealed through observations *in vivo* that thrombin and PAR$_4$-AP, but not PAR$_1$-AP induced leukocyte rolling and adherence in rat mesenteric venules (Vergnolle et al., 2002), and PAR$_4$-deficient mice have no inflammatory responses after injection of soluble tissue factor into hind paw (Busso et al., 2008). *In vitro*, the proinflammatory activity of thrombin is primarily mediated through the activation of PAR$_4$ in human pulmonary artery endothelial cells (Bae and

Rezaie, 2008). The importance of PAR_4 in inflammation was also highlighted in a study from our laboratory by Ramachandran showing that PAR_4 expression can be induced in human lung fibroblasts following treatment with the inflammatory mediators TNF-α and LPS (Ramachandran et al., 2007).

1.5 Physiological role of PAR_1

1.5.1 Cardiovascular system

PARs are expressed by multiple cells in the cardiovascular and circulatory system including vascular endothelial cells and smooth muscle cells. Thrombin has been shown to be a major stimulator of platelets resulting in a series of subordinated events in platelet aggregation *in vitro* or *in vivo* (Eidt et al., 1988, Hung et al., 1992b, Kahn et al., 1999). Activation of PAR_1 and PAR_4 on human platelets is sufficient to trigger calcium mobilization, platelet secretion and aggregation (Kahn et al., 1999). Antibodies targeted against the thrombin interaction site of PAR_1 results in the inhibition of human platelet activation at low thrombin concentrations but not high, while inhibition of PAR_4 had no effect in platelet activation at either thrombin concentration (Brass et al., 1992, Kahn et al., 1999, Hung et al., 1992b). However, inhibition of both receptors markedly reduced platelet activation even when a high thrombin concentration was used (Kahn et al., 1999). The results suggest that PAR_1 appears to be responsible for mediating thrombin-induced responses in human platelets, and in the absence of PAR_1 function, PAR_4 can mediate platelet activation but only at high thrombin concentrations. PAR_1 and PAR_4 have been shown to have different

functions in regulating human platelets. The activation of PAR_1 triggers the release of vascular endothelial growth factor and suppresses the release of endostatin from platelets, whereas PAR_4 acts in a counter-regulatory manner (Ma et al., 2005). They have also been demonstrated to activate human platelets through distinct pathways (Voss et al., 2007, Holinstat et al., 2007, Bilodeau and Hamm, 2007). Moreover, PAR_1 appears to be more important than PAR_4 in regulating platelet aggregation, blockade of PAR_1 in monkeys attenuates thrombus formation even in the presence of PAR_4 (Derian et al., 2003).

PAR_1 has been reported to regulate endothelial nitric oxide production via phosphorylation of endothelial nitric oxide synthase (Watts and Motley, 2009, Suzuki et al., 2009). Activation of endothelial PAR_1 causes endothelium–dependent vasodilation, which is mediated by the release of endothelial nitric oxide. Thrombin and PAR_1-AP result in a relaxation of pre-contracted blood vessels isolated from a number of species such as the human pulmonary artery (Hamilton et al., 2001b), porcine coronary artery (Hamilton and Cocks, 2000) as well as in a few smaller vessels such as the human and porcine intramyocardial arteries (Hamilton et al., 2002). Furthermore, PAR_1 appears to modulate both vasodilator and contractile responses in rat aorta (Magazine et al., 1996). Moreover, PAR_1–AP caused the relaxation of smooth muscle cells in both human internal mammary artery and saphenous vein (Ballerio et al., 2007), and *in vivo* a study in rats has revealed that PAR_1 mainly mediates hypertension (Cicala et al., 2001). Activation of PAR_1 can cause proliferation of cultured rat vascular smooth muscle cells (Chaikof et al., 1995, McNamara et al., 1993). PAR_1 has been implicated in mediating vascular inflammatory responses. Activation of PAR_1 through intraplantar injection of

PAR_1-AP in rat paw caused oedema which was partly due to PAR_1 mediated plasma extravasation to tissue (Vergnolle et al., 1999b). Studies have shown that PAR_1 mediates protective effects of activated protein C in enhancing the integrity of the vascular barrier during inflammation *in vivo* and *in vitro* (Schuepbach et al., 2009, Schuepbach et al., 2008).

1.5.2 Respiratory system

PAR_1 is detectable on numerous airway associated cell lines including pulmonary fibroblasts (Trejo et al., 1996, Chambers et al., 1998) and epithelial cells (Asokananthan et al., 2002), endothelial cells (D'Andrea et al., 1998, Kataoka et al., 2003) as well as smooth muscle cells (Lan et al., 2000, Walker et al., 2005).

An important function of PAR_1 activation in the lung is the mediation of cell mitogenesis (Blanc-Brude et al., 2005, Walker et al., 2005), although some studies indicated that thrombin but not PAR_1-AP mediated mitogenesis, and suggested a PAR-independent mechanism in the mitogenic action of thrombin (Tran and Stewart, 2003, Walker et al., 2005). Thrombin is also shown to stimulate procollagen production in lung fibroblasts and upregulate connective tissue growth factor production during tissue repair via PAR_1 activation (Chambers et al., 1998, Chambers et al., 2000). Work further supporting the importance of PAR_1 in the pulmonary fibrotic response has been carried out in rats using bleomycin-induced pulmonary fibrosis (Howell *et al.*, 2001; Howell *et al.*, 2002). PAR_1 expression is significantly increased in a bleomycin induced model of pulmonary fibrosis, and use of a thrombin inhibitor significantly reduced connective tissue growth factor mRNA level and

collagen accumulation (Howell et al., 2001, Howell et al., 2002). PAR_1 mediated thrombin signalling has been revealed to stimulate IL-6, IL-8, and PGE_2 release from human lung epithelium cells, and as well as the expression of platelet-derived growth factor (Asokananthan et al., 2002). PAR_1 is also involved in regulating airway tone. Thrombin acting via PAR_1 caused the contraction of human bronchial rings *in vitro* and contraction of guinea-pigs *in vivo* (Hauck et al., 1999, Cicala et al., 1999). However, in mouse isolated trachea, PAR_1 has been revealed to mediate the relaxation of tracheal smooth muscle through prostaglandin PGE_2 release (Lan et al., 2001). Activation of PAR_1 by Pen c 13, a mold allergen, induced IL-8 production in human airway epithelail cells, suggesting an important role in the pathogenesis of this form of asthma (Chiu et al., 2007).

1.5.3 Gastrointestinal system

Involvement of PAR_1 in the gastrointestinal system is mainly in ion transport, permeability, motility and inflammation, however, the opposite effects are sometimes observed in different cell type (Vergnolle, 2005, Vergnolle, 2008).

PAR_1 has been found on endothelial cells (Vergnolle et al., 2004), epithelial cells (Buresi et al., 2001, Buresi et al., 2002), smooth muscle cells (Kawabata et al., 2004b), myofibroblasts (Seymour et al., 2003) and on enteric neurons (Corvera et al., 1997).

Activation of PAR_1 stimulates chloride secretion from intestinal epithelial cells in a calcium- and MAP kinase-dependent pathway, thus enhancing the fluid secretion, resulting in diarrhea as well as serving a protective function by flushing away potential pathogens and toxins during intestinal inflammation (Buresi et al., 2001, Buresi et al.,

2002, Vergnolle, 2008). Further, researchers have shown that PAR_1 was overexpressed in the colon of inflammatory bowel disease (IBD) patients, and in a mouse model of IBD, administration of PAR_1-AP into the colon of mice provoked an inflammatory reaction, while PAR_1 antagonists significantly decreased the mortality and severity of colonic inflammation (Vergnolle et al., 2004).

PAR_1 activation is also reported to increase intestinal barrier permeability, and thus regulates the passage of fluids and microorganisms across the gut mucosa (Chin et al., 2003). Activation of PAR_1 in guinea pigs stimulates contraction of the gastric longitudinal smooth muscles (Saifeddine et al., 2001), but causes relaxation of mouse gastric and colonic muscles (Cocks et al., 1999, Mule et al., 2002). Further to this, *in vivo* studies have demonstrated that activation of PAR_1 can enhance gastrointestinal transit in mice (Kawabata et al., 2001). Recently, Lee *et al.* reported that PAR_1 activation causes contraction in the human and guinea-pig gallbladders and may play important roles in the control of gallbladder motility (Lee and Huang, 2008).

1.5.4 Nervous system

PAR_1 has been shown to be expressed throughout the nervous system such as hippocampus, cortex and amygdale (Striggow et al., 2001). PAR_1 expression has further been revealed in human astrocytoma cells (Grishina et al., 2005) and in guinea pig myenteric (Corvera et al., 1999) and submucosal neurons (Reed et al., 2003).

The morphology and proliferation of astrocytes has been shown to be affected by both thrombin and PAR_1-AP. PAR_1 activation has been demonstrated by several groups to stimulate astrocytes to transform from their normally stellate shape into a polygonal

morphology (Nicole et al., 2005). It has been reported that PAR_1 activation induced proliferation of the resting astrocytes after brain injury and is involved in astrogliosis, an astrocyte proliferation-dependent disorder *in vivo* (Nicole et al., 2005). The activation of PAR_1 by thrombin in neuronal cells has also been shown to protect neuronal cells and astrocytes from cell death induced by hypoglycaemia and oxidative stress (Vaughan et al., 1995). Thrombin and PAR_1-AP have also been revealed to affect the morphology of tyrosine hydroxylase-positive neurons resulting in increased neurite elongation and delayed branching (Debeir et al., 1998). *In vitro* studies on cultured neurons and astrocytes indicated that thrombin and PAR_1-AP can attenuate astrocyte stellation and neurotoxicity induced by β-amyloid which is a putative causative agent in Alzheimer's disease, whilst increase β-amyloid induced expression of basic fibroblast growth factor (Pike et al., 1996). In rat primary astrocytes, the activation of PAR_1 mediates thrombin-induced upregulation of matrix metalloprotease-9 which is a zinc-dependent enzyme involved in the breakdown of extracellular matrix during normal physiological processes (Choi et al., 2008). Recently, PAR_1 has been implicated in HIV associated neurodegenerative disorders. Brain sections taken from patient with HIV encephalitis showed increased level of PAR_1 in astrocytes (Boven et al., 2003). PAR_1 is also upregulated in Parkinson disease affected brain tissue (Ishida et al., 2006), and further provides a thrombin mediated protective pre-conditioning against Parkinson disease related behavioural deficits (Cannon et al., 2006). Moreover, PAR_1 has been found in rat olfactory neurons and the addition of thrombin and PAR_1-AP resulted in a rapid neurite retraction suggesting a role of PAR_1 in neuritogenesis (Olianas et al., 2007).

1.5.5 Renal system

Abundant expression of PAR_1 mRNA in the kidney was initially reported by Rasmussen et al (Rasmussen et al., 1991). Since then PAR_1 has been shown to be present in endothelial, mesangial and epithelial cells in normal human kidney tissue (Grandaliano et al., 2000) as well as in primary human renal carcinoma cells (Kaufmann et al., 2002). It was observed that in fibrin associated renal diseases such as thrombotic microangiopathy and extracapillary glomerulonephritis, PAR_1 protein expression reduced with an increase mRNA level within the glomerular lesion (Xu et al., 1995). Another study *in vivo* in a murine model of crescentic glomerulonerphritis revealed that PAR_1 activation enhanced glomerular crescent formation, T cell and macrophage infiltration, fibrin deposition and elevated serum creatinine, which are prominent features of glomerulonephritis (Cunningham et al., 2000), suggesting a important role for PAR_1 in the pathogenesis of crescentic glomerulonephritis. In addition, PAR_1 activation resulted in renal vasodilation and a marked reduction in the glomerular filtration rate in isolated perfused rat kidney, suggesting the involvement of PAR_1 in the regulation of renal haemodynamics (Gui et al., 2003).

1.6 Working hypothesis

The hypothesis of this study is that "**The $hPAR_1$ C-terminal cysteines regulate receptor function**."

1.7 Aims

In order to test this hypothesis the specific aims of this study were to determine:

- the effect of C387, C388 in regulating receptor functions such as receptor cell surface expression, downstream signalling and receptor internalisation
- whether C387, C388 is palmitoylated

2 | GENERAL MATERIALS AND METHODS

2 General materials and methods

2.1 Introduction

$hPAR_1$ has three cysteines located at 387, 388 and 411 in the C-tail as shown in Figure 2.1, C387 and C388 are considered as putative palmitoylation sites of $hPAR_1$. In this chapter, $hPAR_1$ was ligated into plasmid vectors and KNRK cells were transfected with this construct, generating a stably expressing wild type cell line. In order to investigate the roles of these cysteine residues, C387, C388 and C411 were each replaced by site-directed mutagenesis with an alanine to generate single mutants; C387 and C388 were also substituted by alanines simultaneously to create a double mutant. Alanine has a similar chemical structure with cysteines and thus was chosen to replace cysteines in $hPAR_1$ for avoiding the whole protein structure alteration.

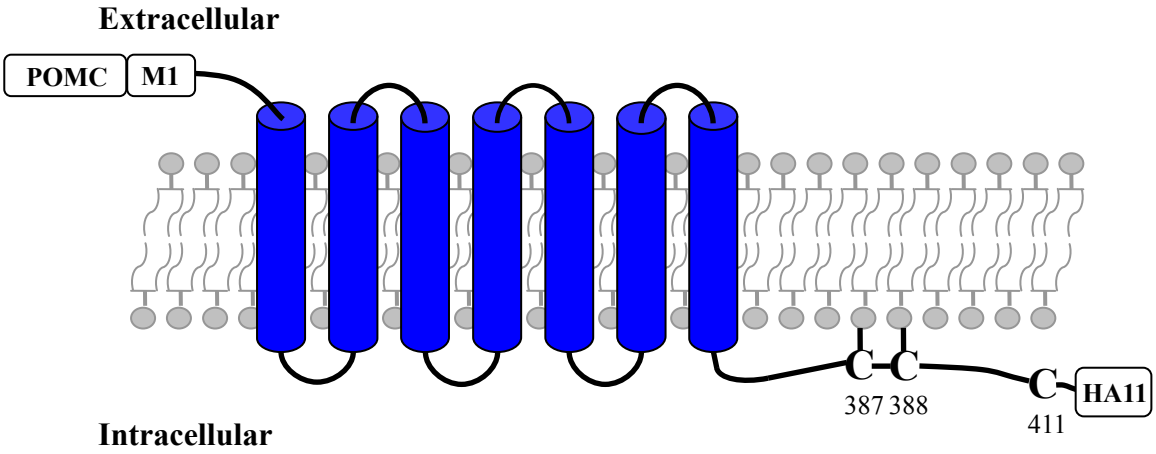


Figure 2.1, Representative model of $hPAR_1$ used in this study. Wild type $hPAR_1$ (POMC-M1-$hPAR_1$-HA11) possessed an N-terminal pro-opiomelanocortin (POMC) singal peptide followed by an M1 epitope, a HA11 epitope was fused to the C-terminal tail. (See Appendix for DNA sequence and protein sequence)

The aim of the chapter was to generate the cell lines perminantly expressing wild type $hPAR_1$, $hPAR_1$C387A, $hPAR_1$C388A, $hPAR_1$C387AC388A and $hPAR_1$C411A to use in

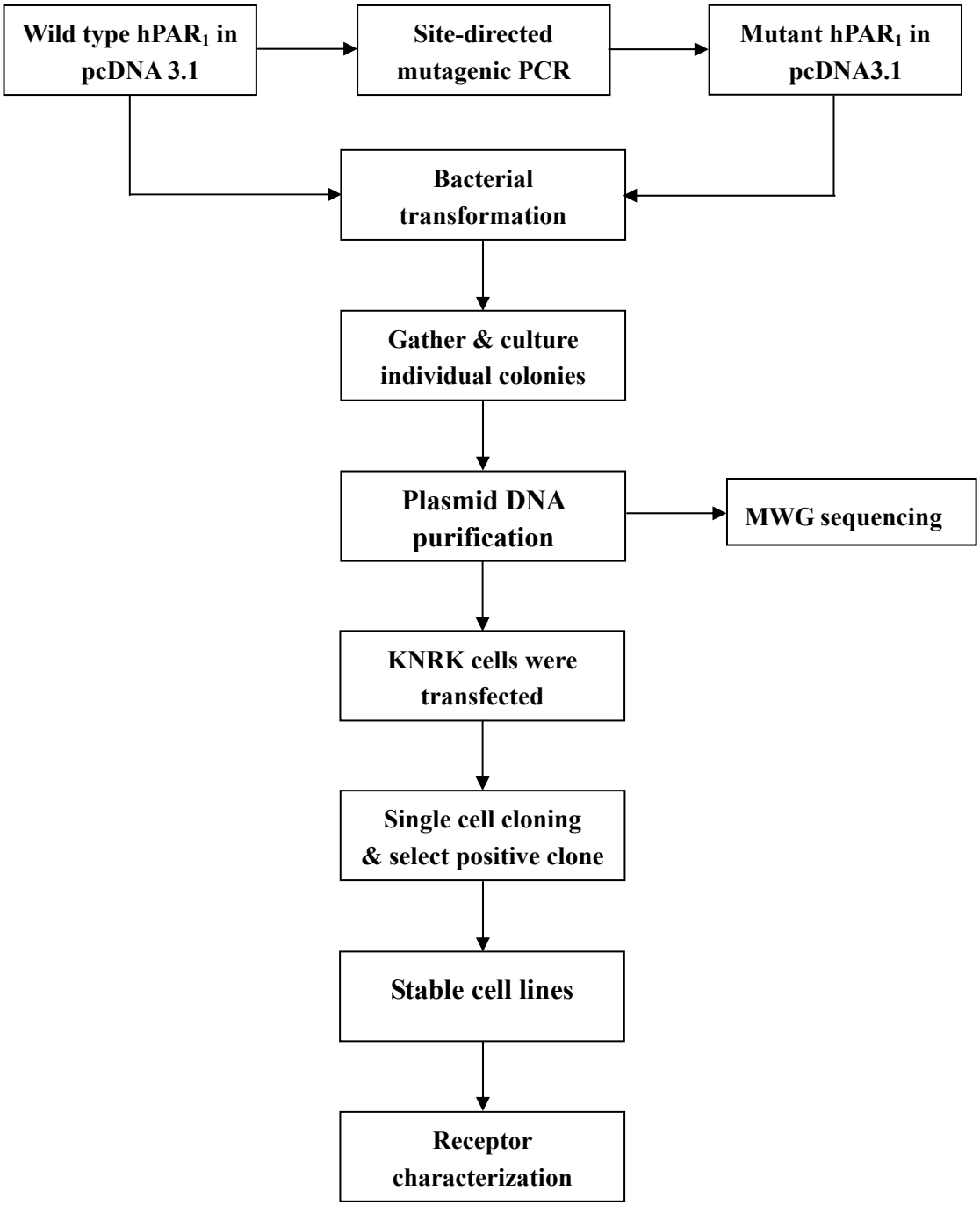
the later chapters.

2.2 Materials and reagents

Primers were purchased from MWG Biotechnology, Ebersberg, Germany. Restriction endonucleases *Hin*dIII and *Xho*I were purchased from New England Biolabs, Hitchin, Hertfordshire, UK. Quick change site-directed mutagenesis kit and XL-1 blue supercompetent *E.coli* were obtained from Stratagene, Amsterdam, The Netherlands. QIAprep miniprep kit was obtained from Qiagen, Crawley, West Sussex, UK. LipofectAMINE, Opti-Mem, Fluo-3 acetoxymethyl ester (Fluo-3 AM), Phosphate Buffer Saline (PBS), geneticin (G418-sulphate), Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, penicillin/streptomycin/L-glutamine (PSG), and Foetal Calf Serum (FCS) were supplied by Invitrogen, Paisley, UK. Anti-PAR_1 ATAP-2 mouse monoclonal antibody was purchased from Zymed Laboratories Inc, San Francisco, USA. Human plasma thrombin was obtained from Calbiochem, UK. Human PAR_1-AP TFLLR-NH_2 was obtained from peptide international, Kentucky, USA. All other chemicals and reagents were purchased from Sigma-Aldrich, Poole, Dorset, UK.

Kirsten virus sarcoma transformed rat kidney epithelial cells (KNRK) were purchased from American Tissue Type Culture Collection (Bethesda, MD, U.S.A). The vector pcDNA 3.1 containing wild type $hPAR_1$ (wt-$hPAR_1$, Figure 2.1) and wt-$hPAR_1$-KNRK cell line were supplied by supervisor Dr. S.J. Compton.

2.3 Methods


Wild type hPAR1 in pcDNA 3.1
Site-directed mutagenic PCR
Mutant hPAR1 in pcDNA3.1
Bacterial transformation
Gather & culture individual colonies
Plasmid DNA purification
MWG sequencing
KNRK cells were transfected
Single cell cloning & select positive clone
Stable cell lines
Receptor characterization

2.3.1 Construction of C-terminal cysteine deleted $hPAR_1$ cDNAs (C387A, C388A, C387AC388A, C411A)

2.3.1.1 Site-directed mutagenesis

The specific oligonucleotide primers for the different $hPAR_1$ mutants were designed between 25-45 base pairs long using primer X (http://bioinformatics.org/primerx) with a melting temperature (T_m) 75-85˚C, a GC content of approximately 40-60%, and a G or C terminator. The sequences of the primers in which the corresponding cysteine was replaced by an alanine are displayed below (the mutations have been highlighted).

C387A primers

5′-CGT CTA CAG TAT CTT AGC CTG CAA AGA AAG TTC CG-3′
3′-GCA GAT GTC ATA GAA TCG GAC GTT TCT TTC AAG GC-5′

C388A primers

5′-CGT CTA CAG TAT CTT ATG CGC CAA AGA AAG TTC CGA TCC-3′
3′-GCA GAT GTC ATA GAA TAC GCG GTT TCT TTC AAG GCT AGG-5′

C387AC388A primers

5′-GGT ACG TCT ACA GTA TCT TAG CCG CCA AAG AAA GTT CCG ATC CC-3′
3′-CCA TGC AGA TGT CAT AGA ATC GGC GGT TTC TTT CAA GGC TAG GG-5′

C411A primers

5′-GCA AGT AAA ATG GAT ACC GCC TCT AGT AAC CTG AAT AAC-3′
3′-CGT TCA TTT TAC CTA TGG CGG AGA TCA TTG GAC TTA TTG-5′

For each $hPAR_1$ mutant, the mutagenesis reaction was prepared in a total volume of 50 µl as follows:

10×reaction buffer	10% (v/v)
Plasmid DNA template	1 ng/µl
Forward primer	2.5 ng/µl
Reverse primer	2.5 ng/µl
dNTP mix	0.5 mM
DNA polymerase (2.5 U/µl)	0.5 U/µl

DEPC H_2O was added to reach a final volume. Afterwards, the mutagenic PCR was placed in a thermal cycler to run the following cycles:

Cycles	**Temperature**	**Time**
1	95˚C	30 sec
16	95˚C	30 sec
	55˚C	1 min
	68˚C	14 min
	4˚C	Forever

Table 2.3.1, Conditions of Mutagenic PCR

Following the PCR reaction, the PCR product was incubated for 1 hour at 37˚C with *Dpn*I restriction enzyme (10 U/µl) to digest the parental vector. Finally, gel electrophoresis was performed to check the presence of the PCR products.

2.3.1.2 Bacterial transformation

1.7 µl of 10% β-mercaptoethanol (β-ME) was added to 50 µl of XL1-Blue supercompetent *E.coli*. The cells were incubated on ice for 10 min with gentle agitation every 2 min. Then 7 µl of mutant DNA from the PCR reaction was added into the

β-ME treated *E.coli* swirled and incubated on ice for a further 30 min. The *E.coli* were heat shocked at 42˚C in a water bath for 45 sec and then immediately returned to ice for a further 2 min. 500 µl of preheated (42˚C) LB Broth was added to the *E.coli* before placing the suspension in a 37˚C, 200 rpm shaking incubator for one hour. Following incubation, 250 µl of the *E.coli* was plated onto an LB agar plate containing 100 µg/ml ampicillin and incubated at 37˚C overnight.

2.3.1.3 Screening colonies and harvesting mutant DNAs

Individual colonies (upto 6 for each mutant) from the transformed *E.coli* plate were randomly picked, and placed into 30 ml universal tubes containing 5 ml of LB broth with 100 µg/ml ampicillin. The universal containers were then incubated at 37˚C at 200 rpm overnight to allow the colonies to grow.

The QIAprep Miniprep Kit was utilized to extract and purify the plasmid DNA from the overnight cultures. 5 µl of the purified DNA was digested by *Hin*dIII and *Xho*I restriction enzymes in NEBuffer 2 at 37˚C for 1 hour, and then run on a 1.3% agarose gel to assess the presence of the insert fragment. The concentration of purified DNA was assessed using a GeneQuant RNA/DNA Calculator (Biochrom LTD, Cambridge, UK) and an aliquot of the sample DNA was sent to MWG (MWG Biotech, London, UK) for sequencing. The construct containing the correct insert sequence was selected for each mutant.

2.3.2 Generation of mutant $hPAR_1$ permanently expressing cell lines

2.3.2.1 Tranfection

KNRK cells were transfected with the mutant $hPAR_1$ DNAs using the lipofectAMINE method. The KNRK cells were plated onto a 65 mm diameter petri dish in normal growth medium and allowed to reach 60-80% confluence by the day of transfection. Two Falcon 5 ml polystyrene round bottom tubes each containing 1 ml of Opti-Mem were prepared. To one tube which was labelled "1", 5 μg of the mutant DNA was added and 20 μl LipofectAMINE was added to the other tube labelled "2". The two tubes were vortexed for upto 4 sec and incubated at room temperature (RT) for 15 min. Meanwhile, the medium was removed from the cells and replaced with 4 ml of Opti-Mem. Before incubating at RT for a further 15 min, the contents of tube 2 were poured directly into tube 1 and vortexed again for upto 4 sec. The Opti-Mem was removed from the KNRK cells, and then the DNA/LipofectAMINE/Opti-Mem mixture was added to the cells. The cells were then incubated at 37˚C, 5% CO_2 for 24 h before replacing the medium with fresh normal growth medium. After incubating for a further 24 h, the cells were harvested and transferred to a 25 cm^2 flask with selective growth medium. A mock transfected KNRK cell line was produced by transfecting KNRK cells with pcDNA 3.1 vectors only using the method described above (empty vector-KNRK, EV-KNRK).

2.3.2.2 Cell culture

KNRK cells were propagated in normal growth medium (DMEM, 10% FCS, 100 U/ml

penicillin, 100 μg/ml streptomycin, 292 μg/ml glutamine and 100 μM sodium pyruvate). The transfected KNRK cell lines stably expressing either wt-$hPAR_1$ or mutant $hPAR_1$ were grown in selective growth medium (DMEM, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml glutamine, 100 μM sodium pyruvate and 0.7 mg/ml G418). All cell lines were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were routinely passaged using 0.5 mM ethylenediamine tetraacetic acid (EDTA).

2.3.2.3 Single cell cloning and clone selection

Once transfected KNRK cells reached 60-80% confluent in the 75 cm^2 flask, they were harvested, and counted using a haemocytometer before diluting to 5 cells/ml in selective growth medium. This diluted suspension was then transferred to a 96 well plate (200 μl per well). The 96 well plate was incubated at 37˚C, 5% CO_2 until colonies of cells became visible in the wells (approximately two weeks). The cells within the wells clearly containing only one colony were transferred to 25 cm^2 flasks with 5 ml of selective growth medium. At least 40 colonies were picked up for the single mutant cell line screening and ~80 colonies were selected for that of the double mutant.

When the cells reached 60-80% confluence in the 25 cm^2 flasks, fluorescence activated cell sorting (FACS, see section 2.3.2.4) was performed against EV-KNRK to identify the expressing mutant $hPAR_1$ colonies. The clone showing the highest level of receptor cell surface expression above control in FACS analysis was selected, and taken through a second round of single cell cloning to ensure that the final cell line was

derived from a single cell. The colonies from the second round were analysed by FACS and calcium signalling assay (see section 2.3.2.5). The procedure therefore for obtaining a single cell line took upto 3 months.

2.3.2.4 Fluorescence activated cell sorting

Cells were harvested, transferred to a 5 ml falcon tube, and centrifuged at 3000 g at 4˚C for 5 min. The supernatant was discarded and the cell pellet was resuspended in 200 µl of ice-cold PBS, 1/300 dilution of mouse anti-PAR1 antibody (ATAP-2) was added to the cell suspension. The sample was then incubated on ice for one hour, gently swirling every 15 min. 1 ml of cold PBS was then added to the sample before centrifuging at 3000 g at 4˚C for 5 min. The supernatant was discarded and the cell pellet was resuspended again in 200 µl of cold PBS. 1/100 dilution of anti-mouse IgG (FITC conjugate) antibody was added and incubated for 45 min on ice with swirling at 15 min intervals. 1 ml of cold PBS was then added to the cell suspension and centrifuged at 3000 g at 4˚C for 5 min. The supernatant was then disposed of and the cell pellet was resuspended in 300 µl of cold PBS. This washing step was repeated one more time, and the cells were suspended in 300 µl of cold PBS. 5 µg/ml of propidium iodide was added to the sample before analysing in a Beckton Dickinson flow cytometer.

2.3.2.5 Calcium signalling assay

Cells were harvested, transferred to a 30 ml universal tube and then centrifuged at

3000 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1ml of normal growth media containing 22 μM Fluo-3 AM (in dimethylformamide) and 250 μM sulphinpyrazone. The cell suspension was then placed on an orbital shaker at 100 rpm at RT for 30 min. After incubation, the cells were washed with 4 ml of PBS by centrifugation. The supernatant was then discarded and the treated cell pellet was resuspended in 1.5 ml of Calcium assay buffer (CAB, 20 mM HEPES, 150 mM NaCl, 3 mM KCl, 10 mM glucose, 250 μM sulphinpyrazone and 1.5 mM $CaCl_2 \cdot 6H_2O$, and then adjusted to pH 7.4.) The cell suspension was then aliquoted in 100 μl amounts into 15 cuvettes. Each cuvette contained a magnetic flea and 1880 μl of CAB. The samples were placed in a fluorospectrometer (Photon Technology International, West Sussex, UK) which was set to emit an excitation wavelength of 480 nm and record emissions at a wavelength of 530 nm. Each cuvette was recorded for about 2 min in order to get a stable baseline. Agonists, either 10 U/ml thrombin or 50 μM TFLLR were added, and the maximum fluorescence response stimulated by the agonists were read and recorded. Calcium ionophore (2 μM) was used to obtain the maximal fluorescence response.

2.3.2.6 Generation of low wt-hPAR$_1$

To generate low expressing wild-type hPAR$_1$ (low wt-hPAR$_1$), the receptor expression was found to decrease with the rise of cell confluence and reach the optimal expression level after about 48 h of splitting. Therefore, all the experiments were carried out using the cells in the second day after passaging.

2.4 Statistical analysis

Results are expressed as mean ± S.E. Comparison of data among groups were performed with one-way ANOVA followed by a Dunnett's multiple comparison test or a Bonferroni's Multiple Comparison test. A paired t test was used for measuring the differences between two groups. An associated probability (*P* value) of <0.05 was considered significant.

2.5 Results

Four mutant $hPAR_1$ stably expressing cell lines were successfully generated by single cell cloning and screened by both FACS and calcium signalling assay. Representative FACS traces are shown in Figure 2.5.1. The solid purple indicates the EV-KNRK and mutant cell lines are represented as green lines (Fig 2.5.1). The mutant cell lines all have a clear rightward shift in fluorescence level against EV-KNRK (Fig 2.5.1). Figure 2.5.2 displays representative $hPAR_1$ mediated calcium signalling in response to thrombin and the internal control calcium ionophore. $hPAR_1$C387A, $hPAR_1$C388A and $hPAR_1$C411A show an increase in the fluorescence level after the addition of 10 U/ml thrombin (Fig 2.5.2a, b, d). $hPAR_1$C387AC388A, EV-KNRK failed to trigger an increase in calcium levels in response to thrombin (Fig 2.5.2c, e). All the cell lines displayed a rapid increase in the fluorescence level in response to 2 μM calcium ionophore (Fig 2.5.2). The cell lines were checked by FACS regularly to assess the stable receptor expression.

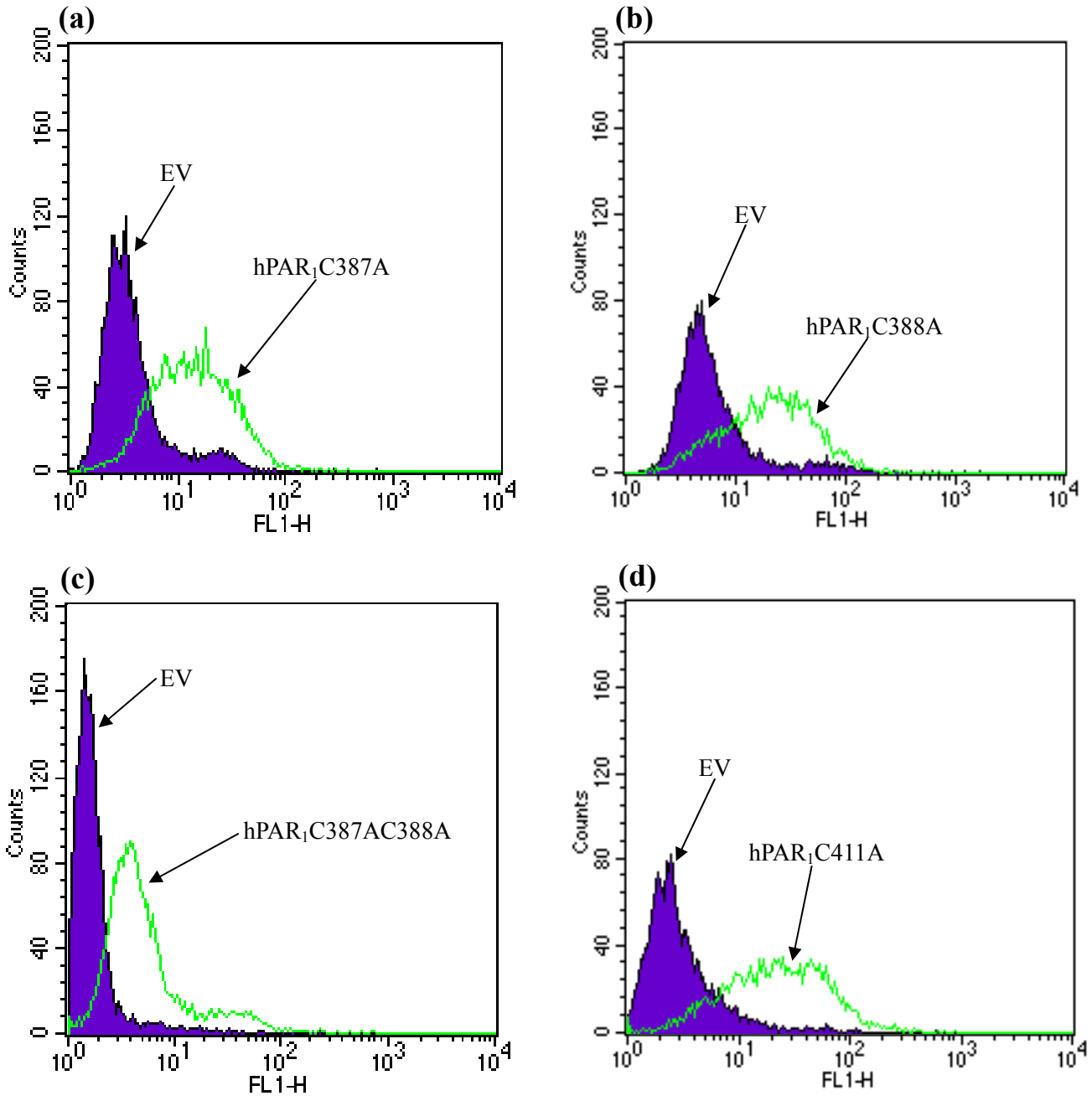


Figure 2.5.1, FACS analysis of the receptor cell surface expression. EV-KNRK is shown as solid purple and mutant cell lines are shown as green lines.
(a) $hPAR_1C387A$, (b) $hPAR_1C388A$, (c) $hPAR_1C387AC388A$, (d) $hPAR_1C411A$

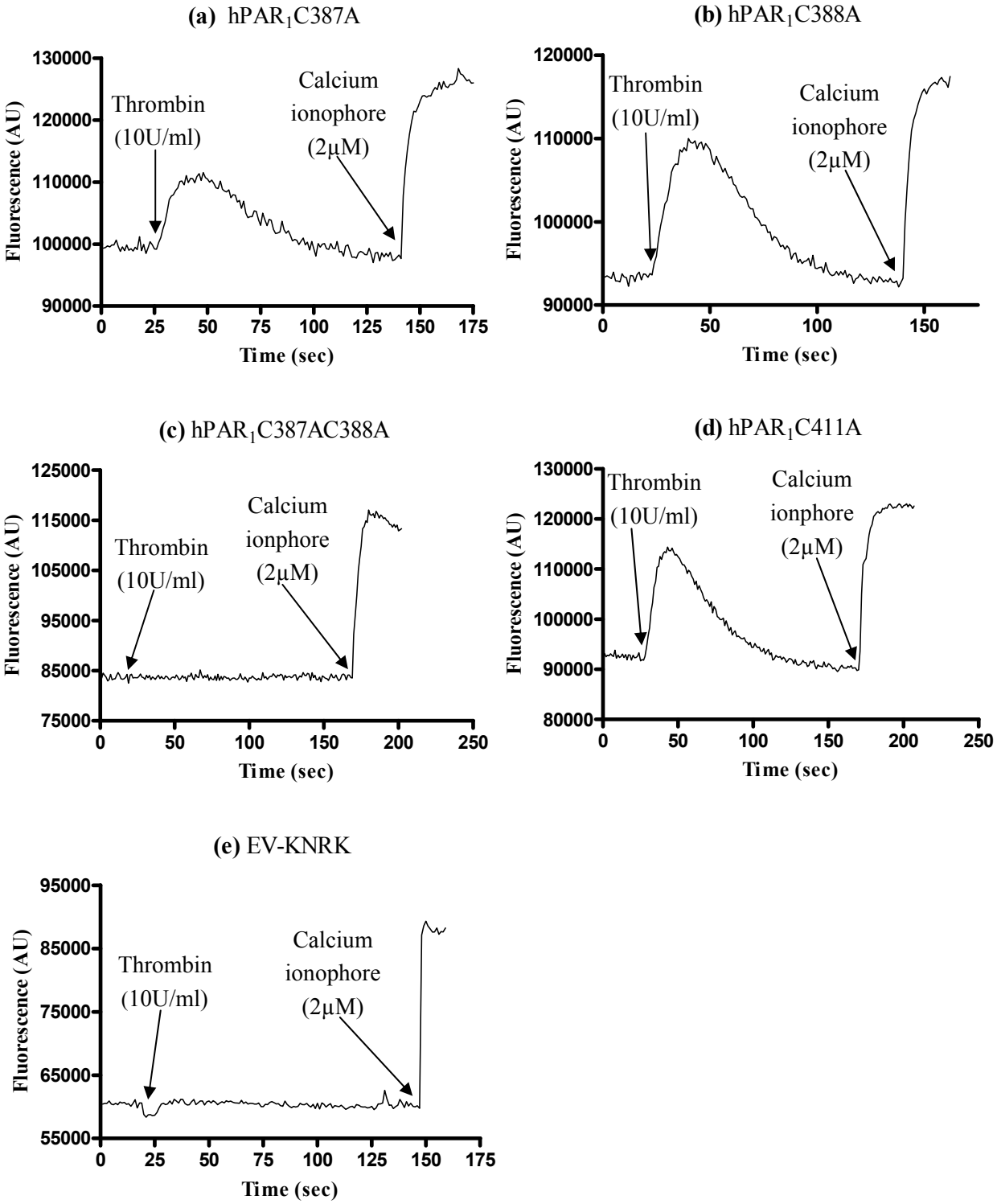


Figure 2.5.2, calcium imaging. Intracellular calcium signalling in the (a) $hPAR_1C387A$ (b) $hPAR_1C388A$ (c) $hPAR_1C387AC388A$ (d) $hPAR_1C411A$ (e) EV-KNRK cell line following activation by an agonist and addition of calcium ionophore

2.6 Discussion

$hPAR_1$ has 3 cysteines in the C-tail—C387, C388 and C411. Using site-directed mutagenesis C387A, C388A, C387AC388A and C411A mutants were successfully generated. Cell lines stabely expressing the mutant receptors were successfully generated and the cell surface expression was observed by FACS. All the $hPAR_1$ mutants responded to thrombin except $hPAR_1$C387AC388A. Moreover, mock transfected KNRK cells failed to respond to thrombin as expected.

These four cell lines stably showing $hPAR_1$ were utilized for future analysis and passaged up into larger flasks before being stored in liquid nitrogen in FCS with 10% dimethyl sulfoxide (DMSO).

3 | THE ROLE OF C-TERMINAL CYSTEINES IN CALCIUM SIGNALLING

3 The role of C-terminal cysteines in calcium signalling

3.1 Introduction

IP_3/DAG pathway is an important GPCR-triggered downstream event which results in an elevation of cytosolic calcium ions (Ca^{2+}). The activation of GPCRs subsequently activates the effector G_q or G_o proteins which in turn activates phospholipase C (PLC). The membrane bound lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is then cleaved by activated PLC into two second messengers: inositol 1,4,5-trisphosphate (IP_3) which can freely diffuse into the cytosol, and 1,2-Diacylglycerol (DAG), a lipophilic molecule that remains associated with the cell membrane. Diffused IP_3 binds to Ca^{2+} channel in the endoplasmic reticulum (ER) membrane and induces an opening of the channel, causing the release of stored Ca^{2+} from the ER into the cytosol and thus a rise of the cytosolic Ca^{2+} concentration. However, the IP_3-mediated cytosolic Ca^{2+} rise is transient since after it's generation IP_3 is rapidly hydrolyzed to inositol 1,4-bisphosphate, which can not bind to the Ca^{2+} channel and thus does not stimulate Ca^{2+} release from the ER. Furthermore, Ca^{2+} pumps in the plasma membrane and ER membrane would transport Ca^{2+} from the cytosol to the cell exterior and back into the ER lumen respectively. PAR_1 as a GPCR has been shown to couple with G_q protein leading to the activation of PLC and calcium mobilization (Figure 3.1). Upon activation by thrombin and PAR_1-AP, PAR_1 is shown to signal through Ca^{2+} (Vu et al., 1991a).

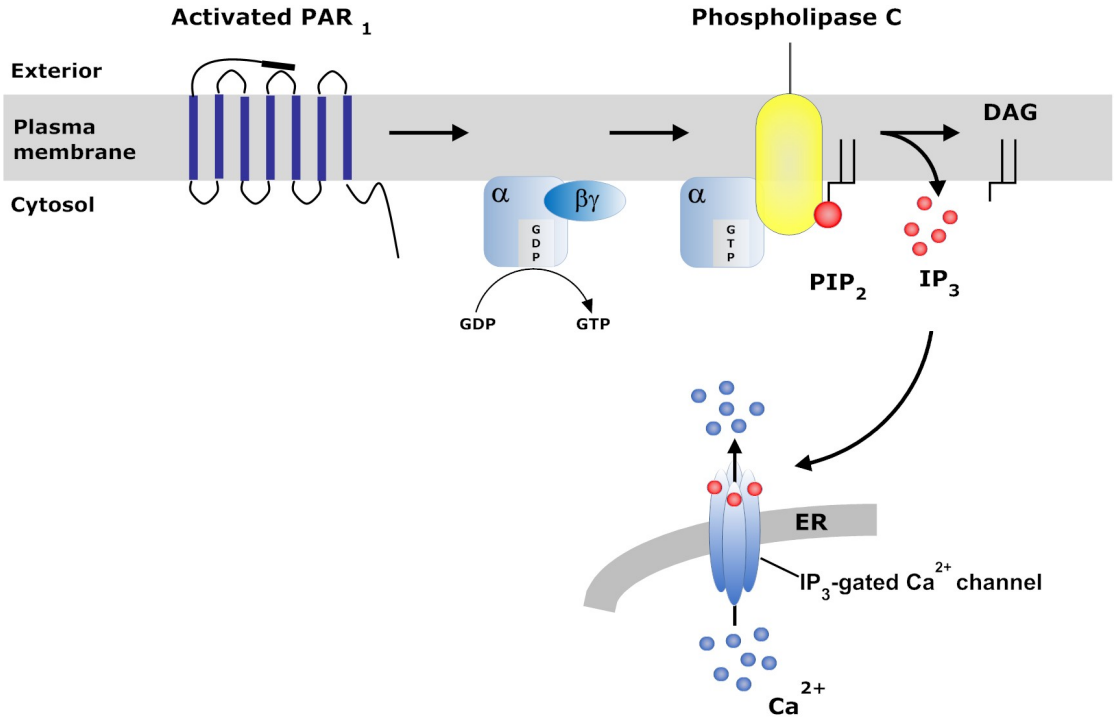


Figure 3.1, **IP_3/DAG pathway.** Intracellular calcium mobilisation mediated by PAR_1 triggered IP_3/DAG pathway.

Aims

The aim of the study in this chapter was to investigate the effect of $hPAR_1$ putative plamitoylation sites—C387 and C388 in the receptor cell surface expression and calcium signalling.

3.2 Materials and Methods

3.2.1 Reagents and materials

All the reagents and the chemicals used in this chapter are described in section 2.1.

3.2.2 Methods

The cell surface expression levels of wt-hPAR$_1$, hPAR$_1$C387A, hPAR$_1$C388A and hPAR$_1$C387AC388A were analysed against EV-KNRK by FACS as described in section 2.3.2.4.

Calcium signalling assays for wt-hPAR$_1$, low wt-hPAR$_1$ and hPAR$_1$ mutant cell lines were performed as detailed in section 2.3.2.5. Agonists, either thrombin (0.03-10 U/ml) or TFLLR (0.316-100 μM) were added, and the maximum fluorescence response stimulated by the agonists were read and recorded. The responses for each cell sample were calculated by subtracting the base fluorescence from the peak fluorescence. The average response for each agonist concentration was expressed as a percentage of the mean response of calcium ionophore (maximum attainable response).

3.3 Results

3.3.1 C-terminal cysteines affect hPAR$_1$ cell surface expression

FACS analysis of wt-hPAR$_1$ and mutant hPAR$_1$ cell surface expression was carried out in order to characterise the effect of C-terminal cysteines on the receptor expression. The data obtained shows that hPAR$_1$C387A and hPAR$_1$C388A cell lines have similar

cell surface expression to that of wt-hPAR$_1$ (80%) (Fig 3.3.1). The statistical analysis shows that there was no significant different in expression level between wt-hPAR$_1$ and the two single mutants ($P>0.05$). However, there was a dramatic decrease in the hPAR$_1$C387AC388A cell surface expression compared to wt-hPAR$_1$ cell line ($P<0.01$); its expression level was found to be approximately 40% of that of wt-hPAR$_1$ (Fig 3.3.1).

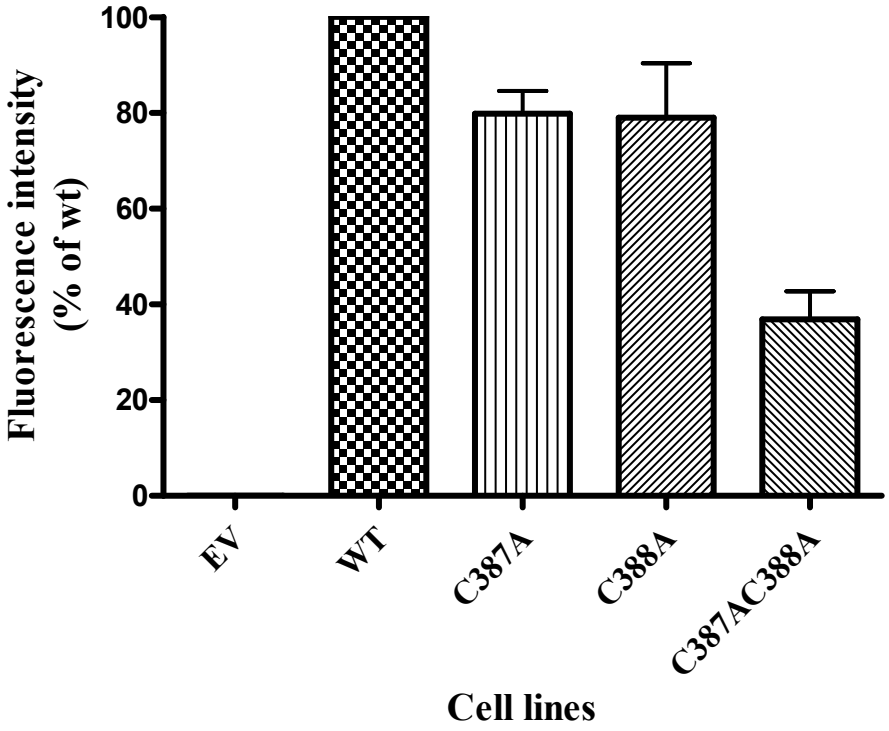


Figure 3.3.1, Comparative FACS analysis of wt-hPAR$_1$ and mutant hPAR$_1$ expressing cell lines. EV was used as a negative control. Histograms shown present the means ± S.E. from four experiments performed in duplicate ($P<0.001$, n=5).

3.3.2 C-teminal cysteines regulate hPAR$_1$ calcium signalling

With the purpose of investigating the effect of deleted cysteines on receptor signalling, agonist triggered calcium signalling assays were performed. The cell lines were treated with different concentrations of PAR$_1$ agonists (either TFLLR or thrombin). The concentration effect curves are shown in Figure 3.3.2 and their EC_{50} values are listed in Table 3.1. The wt-hPAR$_1$, hPAR$_1$C387A, hPAR$_1$C388A and hPAR$_1$C411A triggered calcium responses were concentration dependent in response to thrombin and TFLLR. The thrombin effect curves for wt-hPAR$_1$, hPAR$_1$C387A, hPAR$_1$C388A and hPAR$_1$C411A all plateau at ~45% of maximum obtainable signal (% of A23187). The TFLLR curves for wt-hPAR$_1$, hPAR$_1$C387A, hPAR$_1$C388A and hPAR$_1$C411A all reach ~50% of maximum obtainable signal at 100 μM TFLLR dose. There were no significant differences among the concentration effect curves for hPAR$_1$C387A, hPAR$_1$C388A and hPAR$_1$C411A compared to wt-hPAR$_1$ in response to either TFLLR or thrombin ($P>0.05$). However, hPAR$_1$C387AC388A and EV-KNRK failed to respond to both hPAR$_1$ agonists (Fig 3.3.2).

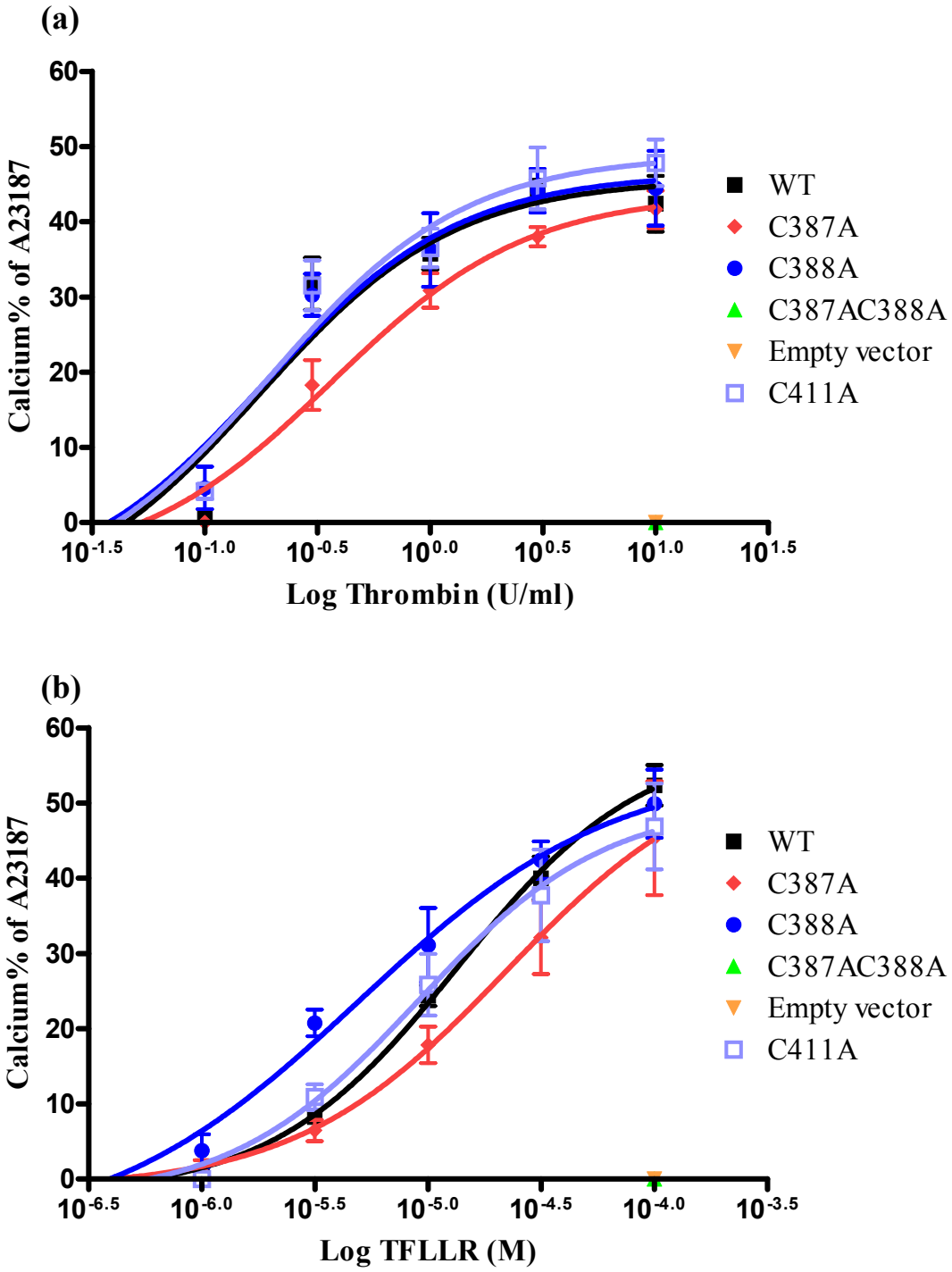


Figure 3.3.2, $hPAR_1$ agonists triggered calcium signalling. (a) Thrombin concentration effect curve for wt-$hPAR_1$, $hPAR_1$C387A, $hPAR_1$C388A, $hPAR_1$C387A C388A, $hPAR_1$C411A and EV. (b) TFLLR-NH_2 concentration effect curve for wt-$hPAR_1$, $hPAR_1$C387A, $hPAR_1$C388A, $hPAR_1$C387A C388A, $hPAR_1$C411A and EV. The results present the means ± S.E. from at least three experiments performed in duplicate.

Cell lines	EC_{50} Thrombin (U/ml)	EC_{50} TFLLR (μM)
WT	0.173 ± 1.411	14.1 ± 1.175
C387A	0.367 ± 1.283	20.5 ± 1.512
C388A	0.183 ± 1.495	4.91 ± 1.365
C411A	0.194 ± 1.417	9.24 ± 1.447

Table 3.1 Mean EC_{50} values ± S.E. of thrombin and TFLLR concentration effect curves for wt-$hPAR_1$, $hPAR_1$C387A, $hPAR_1$C388A and $hPAR_1$C387AC388A

3.3.3 Comparison of low wt-$hPAR_1$ and $hPAR_1$C387AC388A

The expression of low wt-$hPAR_1$ was matched to that of $hPAR_1$C387AC388A (Fig 3.3.3). When analysed using one-way ANOVA and Bonferroni's Multiple Comparison test, low wt-$hPAR_1$ displayed comparable cell surface expression as $hPAR_1$C387AC388A ($P>0.05$), but highly significantly different expression level with wt-$hPAR_1$ ($P<0.001$). The agonist (either TFLLR or thrombin) concentration effect curves for low wt-$hPAR_1$ are shown in Figure 3.3.4. From this data it appears that the response of low wt-$hPAR_1$ is agonist concentration dependent. In response to thrombin low wt-$hPAR_1$ triggered a calcium signal as low at 0.3 U/ml and reached maximal response at 10 U/ml (Fig 3.3.4a), and in response to TFLLR low wt-$hPAR_1$ triggered a calcium signal as low at 3.16 μM and reached maximal response at 100 μM (Fig 3.3.4b). However, $hPAR_1$C387AC388A failed to respond to the maximal dose of either agonist (Fig 3.3.4).

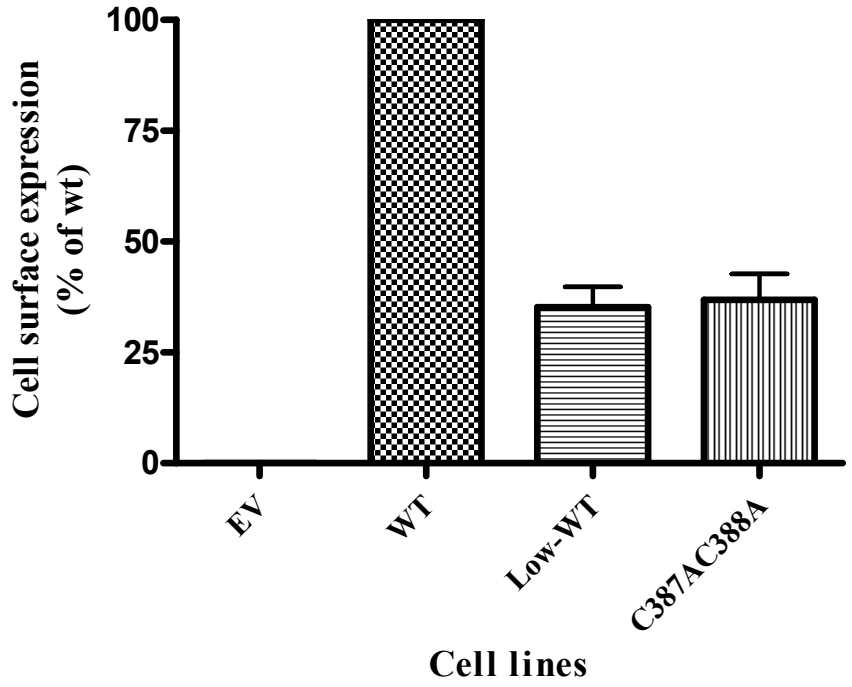


Figure 3.3.3, **FACS analysis of cell surface expression of hPAR$_1$C387AC388A, wt-hPAR$_1$ and low expression wt-hPAR$_1$**. EV was used as the negative control. The results represent the means ± S.E. from four experiments performed in duplicate ($P<0.0001$, n=4).

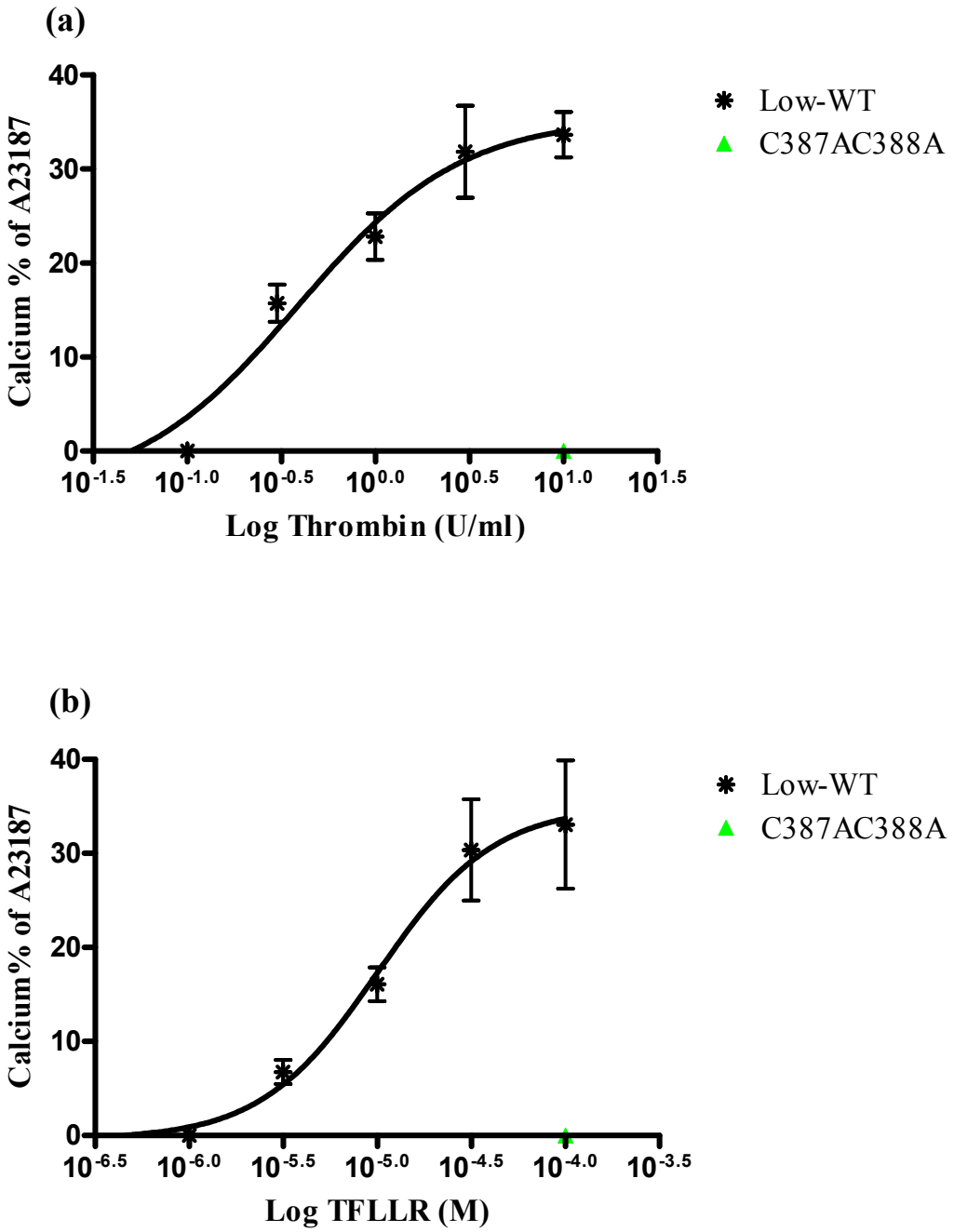


Figure 3.3.4, $hPAR_1$ agonists triggered calcium signalling in $hPAR_1C387AC388A$ and low wt-$hPAR_1$. (a) thrombin concentration effect cure for the low wt-$hPAR_1$ and $hPAR_1C387AC388A$ cell lines. (b) TFLLR concentration effect curve for the low wt-$hPAR_1$ and $hPAR_1C387AC388A$ cell lines. The results represent the means ± S.E. from 3-5 experiments performed in duplicate.

3.4 Discussion

The main finding in this chapter was that the putative palmitoylation sites—Cysteine 387 and 388 within $hPAR_1$ C-tail play an important role in the receptor cell surface expression. Compared to wt-$hPAR_1$, both $hPAR_1$C387A and $hPAR_1$C388A displayed approximately 80% cell surface expression, and $hPAR_1$C387AC388A only displayed approximately 40% cell suface expression. Additionally, these two cystienes are critical for receptor coupling to calcium. $hPAR_1$C387A and $hPAR_1$C388A have similar sensitivity towards PAR_1 agonists (thrombin and TFLLR) as wt-$hPAR_1$, but $hPAR_1$C387AC388A failed to respond to either agonist.

The double substitution of the two cysteines (C387 and C388) in $hPAR_1$ resulted in a 60% decrease in the receptor cell surface expression. Replacement of either cysteines resulted in receptor expression similar (80%) to the wild type. The results strongly suggest that the putative palmitoylation site is important in $hPAR_1$ cell surface expression, but one cysteine is adequate to return the receptor expression level. Our data is consistent with the study of the palmitoylation sites in vasopressin V2 receptor (Sadeghi et al., 1998, Schulein et al., 1996). Elimination of the palmitoylation sites (Cys341 and Cys342) in vasopressin V2 receptor lead to a 30% reduction of cell surface expression, however, palmitoylation of either one of the two cysteines was sufficient to restore cell surface expression to wild type level (Sadeghi et al., 1998, Schulein et al., 1996). Moreover, in the CCR5 receptor, single and double cysteine mutant receptors exhibited altered cell surface expression but the palmitoylation-deficient mutant displayed a more dramatic reduction in cell surface expression (Percherancier et al., 2001, Blanpain et al., 2001). Loss of mutant receptor

cell surface expression may be caused by decreased protein synthesis or export impairment. Prevention of palmitoylation resulted in mutant LH/CG receptors trapped intracellularly (Zhu et al., 1995), and the CCR5 mutant without 3 palmitoylated cysteines was sequestered in intracellular biosynthetic compartments although it was synthesised efficiently (Blanpain et al., 2001). The $hPAR_1$ location could be identified by either confocal microscopy or immunofluorescence microcopy to see whether the receptors were trapped inside the cells.

Once activated by agonists, $hPAR_1$ would couple to multiple G-proteins to transduce signals in different pathways and subsequently result in several downstream intracellular alterations. Transient elevations of intracellular calcium following PAR_1 activation is mediated via the $G_{q/11}$ family (Verrall et al., 1997). $hPAR_1$C387A and $hPAR_1$C388A gave similar agonist concentration effect curves to that of wt-$hPAR_1$, but $hPAR_1$C387AC388A displayed a complete shut-off of calcium signalling. However, the lack of calcium signalling by the double mutant could be attributed to its low expression. To eliminate this possibility, the low wt-$hPAR_1$ which has similar expression to $hPAR_1$C387AC388A was assessed for functionality. The calcium response of low wt-$hPAR_1$ was concentration dependent reaching the maximum 40% of calcium ionophore. Thus, the lack of calcium signalling of $hPAR_1$C387AC388A would be caused by the deficiency of putative palmitoylation sites. In addition, $hPAR_1$C411A showed similar activity to couple to calcium with wt-$hPAR_1$ in response to both agonists. Taken together, it suggests that cysteine 411 does not have an impact on calcium signalling, but cysteines 387 and C388 do influence $hPAR_1$-$G_{q/11}$ triggered calcium signalling. Moreover, although there is some slight variation in the EC_{50}

values for thrombin and TFLLR evoked responses in wt-hPAR$_1$, hPAR$_1$C387A, hPAR$_1$C388A and hPAR$_1$C411A cell lines, comparisons over the entire concentration effect curves shows no statistically significant difference.

One possible explanation for the lack of calcium signalling by hPAR$_1$C387AC388A could be phosphorylation of the receptor. After activation, hPAR$_1$ is desensitised by rapid GRK-phosphorylation at the C-terminus (Paing et al., 2004). hPAR$_1$C387AC388A may expose the otherwise hidden phosphorylation sites or increase the accessibility of phosphorylation sites to GRKs in the absence of the palmitate anchor. Thus, hPAR$_1$C387AC388A would be highly phosphorylated so that it is unable to couple $G_{q/11}$ protein thereby resulting in no fluctuation of intracellular calcium. A human β_2-adrenergic receptor lacking the palmitoylated cysteine 341 has been revealed to be highly phosphorylated under basal conditions and the receptor is unresponsive to agonist (Moffett et al., 1993). However, either one of the two cysteines would replace the function of the hPAR$_1$ palmitoylation and lead to the coupling of $G_{q/11}$ protein. A study in endothelin receptor B concludes that palmitoylation of at least one of the three cysteines is required for the G protein coupling, regardless of the G protein subtypes (Okamoto et al., 1997). $^{32}P_i$ labelling receptor phosphorylation assay would be used to evaluate whether mutant receptors are highly phosphorylated.

PAR$_1$ 7-8-1 activation mechanism could be the other possible reason for the incapacity of hPAR$_1$C387AC388A in calcium signalling. The 8th helix of PAR$_1$ interacts with the transmembrane domain 7 and the intracellular loop 1 to form a H-bonding and ionic network (Swift et al., 2006). This connection is needed for PAR$_1$-G_q signal transference. However, there are not putative palmitoylation sites in hPAR$_1$C387AC388A. Thus the

mutant receptor prevents the formation of the 8th helix, in turn loses the connection of 7-8-1 activation mechanism and consequently is unable to couple to G_q protein to trigger calcium signalling. Either one of the two cysteines could still maintain the formation of the $hPAR_1$ 8th helix and restore $hPAR_1$-G_q coupling capacity. Therefore, $hPAR_1$C387A and $hPAR_1$C388A displayed similar responses to agonists seen with wt-$hPAR_1$ in calcium signalling.

In addition, $hPAR_1$ not only can couple to the $G_{q/11}$ family, but also the G_i family and $G_{12/13}$ family (Hollenberg and Compton, 2002). Evidence is emerging that palmitoylation selectively affects specific signal transduction pathways without affecting others. For example, the palmitoylation-deficient endothelin receptor A receptor fails to couple to G_q but can efficiently couple to G_s (Horstmeyer et al., 1996). The first palmitoylated cysteine (three cysteines) in endothelin receptor B is required for coupling with G_i but not for G_q (Okamoto et al., 1997). Therefore, our results only suggest that palmitoylation plays an important role in coupling to $G_{q/11}$, whether palmitoylation of $hPAR_1$ affect other G protein coupling is still unknown.

4 | THE ROLE OF C-TERMINAL CYSTEINES IN ERK1/2 SIGNALLING

4 The role of C-terminal cysteines in ERK1/2 signalling

4.1 Introduction

Extracellular signal-regulated kinases 1 and 2 (ERK1/2), known as classical MAP Kinases, play a critical role in cell proliferation and differentiation. ERK1/2 are proteins of 44 and 42 kDa respectively and share nearly 85% amino acid homology (Sugden and Clerk, 1997, Pearson et al., 2001). In the ERK1/2 phosphorylation cascade, MEK1 and MEK2 are two direct upstream activators for ERK1/2 and the MEKKs lying above MEK1/2 are Raf-1 isoforms (Sugden and Clerk, 1997, Pearson et al., 2001). GPCRs can couple to at least three G protein subfamilies ($G_{q/11}$, $G_{i/o}$, G_s) to trigger the ERK1/2 activation via different signal pathways and some receptors are able to activate more than one class of G protein. The mechanisms employed by these receptors are widely varied and cell type specific, but generally include modulation of Raf-1 isoforms activity (Pearson et al., 2001, Luttrell, 2002). The G proteins that have been studied in most detail with respect to the regulation of ERK1/2 are $G_{q/11}$ and G_i. In G_i protein triggered ERK1/2 activation, the βγ complex is believed to be the active signal transducer rather than the α subunit (Koch et al., 1994). In COS-7 cells transfected with a mutants G_i α subunit that is constitutively active, no ERK1/2 activation occurred, however, the overexpression of βγ complex are sufficient to activate ERK1/2 (Faure et al., 1994). G_i βγ complex stimulates ERK1/2 activation through the interaction between the monomeric G protein Ras and Raf-1 isoforms. G_i βγ complex activates nonreceptor tyrosine kinases such as the Src family, which may

interact with receptor tyrosine kinase or focal adhesion kinase, and in turn recruit the adaptor protein Shc (Pearson et al., 2001, Luttrell, 2005). Subsequently, Shc binds to and activates the growth-factor-receptor binding protein 2 (Grb2), which in turn recruit the Ras guanine nucleotide exchange factor, mammalian Son of sevenless (mSos), resulting in the exchange of GDP for GTP on Ras (Pearson et al., 2001, Luttrell, 2005). Activated Ras then phosphorylates Raf-1 to initiate ERK1/2 cascade. Moreover, phosphatidylinositide-3 kinase (PI-3 kinase), a heterodimeric enzyme consisting of a 110 kDa (p110) subunit that can be directly activated by $G_i\beta\gamma$ subunits but not α subunits, and a receptor tyrosine kinase associated 85 kDa (p85) subunit, functions as an intermediate in the $G_i\beta\gamma$ proteins induced activation of ERK1/2 in some cell types (Takeda et al., 1999, Bocker and Verspohl, 2001, Touhara et al., 1995, Lopez-Ilasaca et al., 1997). $G_{q/11}$ triggered ERK1/2 activation is often a PKC-dependent process through a Ras-dependent or Ras-independent pathway (Pearson et al., 2001). After $G_{q/11}$ activation, $G_{q/11}$ α subunit activates PLC and leads to the generation of IP_3 and DAG as described previously. DAG can activate PKC which in turn regulates Raf-1 through direct phosphorylation and eventually activates ERK1/2 independent of Ras (Pearson et al., 2001), whereas the intracellular Ca^{2+} release as a result of IP_3 production, would activate the Shc-Grb2-mSos complex and stimulate ERK1/2 via a Ras-dependent pathway (Luttrell, 2002).

PAR_1 is able to couple with $G_{q/11}$ and $G_{i/o}$ in several cells. Although the ERK1/2 regulation induced by PAR_1 is not fully understood, PAR_1 has been shown to incorporate many components of the model of GPCR induced ERK1/2 activation via $G_{q/11}$ and $G_{i/o}$ (Macfarlane et al., 2001). PAR_1-AP was found to rapidly activate the

$p21^{ras}$ which is a 21 kDa mammalian Ras protein in the CCL39 fibroblasts, and the activation was inhibited by the treatment of pertussis toxin, a G_i protein inhibitor and genistein, a tyrosine kinase inhibitor, indicating a G_i protein and a tyrosine kinase are involved in mediating the activation of ERK1/2 via PAR_1 (van Corven et al., 1993). The study in astrocytes has shown that PAR_1 activation of ERK1/2 depends on two branches: one is mediated by $G_i\beta\gamma$ subunit and PI-3 Kinase, and the other is mediated by G_q and PKC (Wang et al., 2002). ERK1/2 phosphorylation in human colon cancer cells was induced by PAR_1 through the Src phosphorylation and the transactivation of epidermal growth factor receptor which is a receptor tyrosine kinase (Darmoul et al., 2004). This result is consistent with that in cardiac fibroblast, PAR_1 mediated ERK1/2 activation requires Src family kinase and EGFR transactivation (Sabri et al., 2002). Additionally, thrombin has been shown to stimulate cell proliferation through activation of Ras/Raf-1/MEK1/2/ERK1/2 pathway in tracheal smooth muscle cells which express PAR_1 and PAR_2 proteins, and the activation was regulated by PKC, Ca^{2+}, PLC, PI-3 kinase and tyrosine kinase (Lin et al., 2002). In CCL2 fibroblast, PKC and Raf-1 have been indicated to involve in the PAR_1-G_q triggered ERK1/2 pathway (Deng et al., 2008).

Aims

This section of the study attempts to determine the effect of $hPAR_1$ putative plamitoylation sites—C387 and C388 in the $hPAR_1$ triggered ERK1/2 signalling.

4.2 Reagents and methods

4.2.1 Reagents

Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody and p44/42 MAP kinase antibody were purchased from Cell Signalling, New England Biolabs, Hitchin, Hertfordshire, UK. ECL western blotting detection reagents and anti-rabbit IgG horseradish peroxidase-linked whole antibody (from donkey) were purchased from Amersham Biosciences, Buckinghamshire, UK. Restore™ western blot stripping buffer was supplied by Pierce, Northumberland, UK. Immun-Blot® PVDF membrane were supplied by Bio-Rad laboratories, Inc, Hertfordshire, UK. All other chemicals and reagents were purchased from Sigma-Aldrich, Poole, Dorset, UK.

4.2.2 Methods

Cells were grown in 6 well plates to ~80% confluence and incubated in serum-free medium overnight. The effect of increasing agonist concentration on MAPK signalling was investigated by incubation with—either thrombin (0.001-3 U/ml) or TFLLR (0.01-31.6 μM) for 5 min. In time course experiments, cells were also challenged with either 0.1 U/ml thrombin or 1 μM TFLLR at different time points (0, 2 min, 5, 10, 20, 30, 60, 120, 240). Treatment of cells with 10% FCS for 5 min was used as a positive control in each experiment. The treatment was halted by removal of media and direct addition of 350 μl of Laemmli's buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl (pH6.8), 5 mM EDTA, 0.008% bromophenol blue and 5% β-ME). The cell lysates were subsequently transferred into 1.5 ml centrifuge tubes and centrifuged at 14,000 g

at 4˚C for 10 min. The samples were then incubated at 4˚C for 20 min with 0.025 U benzonase with end over end mixing to degrade the DNA. The benzonase treated samples were then heated at 100˚C for 5 min and stored at -80˚C until analysis.

The samples were equally loaded and run on a 10% SDS polyacrylamide gel (see table 4.2) in electrode buffer consisting of 25 mM Tris base, 200 mM Glycine and 0.1%(w/v) SDS to separate proteins. The sample proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membrane in blotting buffer containing 25 mM Tris base, 200 mM Glycine, and 20% methanol. A quick reversible Ponceau S (0.25% (w/v) Ponceau S in 5% acetic acid) staining was performed to check the success of protein blotting. The stain was then removed by a quick rinse with dH_2O.

Reagents	**Stacking Gel**	**10% SDS polyacrylamide gel**
Acrylamide solution	1.3 ml	3.33 ml
dH_2O	6.1 ml	4.02 ml
Tris-HCl	pH6.8, 2.5 ml	pH8.8, 2.5 ml
10%SDS	100 µl	100 µl
10%APS	50 µl	50 µl
TEMED	20 µl	5 µl

Table 4.2, Reagents used to make a SDS-PAGE

Once the protein transfer was completed, the membranes were blocked by 5%(w/v) non-fat milk in PBS/0.1% Tween-20 for 1 hour, followed by incubating with phospho-P44/42 MAP kinase antibody (1 in 1000 dilution) in 5% BSA, PBS/0.1% Tween-20 overnight at 4˚C. After washing in PBS/0.1%Tween-20 4×15 min, the membranes were probed with anti-rabbit IgG HRP-linked antibody (1 in 1000 dilution) in PBS/0.1% Tween-20 2% non-fat milk for 60 min at RT. The membranes were then washed again 4×15 min in PBS/0.1%Tween-20 before applying by ECL western

blotting detection reagents. The membranes were photographed using a UVP Laboratory Products Epichem II Darkroom setup to measure chemiluminescence.

The antibodies were stripped from the membranes using Restore^{TM} western blot stripping buffer for 2 hours at 37˚C 200 rpm. Subsequently, the membranes were reprobed with total P44/42 MAP kinase antibody overnight at 4˚C. The membranes were then washed, incubated with secondary antibody and visualized as described above.

4.3 Results

To characterise the effect of deleted cysteines on $hPAR_1$ ERK1/2 (P44/42) MAPK signalling, agonist triggered MAPK signalling assays were carried out in a dose-dependent and time-dependent manner. EV, wt-$hPAR_1$ and $hPAR_1$C387AC388A were rendered quiescent for 18 hours prior to stimulation with the different concentrations of an agonist—either thrombin (0.001 U/ml to 3 U/ml) or TFLLR (0.01 μM to 31.6 μM) for 5min. 10% FCS treatment was utilised as a positive control to stimulate the phosphorylation of P44/42 for 5 min.

The band intensity rapidly increased after the addition of 0.03 U/ml thrombin or 1 μM TFLLR in the wt-$hPAR_1$ phosphorylated P44/42 blot, but there was no obvious fluctuation in band intensity in the EV and $hPAR_1$C387AC388A phosphorylated p44/42 blot (Fig 4.3.1a and 4.3.2a). Figure 4.3.1b shows that 0.03 U/ml thrombin was sufficient to trigger a robust increase in phosphorylated P44/42 in wt-$hPAR_1$ expressing cells, with maximum phosphorylated P44/42 seen by 0.1 U/ml, however there was no significant increase seen in EV or $hPAR_1$C387AC388A. Figure 4.3.2b

shows a TFLLR concentration dependent increase in phosphorylated P44/42 for wt-hPAR$_1$, plateauing after 1 μM. In comparison, the hPAR$_1$C387AC388A triggered MAPK activation curve has a gradual but smaller increase and reached the maximal response at the higher concentration of 31.6 μM, however, the EV triggered MAPK activation curve has a even smaller increase (Fig 4.3.2b). There is not significant difference between EV and hPAR$_1$C387C388A in MAPK activation curve in response either thrombin or TFLLR ($P>0.05$).

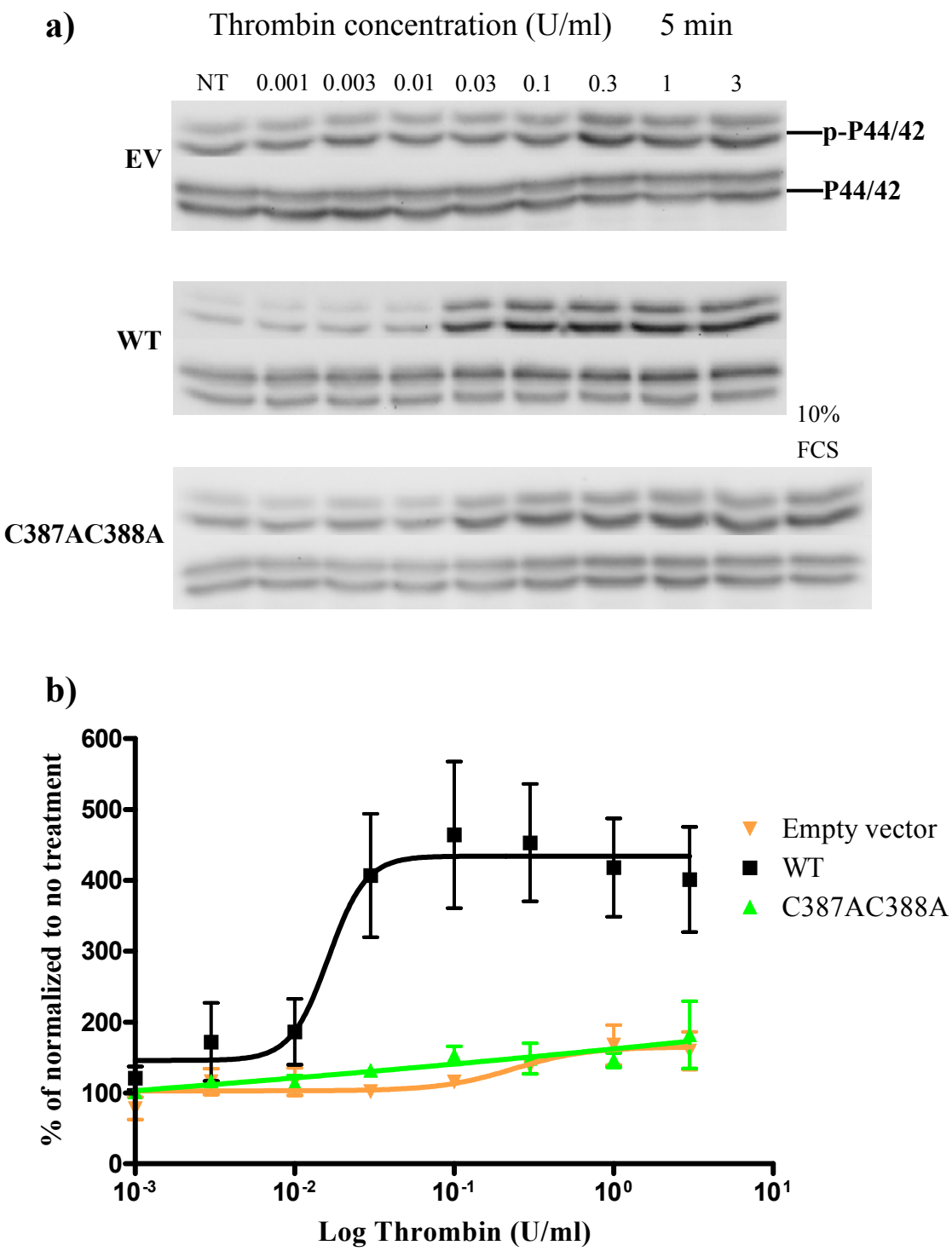


Figure 4.3.1, Thrombin stimulated ERK1/2 phosphorylation. a) Western blots for EV, wt-hPAR$_1$ and hPAR$_1$C387AC388A cells treated with increasing thrombin concentrations. The upper rows are the blots probed with anti-phosphorylated P44/42 antibody and the lower rows are the blots re-probed with anti-total P44/42. 10% FCS was used as a positive control. The blots shown are representative of 3 repeats.**b)** Graphs showing densitometric values for bands produced by western blots. Values are expressed as a percentage of total P44/42 and then normalised to no treatment. The results are expressed as the means ± S.E. from 3 separate experiments.

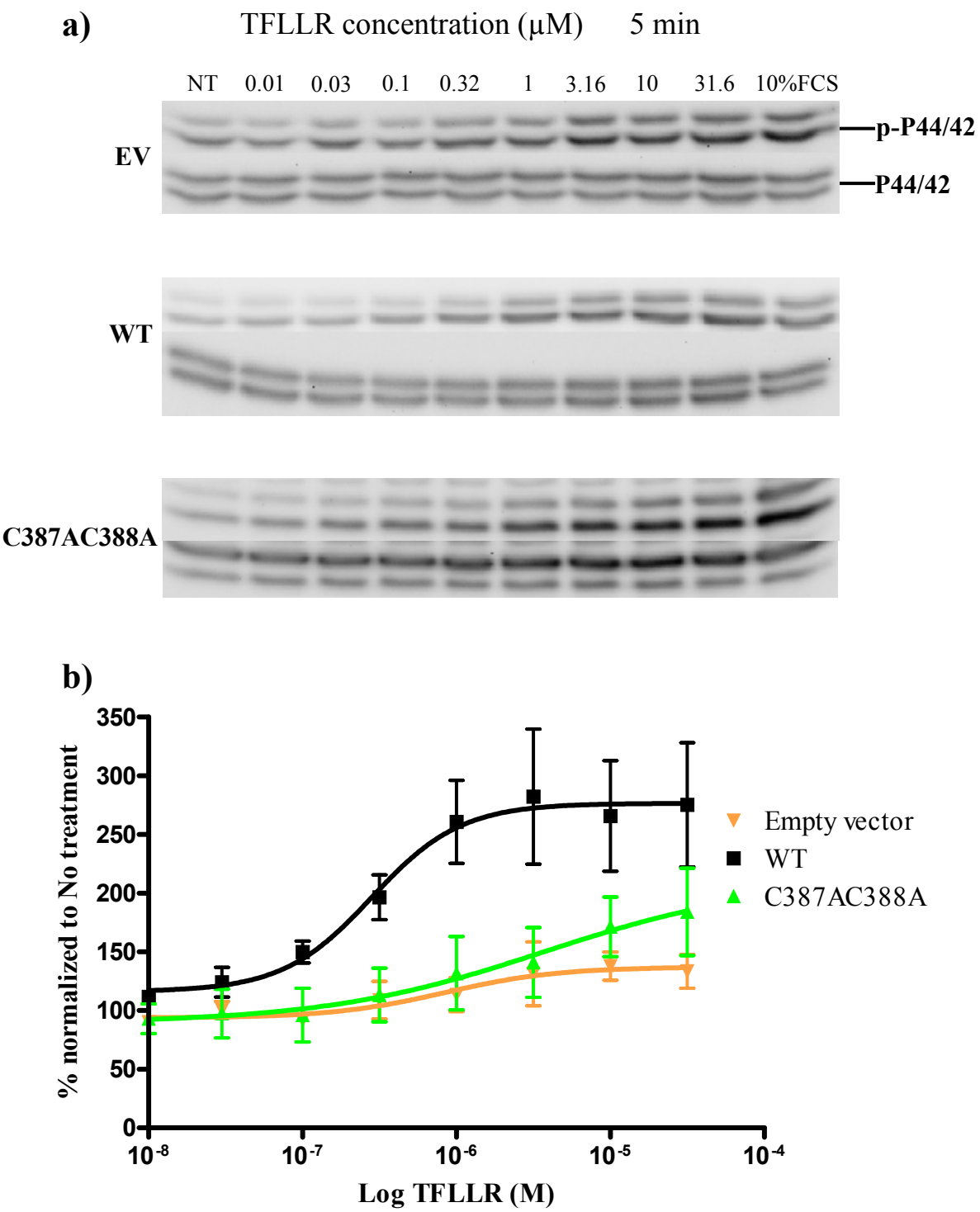


Figure 4.3.2 TFLLR stimulated ERK1/2 phosphorylation. **a)** Western blots for EV, wt-hPAR$_1$ and hPAR$_1$C387AC388A cells treated with increasing TFLLR concentrations. The upper rows show the blots probed with anti-phosphorylated P44/42 antibody and the lower rows are the blots re-probed with anti-total P44/42. 10% FCS was used as a positive control. The blots shown are a representation of 3 repeats.**b)** Graphs showing densitometric values for bands produced by western blots. Values was expressed as a percentage of total P44/42 and then normalised to no treatment. The results present the means ± S.E. from 3 separate experiments.

EV, wt-hPAR$_1$ and hPAR$_1$C387AC388A were then treated with either 0.1 U/ml thrombin or 1 μM TFLLR over a period of 240 min. 10% FCS was utilised as a positive control to stimulate the phosphorylation of P44/42 at 5 min. Thrombin stimulated the phosphorylation of P44/42 through wt-hPAR$_1$ activation reaching the maximal activity at 5 min, and then gradually returned to the basal level by 240 min (Fig 4.3.3b). However, thrombin induced MAPK activation through either EV or hPAR$_1$C387AC388A has no clear fluctuation during 240 min (Fig 4.3.3b). Similarly, TFLLR triggered MAPK activation through wt-hPAR$_1$ activation producing a rapid increase in the first 5 min reaching a plateau, then decreasing after 60 min (Fig 4.3.4b). TFLLR triggered MAPK activation through either EV or hPAR$_1$C387AC388A with a slow rise in the first 20 min returning to basal levels, and then increasing again after 60 min (Fig 4.3.4b). The statistical analysis shows that there is no significant difference between EV and hPAR$_1$C387AC388A triggered MAPK signalling time course in response to either thrombin or TFLLR ($P>0.05$).

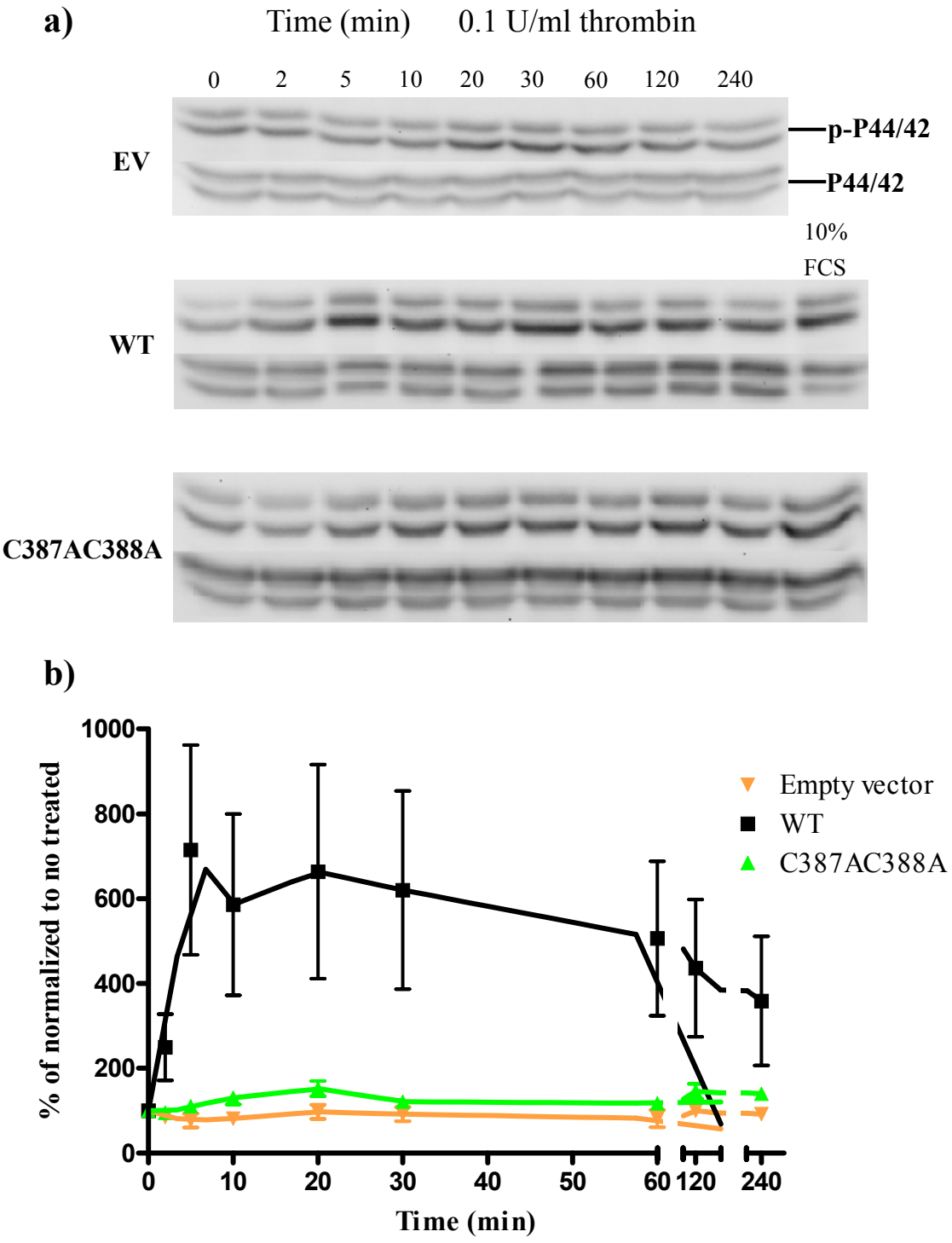


Figure 4.3.3 Thrombin stimulated ERK1/2 phosphorylation in a time course. a) Western blots for EV, wt-hPAR$_1$ and hPAR$_1$C387AC388A cells treated with 0.1 U/ml thrombin at different time points. The upper rows are the blots probed with anti-phosphorylated P44/42 antibody and the lower rows are the blots re-probed with anti-total P44/42. 10% FCS was used as a positive control. The blots shown are a representation of 3 repeats.**b)** Graphs showing densitometric values for bands produced by western blots. Values are expressed as a percentage of total P44/42 and then normalised to no treatment. The results present the means ± S.E. from 3 separate experiments.

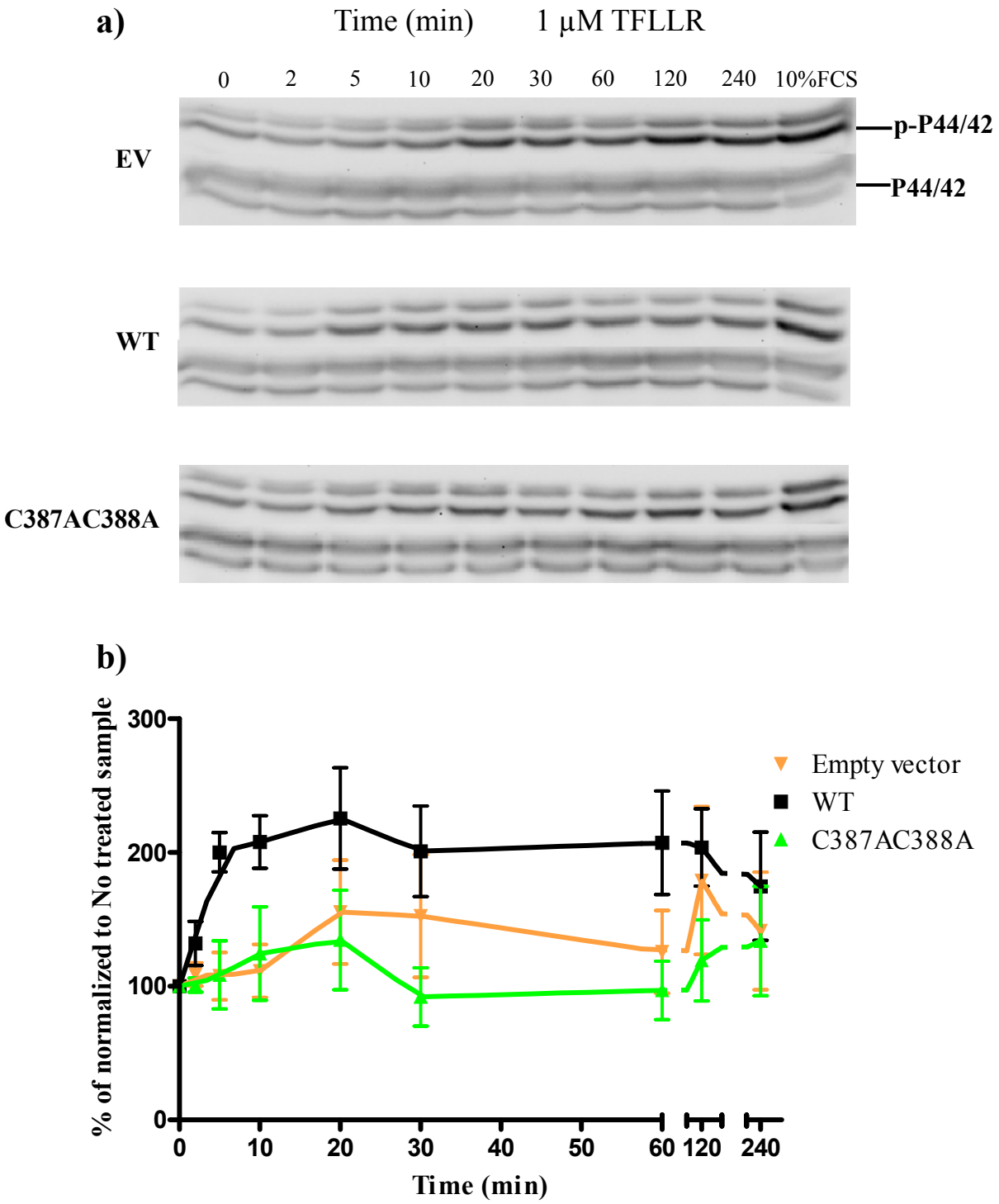


Figure 4.3.4, TFLLR stimulated ERK1/2 phosphorylation in a time courese. a) Western blots for EV, wt-hPAR$_1$ and hPAR$_1$C387AC388A cells treated with 1µM TFLLR at different time points. The upper rows are the blots probed with anti-phosphorylated P44/42 antibody and the lower rows are the blots re-probed with anti-total P44/42. 10% FCS was used as a positive control. The blots shown are a representation of 3 repeats.**b)** Graphs showing densitometric values for bands produced by western blots. Values are expressed as a percentage of total P44/42 and then normalised to no treatment. The results present the means ± S.E. from 3 separate experiments.

4.4 Discussion

The results in this section show that $hPAR_1$C387AC388A was unable to trigger agonist induced activation of ERK1/2 in a dose-dependent manner and time course as wt-$hPAR_1$. It is partially consistent with the study in the palmitoylation-deficient V2 vasopressin. The mutant receptor displayed a delayed and decreased agonist triggered ERK1/2 activation (Charest and Bouvier, 2003). However, palmitoylation was shown to have a contrary impact on $hPAR_2$, palmitoylation-deficient mutant triggered greater and more prolonged ERK1/2 activation in response to agonists (Botham, 2007). The lack of ERK1/2 signalling in $hPAR_1$C387AC388A could be due to the uncoupling of palmitoylation-less $hPAR_1$-G protein. As hypothesized in section 3.4, $hPAR_1$C387AC388A would be highly phosphorylated leading to the failure of coupling to G protein. Both G_q and G_i proteins have been shown to be involved in ERK1/2 activation by PAR_1 in different cell types. However, the mechanisms employed by GPCRs in ERK1/2 activation are varied and cell specific. It is difficult to conclude that the lack of ERK1/2 signalling is caused by the deficiency in $hPAR_1$-G_q coupling, $hPAR_1$-G_i coupling or both in KNRK cells. PAR_1 7-8-1 activation mechanism is required for PAR_1-G_q coupling, but whether this mechanism is also essential for the coupling to other G proteins is still unknown (Swift et al., 2006). G protein inhibitor would be used to determine which signalling pathway PAR_1 use to trigger ERK1/2 activation in KNRK, and $hPAR_1$C387A and $hPAR_1$C388A would be analysed to investigate the role of single cysteine in the $hPAR_1$ induced ERK1/2 signalling.

5 THE ROLE OF C-TERMINAL CYSTEINES IN AGONIST TRIGGERED hPAR$_1$ INTERNALISATION

5 The role of C-terminal cysteines in agonist triggered receptor internalisation

5.1 Introduction

Following activation, most GPCRs are rapidly phosphorylated by GRKs leading to β-arrestin binding which subsequently recruits clathrin and adaptor protein complex-2 (AP2). Receptors are then internalised via clathrin-coated pits mediated by dynamin to endosomes. Dynamin is a GTPase that regulates the detachment of clathrin-coated pits from the plasma membrane (Wolfe and Trejo, 2007). PAR_1 as a GPCR member has also been reported to utilize a clathrin and dynamin dependent pathway for receptor internalization upon activation (Trejo et al., 2000, Paing et al., 2002). Activated PAR_1 is rapidly recruited to clathrin-coated pits and the internalisation is inhibited in dynamin and clathrin mutants (Trejo et al., 2000). Apart from the agonist triggered receptor internalisation, PAR_1 internalises constitutively between the cell surface and an intracellular compartment in the absence of a ligand. However, β-arrestins which are essential for internalisation of many GPCRs are not required by either type of PAR_1 internalisations. In the mouse embryonic fibroblasts (MEFs) derived from β-arrestin knockouts, activated PAR_1 was internalised similarly with wild type via a clathrin and dynamin dependent pathway, and constitutive internalisation also remained intact (Paing et al., 2002). Coexpressing of PAR_1 and β-arrestin in COS-7 cells also failed to enhance PAR_1 internalisation induced by agonist (Chen et al., 2004). Clathrin adaptor AP2 and its binding site tyrosine-base motif Y*XX*L within the C-tail are required for constitutive PAR_1 internalisation, but not agonist triggered internalisation. In HeLa cells depleted of

AP2 by RNA interference, the non-activated receptor failed to constitutively internalise and accumulate in the intracellular store, in contrast, activated receptor internalised irrespective of siRNA treatment (Paing et al., 2006). However, activated PAR_1 internalisation still occurred through a clathrin and dynamin dependent pathway in AP2 depleted cells (Paing et al., 2006). There are two tyrosine-based motifs $Y^{383}SIL^{386}$ and $Y^{420}KKL^{423}$ found in the C-tail of PAR_1. The replacement of tyrosine 383 and leucine 386 by alanines caused a significant impairment in agonist triggered PAR_1 internalisation, but did not affect the constitutive internalisation of unactivated PAR_1 (Paing et al., 2004). However, this group reported that the PAR_1 $A^{383}SIA^{386}$ mutant exhibited levels of constitutive and agonist induced internalization similar to wild-type two years later (Paing, 2006). The other tyrosine-based motif $Y^{420}KKL^{423}$ has been indicated to directly interact with AP2 and be critical to PAR_1 constitutive internalization, but not agonist activated receptor internalisation (Paing et al., 2006). Taken together, it suggests that PAR_1 may utilize distinct molecular machinery in regulating constitutive internalisation and agonist induced internalisation. Recently, a study indicated that ubiquitination, a covalent attachment of ubiquitin to a protein, negatively regulates constitutive PAR1 internalisation, and K^{421} and K^{422} located within the C-tail tyrosine-based motif are the major sites of ubiquitination (Wolfe et al., 2007). Constitutive internalisation of ubiquitin-deficient PAR_1 was significantly increased and inhibited by the fusion of ubiquitin to the C-tail (Wolfe et al., 2007). Interestingly, AP2 was required for the agonist triggered ubiquitin-deficient receptor internalisation, unlike the activated wild-type receptor that is internalised independent of AP2 (Wolfe et al., 2007).

The role of β-arrestin, AP2, tyrospin-based motif in the PAR_1 C-tail and ubiquitination in PAR_1 internalisation has been well studied. However, the relationship between palmitoylation and PAR_1 internalisation is still unclear. Here, flow cytometry was used to asses the rate and the extent of receptor internalisation following agonist stimulation.

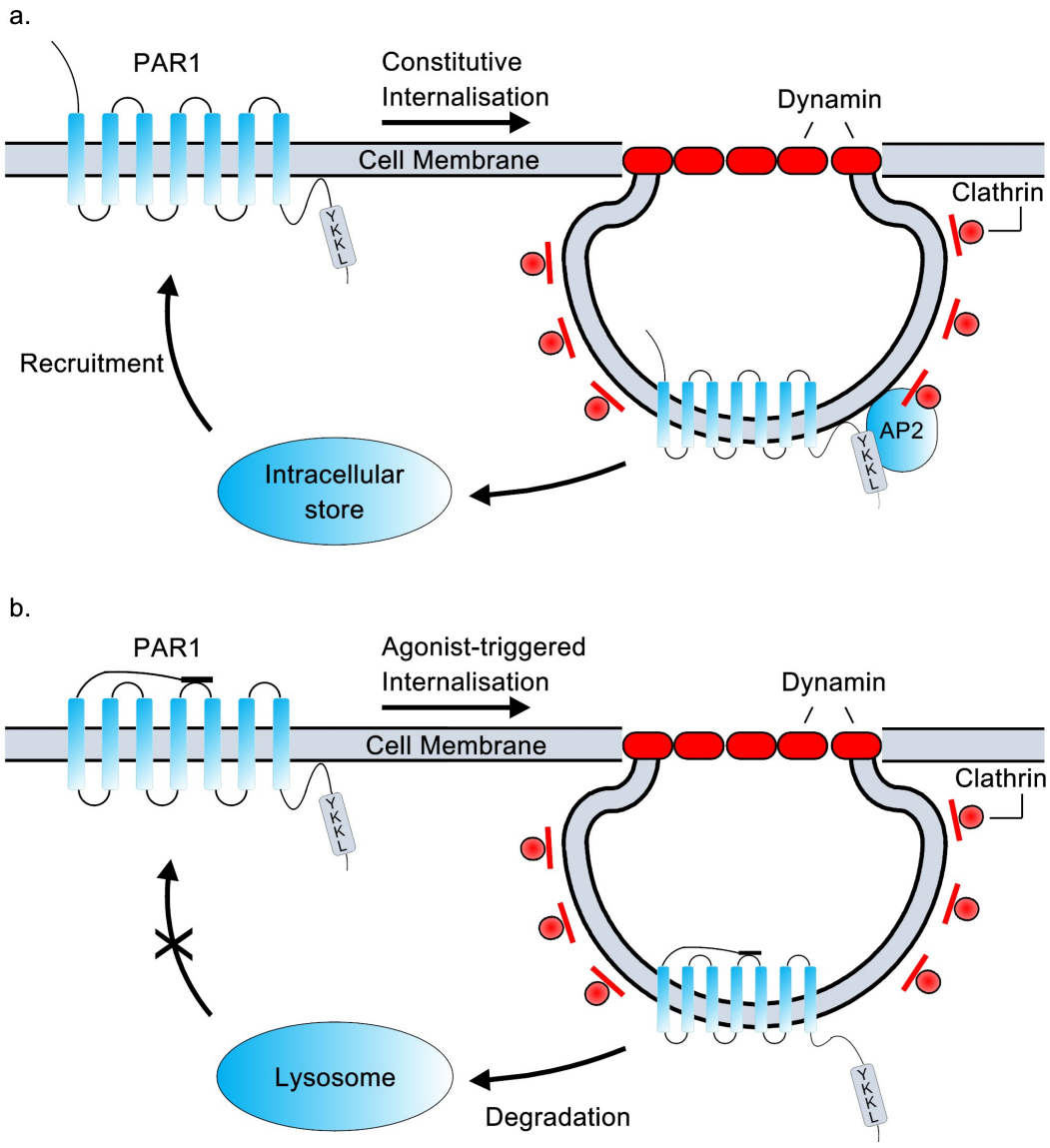


Figure 5.1, Modes of PAR_1 internalisation. a) Constitutive internalisation, intact PAR_1 cycles between the plasma membrane and the intracellular store by a clathrin, dynamin and AP2 mediated mechanism. b) Agonist triggered internalisation using a clathrin and dynamin dependent pathway, activated PAR_1 is then degraded in the lysosome.

Aims

The aim of the study in this chapter is to investigate the effect of $hPAR_1$ putative plamitoylation sites—C387 and C388 in the agonist triggered receptor internalisation.

5.2 Materials and Methods

5.2.1 Materials and reagents

All the reagents and chemicals used here are described in section 2.2.

5.2.2 Methods

5.2.2.1 Construction of hPAR$_1$R41A DNA and hPAR$_1$R41A expressing cell line

The KNRK cell line expressing hPAR$_1$R41A in which the arginine located at 41 in the N-terminal was replaced with an alanine was generated as described in section 2.3. Briefly, the mutant DNA construction was produced by mutagenic PCR using the following primers and wt-hPAR$_1$ in pcDNA3.1 vector as a DNA template.

R41A primers

5′-GCC ACC TTA GAT CCC GCG TCA TTT CTT CTC AGG-3′
3′-CGG TGG AAT CTA GGG CGC AGT AAA GAA GAG TCC-5′

Then, KNRK cells were transfected with the amplified and purified hPAR$_1$R41A DNA and taken through single cell cloning. FACS and calcium signalling assay were employed to pick up the best hPAR$_1$R41A expressed cell line out of ~40 colonies.

5.2.2.2 Flow cytometry detection of PAR$_1$ internalisation

Cells were harvested from 75 cm^2 flasks then divided into 5 ml falcon tubes (~30000 cells per tube) before centrifugation at 3000 g at 4˚C for 5 min. Cell pellets were resuspended in 300 µl of ice-cold CAB. The agonist (either 10 U/ml thrombin or 50 µM TFLLR) was added to the cells at the different time points (0, 30 sec, 1, 5, 10, 20,

40 min for wild type and mutant PAR_1 cell lines, 0 and 40 min for EV-KNRK) and mixed well before incubation at 37˚C with gentle orbital shaking (100 rpm) for the time specified. The cells were then placed on ice for 5 min and centrifuged at 3000 g at 4˚C for 5 min. Receptor expression was then assessed by FACS as described in section 2.3.2.4. The percentage of receptors on the cell surface was calculated using the mean of the fluorescence level and this way plotted to represent a receptor internalisation.

5.3 Results

5.3.1 Generation of hPAR$_1$R41A expressing cell line

Thrombin recoginizes the LDPR^{41}/S^{42} in the N-terminal of hPAR$_1$ and cleaves the peptide bond between arginine and serine to activate hPAR$_1$, whereas TFLLR binds to the ECL2 of hPAR$_1$ directly to activate the receptor without interacting with the N-terminus. Since the alanine substituted for the arginine41 in hPAR$_1$, thrombin is believed to be incapable of recognising/cleaving the receptor, but TFLLR is still able to activate the receptor. The hPAR$_1$R41A expressing cell line was successfully generated and screened by FACS and calcium signalling assay. The results are shown in Figure 5.3.1. hPAR$_1$R41A has a clear rightward shift compared to EV-KNRK (Fig 5.3.1a). Figure 5.3.1 b and c display that hPAR$_1$R41A triggered intracellular calcium signalling in response to TFLLR and calcium ionphore, but failed to respond to the other hPAR$_1$ agonist—10 U/ml thrombin.

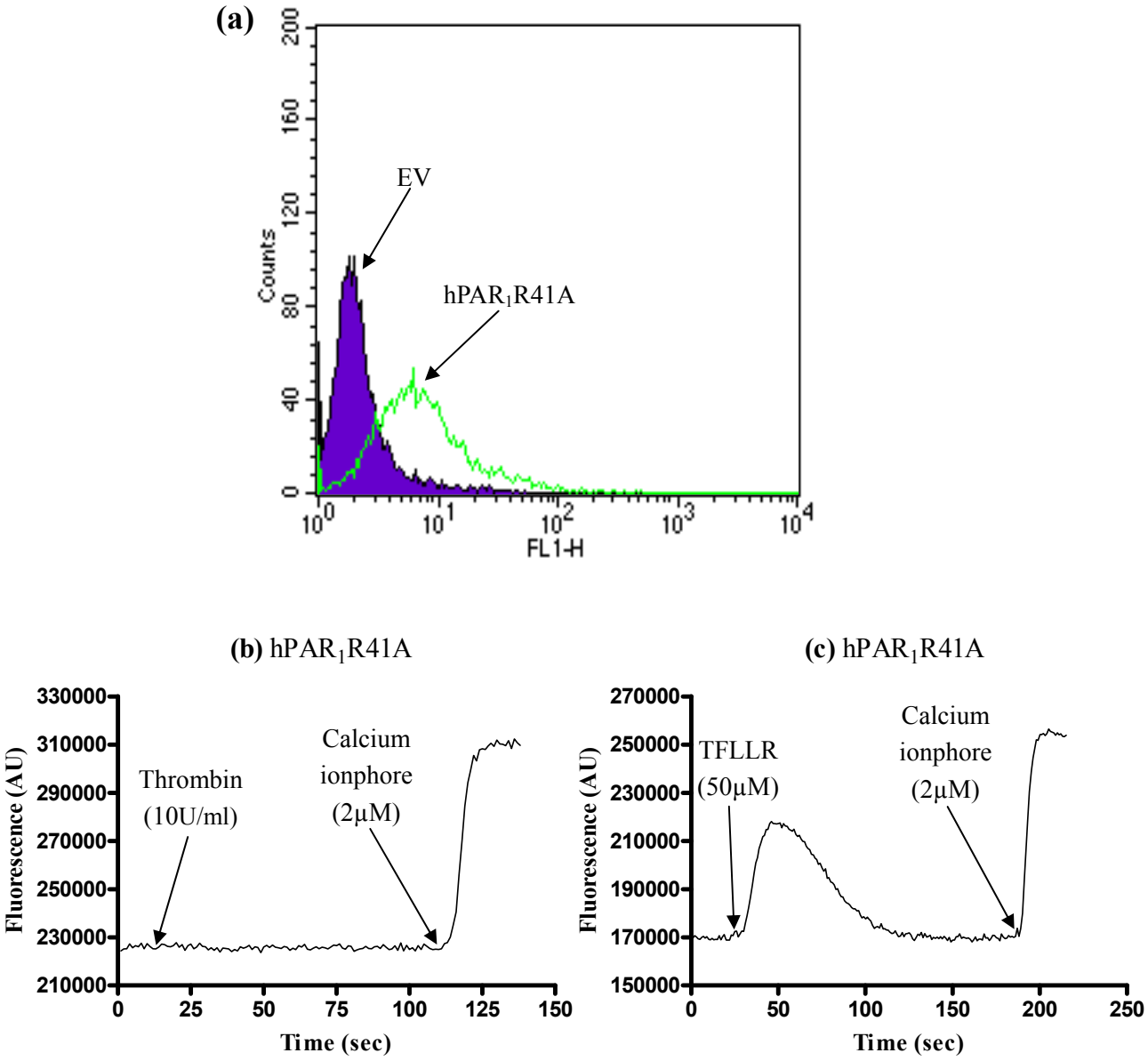


Figure 5.3.1, FACS analysis and calcium imaging of hPAR$_1$R41A. (a) FACS trace of hPAR$_1$R41A cell surface expression against EV-KNRK. hPAR$_1$R41A is shown as a green line and EV-KNRK is shown as solid purple. (b) Intracellular calcium mobilisation in hPAR$_1$R41A after agonist activation and addition of calcium ionphore.

5.3.2 Agonist triggered receptor internalisation

$hPAR_1$ internalisation in response to TFLLR and thrombin are shown in Figure 5.3.2. Internalisation was determined by the agonist-promoted decrease in cell surface immunoreactivity against $hPAR_1$. After the agonist treatment for different time period, the treated cells were placed on ice for 5 min in order to stop the receptor internalization from the cell surface. The remaining $hPAR_1$ receptors on the cell surface were then labelled with anti-PAR_1 antibody and further analysed at 4˚C.

All the cell lines internalised after treatment with 10 U/ml thrombin except $hPAR_1$R41A. For wild type and C-terminal cysteine mutant $hPAR_1$ cell lines, thrombin triggered internalisation of approximately 50% of the receptor in the first 30 sec reaching a maximal internalisation of 70% by 20 min, and thereinto $hPAR_1$C387AC388A has the fastest internalisation rate (Fig 5.3.2a). The wt-$hPAR_1$ failed to internalise by 40 min of 50 μM TFLLR treatment (Fig 5.3.2b). However, all mutant $hPAR_1$ cell lines internalised in response to TFLLR including $hPAR_1$ R41A reaching maximal internalisation of 60% by 40 min (Fig 5.3.2b). Seventy percent of TFLLR treated $hPAR_1$C387A and $hPAR_1$C388A remained at the surface after 10 min (Fig 5.3.2b). The internalisation rate of $hPAR_1$C387AC388A is slower than that of the other mutant cell lines, but the maximal internalisation levels are similar after 40 min (Fig 5.3.2). In general, thrombin triggered internalisation more rapidly and to a greater extent compared to TFLLR (compare Fig 5.3.2a and b).

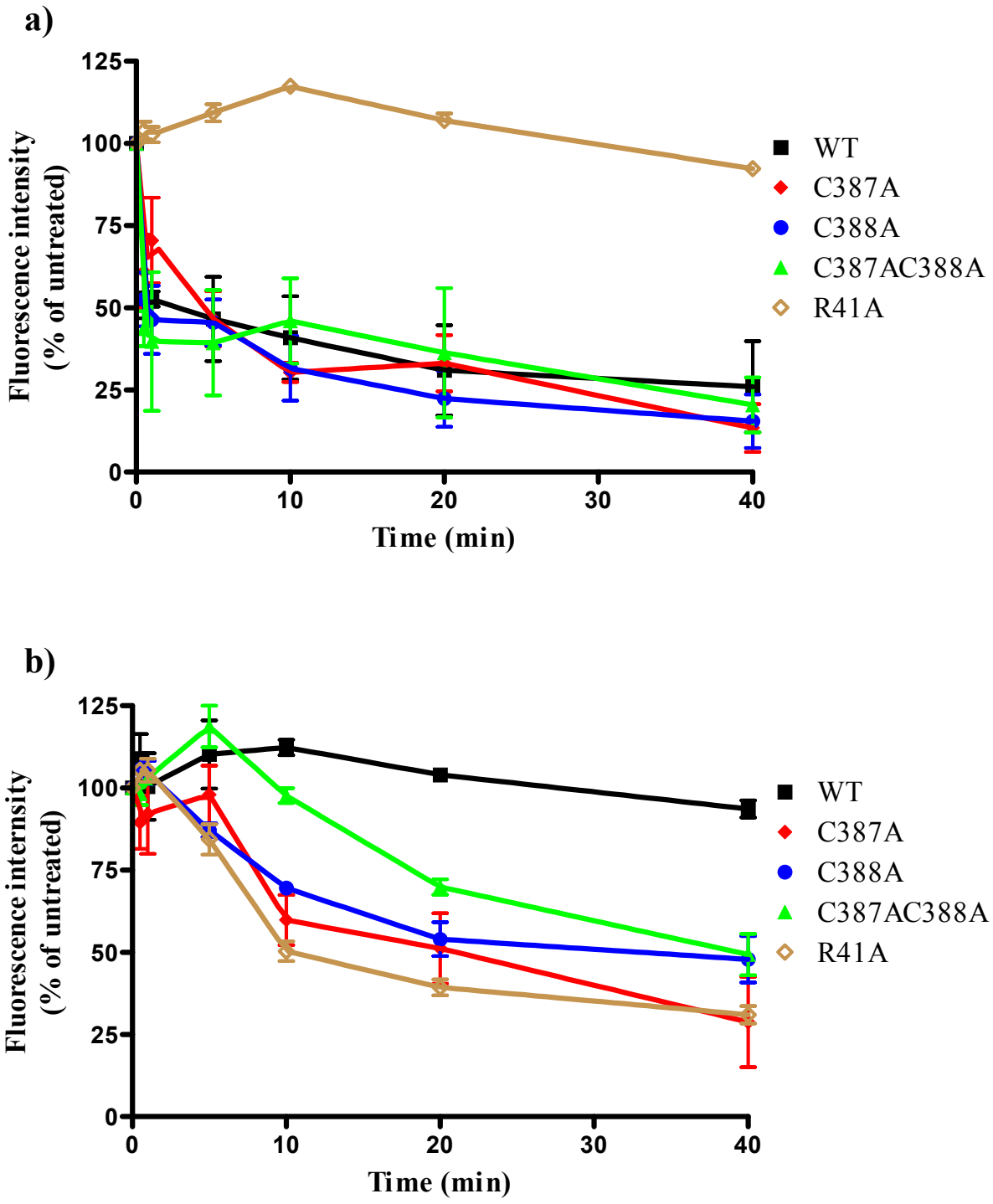


Figure 5.3.2, Agonists triggered receptor internalisation. a) The percentage of either wild type or mutant $hPAR_1$ receptors left on the cell surface after the treatment of 10 U/ml thrombin at different time points. b) The percentage of either wild type or mutant $hPAR_1$ receptors left on the cell surface after the treatment of 50 µM TFLLR-NH_2 at different time points. The results represent the means ± S.E. from three experiments performed in duplicate.

5.4 Discussion

The results in this section shows that in agonist triggered internalisation, $hPAR_1C387A$, $hPAR_1C388A$ and $hPAR_1C387AC388A$ all internalised in response to thrombin and TFLLR, except for wt-$hPAR_1$ which only internalised in response to thrombin. The thrombin recoginition site deficient mutant $hPAR_1R41A$ as expected failed to internalise following thrombin treatment, but internalised with TFLLR.

The role of palmitoylation in regulating GPCR internalisation varies. It has been reported that lacking palmitoylation sites can lead to an increase, a decrease or even no affect on receptor internalisation in different GPCRs. For example, an unpalmitoylated CCR5 mutant does not influence the receptor internalisation (Blanpain et al., 2001), while an unpalmitoylated human β_2-adrenergic receptor mutant increases the receptor internalisation (Moffett et al., 1993), and an unpalmitoylated human bradykinin B_2 receptor has a 30% reduction in the internalisation capacity (Pizard et al., 2001).

There is no remarkable difference among the thrombin triggered internalisation rate of wt-$hPAR_1$, $hPAR_1C387A$, $hPAR_1C388A$ and $hPAR_1C387AC388A$. Surprisingly, wt-$hPAR_1$ treated with TFLLR failed to internalise, but TFLLR triggered internalisation occurred in all mutant cell lines. Therefore, a KNRK cell line expressing the mutant $hPAR_1R41A$ which lacks the thrombin recoginition site was generated and utilized as a control. This mutant would be unable to be recognised by thrombin and thus can not be activated, but TFLLR can still activate it by direct binding to the mutant receptor. As expected $hPAR_1R41A$ internalised following TFLLR treatment but not thrombin. However, unlike wt-$hPAR_1$ $hPAR_1R41A$ internalised with TFLLR treatment. It may suggest the R41 is critical for the $hPAR_1$

internalisation, the existence of R41 disrupt the agonist-induced receptor internalisation. The internalisation would take place only when R41 is removed by either thrombin or a point mutation. Thrombin and TFLLR may utilize different mechanisms to promote $hPAR_1$ internalisation in the KNRK cell system. TFLLR as an intermolecular PAR_1 peptide agonist does not activate the PAR_1 in the same mechanism as thrombin (Blackhart et al., 2000, Swift et al., 2006). The receptor conformational change induced by TFLLR binding may not be sufficient to trigger the depalmitoylation or internalisation of wt-$hPAR_1$. It may also explain why thrombin-induced internalisation is more efficient than TFLLR. $hPAR_1$C387A and $hPAR_1$C388A have a weaker palmitoylation anchor and thus would still depalmitoylate/internalise with TFLLR. As a result, the data may suggest that both R41A and palmitoylation are important in TFLLR-triggered $hPAR_1$ internalisation. Additionally, it needs to be noted that PAR_1AP doesn't lead to receptor internalisation which may be due to the specific peptide used in this study—TFLLR.

In the thrombin triggered internalisation, as hypothesized in section 3.4, either one of the two cysteines would restore the palmitoylation function, thus $hPAR_1$C387A and $hPAR_1$C388A have the same internalisation rate as wt-$hPAR_1$. Interestingly, $hPAR_1$C387AC388A displayed a faster internalisation rate compared to wt-$hPAR_1$. The removal of the C-terminus region including palmitoylation sites of PAR_1 resulted in constitutive receptor internalisation in a agonist-independent manner (Paing et al., 2004), which would be caused by excessive phosphorylation of the palmitoylation-deficient receptors. Thus, $hPAR_1$C387AC388A would internalise constitutively and accordingly exhibited a low cell surface expression. The small

amount of the receptors expressed on the cell surface would be activated and further internalised after thrombin treatment. As a result, the internalisation rate of $hPAR_1C387AC388A$ was faster than wt-$hPAR_1$ in the first 30 sec as shown in Figure 5.3.2.

Immunofluorescence microcopy has been utilized to attempt to give a detailed image of agonist-triggered internalisation on an individual cell, but the laser bleach out the fluorescent staining in a few seconds. If time had permitted, the technique would be improved or new technique would be employed.

6 | THE ROLE OF C-TERMINAL CYSTEINES IN hPAR$_1$ PALMITOYLATION

6 The role of C-terminal cysteines in $hPAR_1$ palmitoylation

6.1 Introduction

Palmitoylation is one of the most common post-translational modifications occurring in proteins. A 16 carbon palmitic acid is covalently attached via a thioester bond to cysteine residues of the targeting protein, associating it with the plasma membrane. The reaction is catalysed by palmitoyl transferases (PATs).

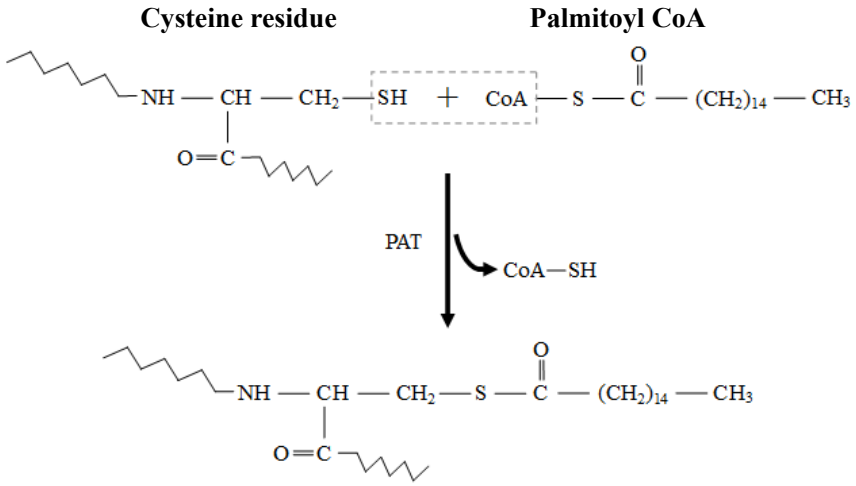


Figure 6.1, Protein palmitoylation process.

This covalent attachment of palmitic acid is reversible. The rapid palmitic acid turnover (depalmitoylation) has been observed *in vivo* and is mediated by palmitoyl-thiolesterases (APTs) (Escriba et al., 2007). In a variety of GPCRs, an increase in the palmitate turnover rate has been detected in response to extracellular agonist stimulation. For example, the pretreatment of β_2-adrenergic receptor with agonist resulted in a dramatic (~80%) decrease in the [^{3}H] palmitate incorporation (Loisel et al., 1996). The half-life of the β_2-adrenergic receptor-bound palmitate was

reduced 1.8 fold after the agonist stimulation without affecting the turnover rate of the receptor itself (Loisel et al., 1996).

The most frequently used experimental approach to demonstrate if a protein is palmitoylated is to chemically dissociate radio-labelled palmitate from the protein using dithiothreitol or hydroxylamine at a neutral pH (Qanbar and Bouvier, 2003).

Here, cells were labelled with [^{3}H] palmitic acid, the hPAR$_1$ receptors were immunoprecipitated using anti-HA11 antibody and then analysed on SDS-PAGE to represent [^{3}H] palmitic acid incorportaion. Unfortunately the immunoprecipitation using HA11 epitope failed to precipitate the receptors from hPAR$_1$ cell lines. However, fusing enhanced yellow fluorescent protein (eYFP) after HA11 epitope at the end of hPAR$_1$ receptors restored the ability of HA11 binding (Figure 6.2). Thus the wild type and mutant hPAR$_1$ were regenerated with eYFP.

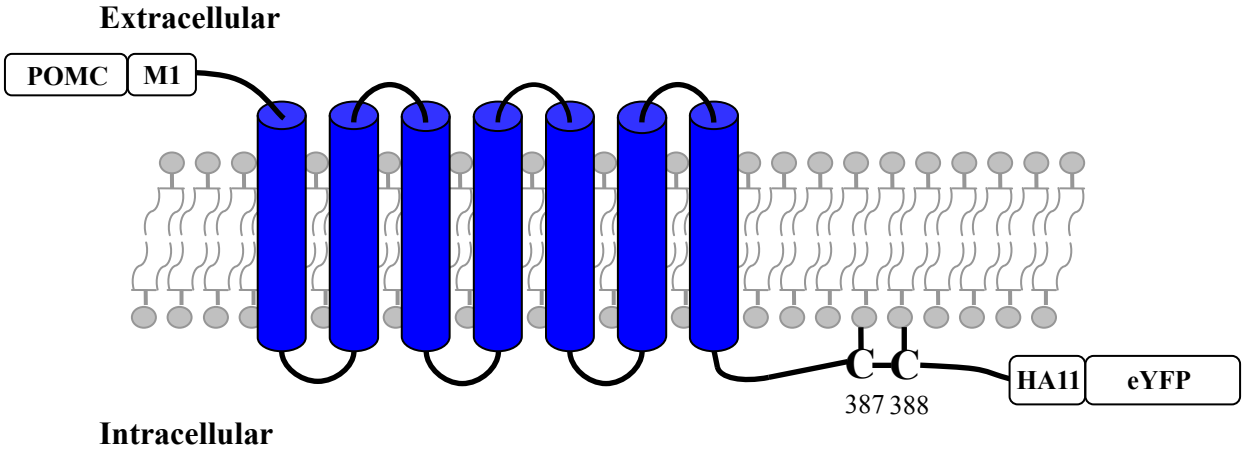


Figure 6.2, Representative model of hPAR$_1$eYFP. Wild type hPAR$_1$eYFP (POMC-M1-hPAR$_1$-HA11-eYFP) possessed an N-terminal POMC singal peptide followed by an M1 epitope, a HA11 epitope was fused to the C-terminal tail followed by an enhanced yellow fluorescent protein. See Appendix for DNA sequence and protein sequence.

Aims

This section of the study attempts to determine whether cysteines 387, 388 in the C-terminus of $hPAR_1$ are palmitoylated.

6.2 Materials and methods

6.2.1 Reagents and materials

Primers were purchased from MWG Biotechnology, Ebersberg, Germany. 1Kb DNA ladder was purchased from Invitrogen, Paisley, UK. ACCUZYME™ DNA polymerase and 10×AccuBuffer were purchased from Bioline, London, UK. Restriction endonucleases *Xho*I and *Bam*HI were purchased from New England Biolabs, Hithchin, Hertfordshire, UK, along with their specific NEBuffer 3 (10×). The rapid DNA ligation kit was supplied by Roche, East Sussex, UK. QIAquick® gel extraction kit was supplied by Qiagen, Crawley, West Sussex, UK. pEYFP vector was supplied by BD biosciences clontech, Erembodegem,Belgium. PageRuler™ plus prestained protein ladder was obtained from Fermentas, York, UK. M-PER® mammalian protein extraction reagent was obtained from Pierce, Northumberland, UK. μMACs HA tagged protein isolation kit was purchased from Miltenyi Biotech, Bergisch Gladbach, Germany. Purified mouse antibody mono HA.11 was purchased from Covance, Berkshire, UK. Sheep anti-mouse IgG-horseradish peroxidise, [9,10(n)-^{3}H] palmitic acid, Amplify™ fluorographic reagent and Hyperfilm™ MP were all supplied by Amersham Biosciences, Buckinghamshire, UK. All the other chemicals and reagents used in this chapter were the same with those in section 2.2. pcDNA 3.1 containing

wild type hPAR$_1$eYFP was supplied by Dr S.J.compton.

6.2.2 Methods

6.2.2.1 Constructions of C-terminal cysteine deleted hPAR$_1$eYFP DNAs (hPAR$_1$C387AeYFP, hPAR$_1$C388AeYFP, hPAR$_1$C387AC388AeYFP) and eYFP-HA11 in pcDNA3.1 vector

C-terminal cysteine deleted hPAR$_1$eYFP constructs were generated as described in section 2.2.1.1. Briefly, the mutant DNAs were produced by mutagenic PCR using the same C387A, C388A, C387AC388A oligonucleotide primers (as shown in 2.3.1.1), the pcDNA3.1 vector containing wt-hPAR$_1$eYFP was utilized instead as a plasmid DNA template.

6.2.2.2 Generation of eYFP-HA11 DNA fragment

The generation of eYFP-HA11 was performed by PCR of eYFP from the eYFP vector with the specific oligonucleotide primers (as below). eYFP F primer possessed an *Xho*I restriction site and a Kozak sequence prior to the start codon of the eYFP sequence. eYFP/HA R primer possessed a *Bam*HI restriction site and a 24 nucleotides HA11 sequence, which located between the stop codon and the main body of the eYFP sequence. Both primers were designed to terminate with several G or C bases.

eYFP F primer

5′-GCG GCC CTC GAG CCA CCA TGG TGA GCA AGG GCG AGG AGC TGT TCA CCG GGG TGG TGC CC-3′

eYFP/HA R primer

5′-GGC CGC GGA TCC TTA GGC ATA ATC GGG AAC ATC ATA GGG ATA CTT GTA CAG CTC GTC CAT GCC-3′

PCR reactions were prepared as follows; water blank was used instead of DNA template as a control.

10×AccuBuffer	10% (v/v)
eYFP vector (1 ng/μl)	0.02 ng/μl
eYFP F (20 ng/μl)	0.4 ng/μl
eYFP/HA R (20 ng/μl)	0.4 ng/μl
dNTP mix	0.5 mM
ACCUZYME^{TM} DNA polymerase	0.5 U/μl

40 μl of DEPC H_2O was added to reach a total volume of 50 μl. The PCR reactions were then placed in a thermal cycler to run the following cycles:

Cycles	Process	Temperature (˚C)	Time
1	Pre-heat	95	2 min
35	DNA denaturation	95	30 sec
	Primer Annealing	55	30 sec
	Extension	68	1 min
1	Final extention	68	10 min

Table 6.2.1, Conditions of PCR

The PCR products were run on 1.3% agarose gel alongside a 1Kb DNA ladder to allow the identification of band size. The eYFP-HA11 DNA fragment was isolated from the gel under UV light and the inclusive PCR product was extracted using QIAquick® gel extraction kit.

6.2.2.3 Digestion and ligation of eYFP-HA11 and pcDNA3.1 vector

pcDNA 3.1 and the PCR product were digested with *Xho*I and *Bam*HI restriction enzymes in order to create *Xho*I and *Bam*HI cohesive ends. Both *Xho*I and *Bam*HI exhibit optimum activity at 37˚C. In a total volume of 20 µl, 5 µl pcDNA3.1 vector or 10 µl PCR product was incubated with NEBuffer 3, BSA, *Xho*I, *Bam*HI at 37˚C for 3 hours. The vector and PCR product were then gel purified to remove the unwanted, cleaved portion of DNA.

After gel purificiation, eYFP-HA11 was ligated into pcDNA3.1 using a rapid DNA ligation kit to generate eYFP-HA11 in pcDNA3.1. The ligation reaction contained 10 µl of buffer, 1 µl of DNA ligase, 1 µl of pcDNA 3.1 vector, 5 µl of eYFP-HA11 and 4 µl dH_2O, and was performed at room temperature for 5min.

6.2.2.4 Bacterial transformation

Bacterial transformation was carried out as described in section 2.3.1.2. Briefly, either 7 µl of $hPAR_1$eYFP DNA (wild type/mutants) or 3 µl of the ligation product was incubated with β-ME treated XL-1 blue supercompetent *E.coli*, and then heat shocked at 42˚C. After incubating with 500 µl of LB broth at 37˚C, 200 rpm for 1 hour, the

E.coli was spread onto a LB agar plate containing ampicillin (100 μg/ml) and incubated at 37˚C overnight.

6.2.2.5 Harvesting hPAR$_1$eYFP DNAs and eYFP-HA11 in pcDNA3.1

hPAR$_1$eYFP DNA (wild type/mutants) and eYFP-HA11 in pcDNA3.1 were amplified, extracted and purified as described in section 2.3.1.3, except that *Xho*I and *Bam*HI restrition enzymes were utilized to digest the purified DNAs in order to assess the presence of insert. A small amount of the sample DNAs was then sent to MWG for sequencing.

6.2.2.5 Generation of wt-hPAR$_1$eYFP, hPAR$_1$C387AeYFP, hPAR$_1$C388AeYFP, hPAR$_1$C387AC388AeYFP and eYFP-HA11 permanently expressing cell lines

The procedure was the same as that described in section 2.3.2. Briefly, KNRK cells were transfected with the hPAR$_1$eYFP DNA (wild type/mutants) and eYFP-HA11 in pcDNA 3.1 respectively. The resulting cells were taken through two rounds of single cell cloning and analysed by FACS as described in section 2.3.2.4. The clone permanently showing the highest level of receptor expression was selected and utilised for further analysis.

6.2.2.6 [^{3}H] palmitic acid incorporation

Cells were grown in T75 flasks to ~80% confluence and then incubated in serum-free medium overnight. Toluene containing [^{3}H] palmitic acid was evaporated overnight

under vacuum. Dried [^{3}H] palmitic acid was dissolved in DMSO. [^{3}H] palmitate labelling medium was then made and comprised of DMEM, 1 mCi/3ml [^{3}H] palmitic acid, 1% DMSO and 2.5% FCS. The serum-free medium was removed from the flasks and [^{3}H] palmitate labelling medium was introduced to the cells. After 4 hours incubation at 37˚C, the [^{3}H] palmitate labelling medium was disposed of. The cells were then washed with PBS prior to dissociation by a cell scrapper in 0.5 mM EDTA. The cell suspensions were transferred into pre-chilled 15 ml falcon tubes and pelleted by centrifugation at 3,000 g for 5 min at 4˚C. The supernatant was discarded and the cell pellet was resuspended in 1ml of M-PER® mammalian protein extraction reagent (PIERCE). The mixture was then mixed at 100 rpm, RT for 10-15 min, followed by centrifuging at 14,000g for 20 min at 4˚C. The resulting supernatant was retained and purified by μMACs HA tagged protein isolation kit. The purified sample was stored at -80˚C until analysis.

Half of the sample was resolved on a 10% SDS gel alongside PageRuler™ plus prestained protein ladder. The gel was then fixed in gel fixing solution (isopropanol: dH_2O: acetic acid, 25: 65: 10) for 30min before treating with Amplify fluorographic reagent for 20 min. Subsequently, the gel was dried under vacuum at 80˚C for 40 min and the dried gel was exposed to Hyperfilm™ MP at -80˚C for 6 weeks before developing. The remaining half of the sample was also run on a 10% SDS gel with protein ladder. Similar to section 4.2.2, the sample proteins were transferred to a PVDF membrane. The membrane was then blocked by 5% non-fat milk in PBS/0.1% Tween-20 for 1 hour. The membrane was incubated with purified mouse antibody mono HA.11 (1 in 1000 dilution) in PBS/0.1% Tween-20 2% non-fat milk overnight at

4˚C. Followed by washing with PBS/0.1% Tween-20 for 4×15min, the membrane was probed with sheep anti-mouse IgG-horseradish peroxidise (1 in 1000 dilution) in PBS/0.1% Tween-20 2% non-fat milk. Visualization of the sample proteins was carried out by ECL western blotting detection reagents after washing with PBS/0.1% Tween-20.

6.3 Results

6.3.1 Generation of wild type and C-terminal cysteine mutant hPAR$_1$eYFP constructs and eYFP-HA11 construct

Three C-terminal cysteine mutant hPAR$_1$eYFP DNAs were generated by site-directed mutagenesis: hPAR$_1$C387AeYFP, hPAR$_1$C388AeYFP and hPAR$_1$C387AC388AeYFP. eYFP-HA11 in pcDNA3.1 was also generated. All the constructs were verified by automated DNA sequencing (MWG), and the sample containing the correct insert sequence was selected for each mutant.

6.3.2 Generation of wild type, C-terminal cysteine mutant hPAR$_1$eYFP and eYFP-HA11 permanently expressing cell lines

Both wild type and mutant hPAR$_1$eYFP stably expressing cell lines were successfully generated by single cell cloning as well as eYPF-HA11. The FACS analysis is shown in Figure 6.3.1. All the transfected cell lines display a clear rightward shift in fluorescence level against parental KNRK cells. The FACS traces of hPAR$_1$C387AeYFP and hPAR$_1$C388AeYFP match that of wt-hPAR$_1$eYFP, however,

hPAR$_1$C387AC388AeYFP displayed a smaller rightward shift than the others.

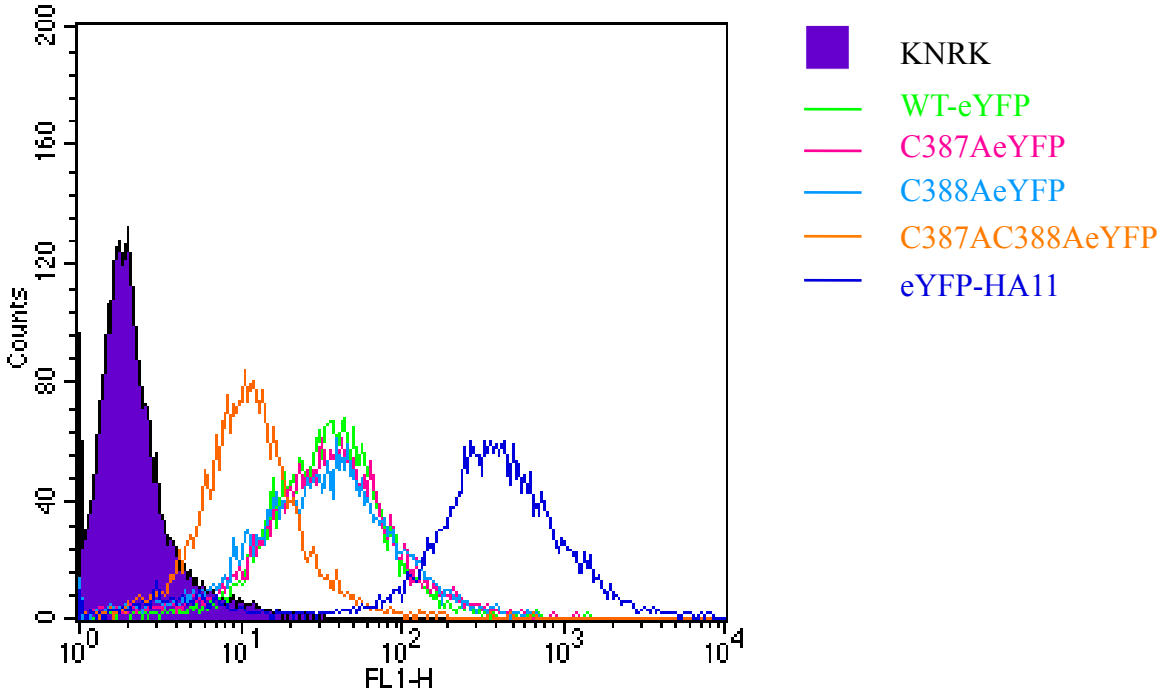


Figure 6.3.1, FACS traces of hPAR$_1$eYFP receptors cell surface expression. Parental KNRK cell line is shown as solid purple with the transfected cell lines shown in different colours. The histogram is a representative of at least 3 experiments.

6.3.3 C-terminal cysteines affect receptor palmitoylation

To ascertain whether hPAR$_1$ is palmitoylated on C387 and C388, eYFP-HA11, wt-hPAR$_1$eYFP, hPAR$_1$C387AeYFP, hPAR$_1$C388AeYFP and hPAR$_1$C387AC388AeYFP cell lines were labelled with [^{3}H] palmitate prior to immunoprecipitation of HA11 epitope-tagged receptors. More hPAR$_1$C387AC388AeYFP cells were used to gain the same receptor concentration as the other hPAR$_1$eYFP cell lines (assessed by western blot analysis, data not shown). Autoradiographical and western blot for [^{3}H] palmitate labelled cell lines are shown in

Figure 6.3.2., there is a band at ~150 kDa in the eYFP-HA11 lane. Similar bands are also shown in the wt-hPAR$_1$eYFP, hPAR$_1$C387AeYFP and hPAR$_1$C388AeYFP lanes, but a lot stronger in the former two lanes. It can be seen that a spread of bands appear from 65 to 100 kDa in wt-hPAR$_1$eYFP, hPAR$_1$C387AeYFP and hPAR$_1$C388AeYFP lanes, however, hPAR$_1$C387AC388AeYFP displayed no observable radiation signal (Fig 6.3.2a). The western blot (Figure 6.3.2b) shows multiple bands ranging from 65 to 150 kDa as well as bands at ~27, 28, 29, 36 and 42 kDa for wt-hPAR$_1$eYFP, hPAR$_1$C387AeYFP, hPAR$_1$C388AeYFP and hPAR$_1$C387AC388AeYFP lanes, however, the banding pattern of hPAR$_1$C387AC388AeYFP is much weaker. In the eYFP-HA11 lane, there are just two detectable bands at 27 and 40 kDa.

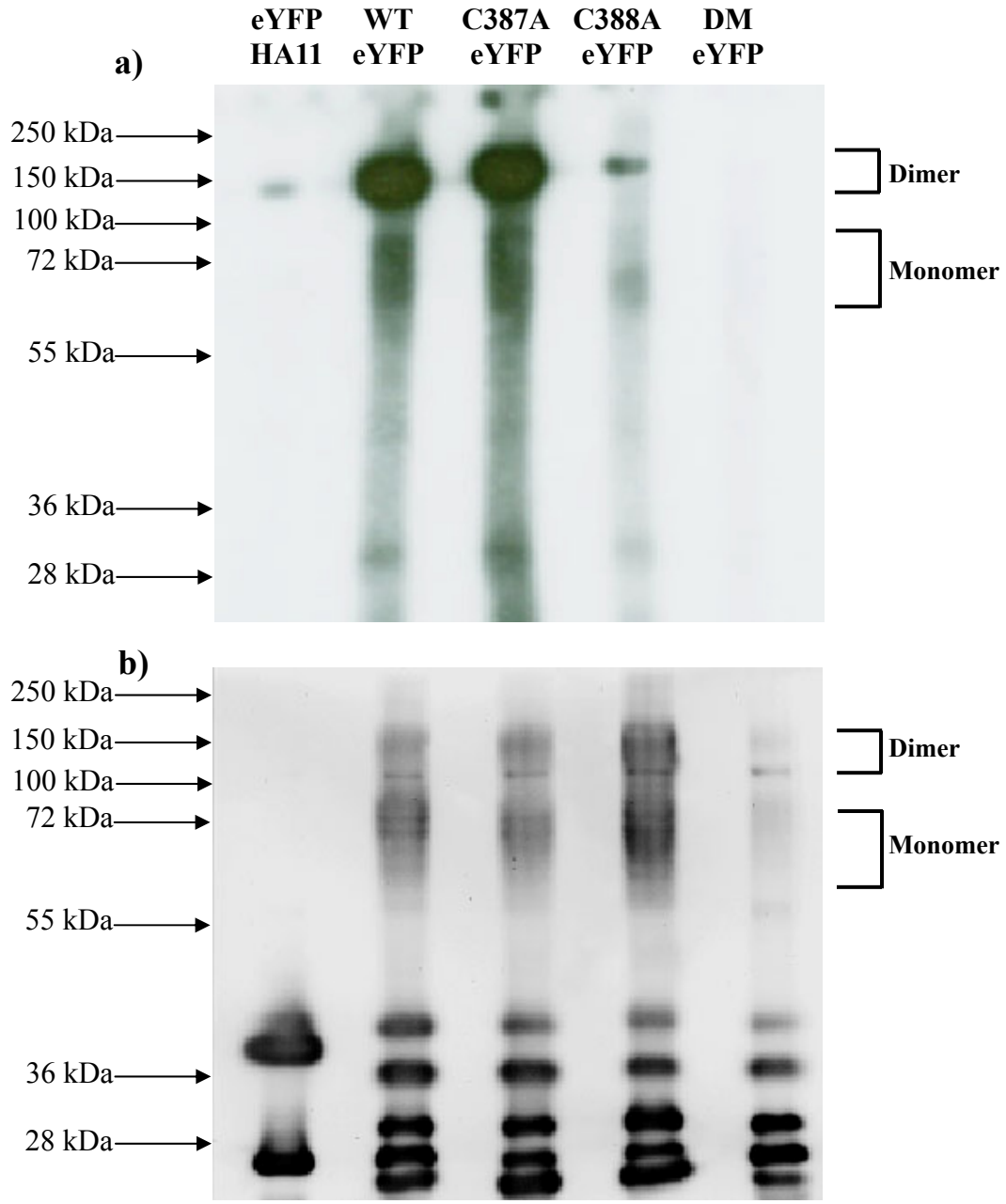


Figure 6.3.2, Incorporation of [^{3}H] palmitic acid. a) EV, wild type and mutant hPAR$_1$ receptors each labelled with 1mCi [^{3}H] palmitic acid for 4 hours and HA11-epitope immunoprecipitated run on a 10% SDS-PAGE. Gel dried and exposed to X-ray film at -80˚C for 6 weeks. b) a portion of the same samples were analysed on western blot. DMeYFP stands for double mutant, namely hPAR$_1$C387AC388A eYFP.

6.4 Discussion

The results in this chapter suggest that $hPAR_1$ is palmitoylated. wt-$hPAR_1$eYFP, $hPAR_1$C387AeYFP and $hPAR_1$C388AeYFP all have shown the incorporation of [^{3}H] palmitic acid, thus suggesting the three $hPAR_1$ receptors undergo palmitoylation (Fig 6.3.2). $hPAR_1$C388AeYFP shows a weaker radiation signal than the other two cell lines, and $hPAR_1$C387AC388AeYFP has no signal at all suggesting no palmitoylation. It may imply that C388 is a primary palmitoylation site and C387 is minor palmitoylation site, one cysteine is sufficient to keep the receptor palmitoylation. However, despite loading more proteins, the bands for $hPAR_1$C387AC388AeYFP are not as dark as the other samples in this experiment, suggesting that the lack of [^{3}H] palmitate visualised is caused by the insufficient protein amount (Figure 6.3.2b).

$hPAR_1$ is known to be heavily glycosylated explaining the smear of multiple bands seen in the western blot. Similar banding patterns have been seen in the glycosylation study of $hPAR_1$ and the glycosylation and palmitoylation study of $hPAR_2$ (Compton et al., 2002, Botham, 2007, Xiao, 2008). According to the size of $hPAR_1$-eYFP ($hPAR_1$=47kDa, eYFP=27kDa, 74kDa in total), the banding pattern between 65 kDa and 100 kDa would indicate the $hPAR_1$-eYFP monomer and the bands at ~150 kDa are possibly as a result of $hPAR_1$ dimerization. The multiple bands on the bottom half of the immunoblot in all four $hPAR_1$ lanes could be due to the protein degradation or contamination during immunoprecipitation. The band located at ~150 kDa in the eYPF-HA11 lane of the autoradiographic blot does not represent in the immunoblotting. It suggests the band is caused by the contamination or spillover of the sample in the adjacent lane, and eYPF-HA11 is not palmitoylated, thus would not

affect the radiation signal in the $hPAR_1$ samples.

This traditional protein palmitoylation assay is time consuming and takes at least 8 weeks to finish one experiment. There is a new method to purify and identify palmitoylated proteins based on an acyl-biotinyl exchange published recently (Wan et al., 2007). In this 3 step protocol, free thiols are blocked by *N*-ethylmaleimide (NEM), and then the Cys-palmitoyl thioester linkage is cleaved by hydroxylamine treatment, resulting in the release of palmitoyl moieties and the restore of cysteines/thiols (Wan et al., 2007). The newly exposed thiols are subsequently labelled with biotin-HPDP post via biotinylation (Wan et al., 2007). The biotinylated proteins are purified using agarose beads and analysed. If time had permitted, it would have been useful to repeat the palmitoylation studies using this technique.

7 | GENERAL DISCUSSION

7 General discussion

The major hypothesis of this thesis was that "*The $hPAR_1$ putative palmitoylation sites—C-terminal cysteines located at 387and 388 regulate receptor function"*. The major findings in this research supporting this hypothesis are outlined below:

1) Either of C387 and C388 is required for the optimal cell surface expression of $hPAR_1$. Loss of both cysteines caused a significant decrease in $hPAR_1$ cell surface expression.
2) Either of C387 and C388 is required for the optimal agonist triggered calcium signalling ($hPAR_1$-G_q coupling). Loss of both cysteines led to the complete ablation of agonist triggered calcium signalling.
3) C387 and C388 do not affect thrombin triggered receptor internalisation, however, in our experiments, wt-$hPAR_1$ does not internalise in response to TFLLR, but the mutant cell lines all internalise with TFLLR in a similar way.
4) Loss of both C387 and C388 in $hPAR_1$ led to the failure in agonist triggered ERK1/2 activation.

Taken together, the findings in this thesis clearly show that cysteines located at 387 and 388 play an important role in regulating the cell surface expression and the downstream signalling of $hPAR_1$.

Despite nearly all GPCRs being palmitoylated at cysteines present in the C-terminal tail, previous studies have suggested that the influence of palmitoylation depends on the receptor subtype. The observation also applies to the PAR family. Wt-$hPAR_2$ has a putative palmitoylation site on the C-terminal (cysteine361). Palmitoylation-deficient

$hPAR_2$ has an increased cell surface expression, decreased calcium signalling triggered by trypsin and PAR_2AP via G_q protein coupling, and an increased agonist stimulated ERK1/2 activation via G_i protein coupling, suggesting that palmitoylation would switch the coupling of the G protein. In contrast, palmitoylation-deficient $hPAR_1$ showed a decreased cell surface expression, and a complete shutoff of calcium signalling and ERK1/2 activation induced by thrombin and PAR_1AP implying the importance of palmitoylation in $hPAR_1$-G protein coupling. Interestingly, similar to $hPAR_1$, wt-$hPAR_2$ has also been shown not to internalise in response to its AP and internalises slower than palmitoylation-less mutant following proteinase agonist activation. In addition, $hPAR_2AP$ stimulation failed to induce any alteration in [^{3}H] palmitate incorporation with wt-$hPAR_2$, suggesting that wt-$hPAR_2$ did not undergo depalmitoylation after $hPAR_2$-AP activation.

Palmitoylation allows $hPAR_1$ to adopt a conformation that prevents phosphorylation by concealing the phosphorylation sites from GRKs. Upon agonist activation, the palmitoylated receptor would depalmitoylate, and thus exposes the phosphorylation sites to GRKs, resulting in the receptor phosphorylation, desensitisation and β-arrestin binding. Subsequently, the receptor internalisation occurs. TFLLR activation may be incapable of triggering $hPAR_1$ depalmitoylation like $hPAR_2$, and thus fail to expose the phosphorylation sites, in turn, the receptor can not be phosphorylated and the internalisation can not take place. However, the failure in receptor phosphorylation would cause the prolonged downstream signalling because $hPAR_1$ is desensitised by rapid phosphorylation. It is obviously inconsistent with our observations in $hPAR_1$ triggered calcium signalling and ERK1/2 signalling in response to TFLLR. However,

the study by Chen *et al* revealed that β-arrestins were able to bind and desensitise the activated PAR_1 independent of receptor phosphorylation (Chen et al., 2004). β-arrestins have also been shown not to be involved in PAR_1 internalisation (Paing et al., 2002). Consequently, TFLLR evoked calcium signalling and ERK1/2 signalling of $hPAR_1$ would be terminated by β-arrestin binding without the receptor phosphorylation. It also implies that receptor depalmitoylation/phosphorylation is required for $hPAR_1$ internalisation. Thrombin activation would be sufficient to induce $hPAR_1$ depalmitoylation and then phosphorylation, the receptor internalisation subsequently occurs. Nevertheless, TFLLR activation would be unable to elicit $hPAR_1$ depalmitoylation and thus inhibit the receptor phosphorylation and internalisation.

$hPAR_1$ lacking palmitoylation would be highly phosphorylated under basal conditions and behave as a constitutively desensitised/internalised receptor, owing to the increased accessibility of phosphorylation sites which were hidden by palmitoylation to GRKs. As a result, $hPAR_1$ is unable to couple to G proteins to trigger any downstream signalling pathways. $hPAR_1$ requires an intracellular network so-called 7-8-1 activation mechanism which is formed by the motif on the 7^{th} transmembrane helix, intracellular 8^{th} helix and intracellular loop 1 in coupling to G_q protein. The mechanism may also be essential for coupling of $hPAR_1$ to other G protein subtypes. The intracellular 8^{th} helix of $hPAR_1$ terminates with cysteines 387 and 388. Accordingly, loss of palmitoylation would destroy the 8^{th} helix, in turn the 7-8-1 connection. Palmitoylation-deficient $hPAR_1$ failed to couple to G proteins and trigger downstream signalling pathway. Additionally, alanine was chosen as an amino acid substitution for cysteine because they are structurally similar with the thiol group of

cysteine being replaced with a methyl group in alanine. This substitution should cause minimal disruption to the structure of the protein. Nonetheless, it is notable that all the deductions based on that the replacement of cysteines by alanines has no affect the $hPAR_1$ protein structure. The potential conformational change caused by the alanine replacement may leave the tethered ligand and $hPAR_1$-AP incapable of correctly binding to the ligand binding site in ECL2.

PAR_1 as described above regulates many physiological processes and participates in a variety of disease states including thrombosis, inflammation of cardiovascular, respiratory and gastrointestinal systems, neurodegenerative disorders and cancer, thus represents an attractive target for therapeutic drug development. Actually, substantial success has been achieved to develop PAR_1 inhibitors. Several PAR_1 antagonists have been developed for pharmaceutical use in humans and some of them are currently in the phase II&III clinical trails (Oestreich, 2009, Serebruany et al., 2009, Chackalamannil et al., 2005). A number of PAR_1 functions including receptor expression, downstream signalling and receptor internalisation are dynamically regulated by palmitoylation. Therefore, the palmitoylation sites in the C-terminal of PAR_1 may provide an alternative target of PAR_1 drug development.

Future work

Two more $hPAR_1$ [^{3}H] palmitate incorporation experiments are needed to be carried out in order to confirm whether C387 and C388 are $hPAR_1$ palmitoylation sites. If $hPAR_1$C387AC388A is highly phosphorylated and replacement of either cysteines would restore the receptor palmitoylation, [^{32}P] phosphorylation assay would be utilized to assess the receptor phosphorylation level under basal conditions.

$hPAR_1C387A$ and $hPAR_1C388A$ are expected to have a similar phosphorylation level with wt-$hPAR_1$, and $hPAR_1C387AC388A$ are expected to show a strong [^{32}P] incorporation. $hPAR_1S391Z$ which lacks the major phosphorylation site signalled well in calcium mobilization but can not shut down properly (data not shown). Thus, it leads to a hypothesis that the additional mutation of $hPAR_1$ phosphorylation site ($hPAR_1C387Z$) can restore cell surface expression and be able to mediate intracellular calcium mobilization or ERK1/2 activation, but can not desensitised appropriately. However, in terms of PAR_1 7-8-1 activation mechanism, $hPAR_1C387Z$ is incapable of triggering any downstream signalling pathways. It would be interesting to complete these investigations.

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Appendix: hPAR$_1$eYFP DNA sequence and Protein sequence

ATGCCGAGATCGTGCTGCAGCCGCTCGGGGGCCCTGTTGCTGGCCTTACTGCTTCAGGC
CTCCATGGAAGTGCGTGGTGACTACAAGGACGACGACGACCGCAGGCCAGAATCAAAA
GCAACAAATGCCACCTTAGATCCCCGGTCATTTCTTCTCAGGAACCCCAATGATAAATAT
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CATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAAC
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AGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAA
CACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGT
CCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGT
GACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG**TAA**GGATCCGAGCTC
GGTACCAAGCTTAAGTTTAAACCCGCTGATCAGCCTCGACTGTGC

MPRSCCSRSGALLLALLLQASMEVRGDYKDDDDRRPESKATNATLDPRSFLLRNPNDKYE
PFWEDEEKNESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLTSSWLTLFVPSVYTGVFVVS
LPLNIMAIVVFILKMKVKKPAVVYMLHLATADVLFVSVLPFKISYYFSGSDWQFGSELCRFV
TAAFYCNMYASILLMTVISIDRFLAVVYPMQFLSWRTLGRASFTCLAIWALAIAGVVPLLLK
EQTIQVPGLNITTCHDVLNETLLEGYYAYYFSAFSAVFFFVPLIISTVCYVSIIRCLSSSAVANR
SKKSRALFLSAAVFCIFIICFGPTNVLLIAHYSFLSHTSTTEAAYFAYLLCVCVSSISCCIDPLIY
YYASSECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLTYPYDVPD
YAMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTFGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDT
LVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA
DHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK.

Blue: POMC
Pink: M1 epitope
Black: $hPAR_1$ sequence
Grey: HA11 epitope
Red: eYFP sequence